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Molecular-level characterization of elastin-like constructs and human aortic elastin

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ABSTRACT

This study aimed to characterize the structures of two elastin-like constructs, one composed of a cross-linked elastin-like polypeptide and the other one of cross-linked tropoelastin, and native aortic elastin. The structures of the insoluble materials and human aortic elastin were investigated using scanning electron microscopy. Additionally, all samples were digested with enzymes of different specificities, and the resultant peptide mixtures were characterized by ESI mass spectrometry and MALDI mass spectrometry. The MS^2 data was used to sequence linear peptides, and cross-linked species were analyzed with the recently developed software PolyLinX. This enabled the identification of two intramolecularly cross-linked peptides containing allysine aldols in the two constructs. The presence of the tetrafunctional cross-link desmosine was shown for all analyzed materials and its quantification revealed that the cross-linking degree of the two *in vitro* cross-linked materials was significantly lower than that of native elastin. Molecular dynamics simulations were performed, based on molecular species identified in the samples, to follow the formation of elastin cross-links. The results provide evidence for the significance of the GVGTP hinge region of domain 23 for the formation of elastin cross-links. Overall, this work provides important insight into structural similarities and differences between elastin-like constructs and native elastin. Furthermore, it represents a step toward the elucidation of the complex cross-linking pattern of mature elastin.

1. Introduction

Elastin is an essential extracellular matrix protein of vertebrates and possesses unique properties including elasticity and extreme durability, which make it critical for the long-term function of tissues and organs such as aorta, skin and cartilage. Elastin is formed through K-mediated cross-linking of its monomeric precursor tropoelastin, which results in the formation of a variety of cross-links such as allysine aldol (AA), desmosine (DES) and its isomer isodesmosine (IDES) (Eyre et al., 1984; Reiser et al., 1992; Akagawa and Suyama, 2000; Mithieux and Weiss, 2005). However, even though the types of cross-links in elastin have been identified decades ago, almost nothing is known about the cross-linking pattern of elastin in different tissues. Elastin not only provides mechanical integrity to various tissues, but also plays an important role in the regulation of the cell behavior *via* bioactive elastin peptides (elastokines) that result from enzymatic degradation of

Abbreviations: AA, allysine aldol; ACN, acetonitrile; ANAO, *Aspergillus nidulans* amine oxidase; ANAO-TE, tropoelastin cross-linked *in vitro* after addition of ANAO; CTR, chymotrypsin; DES, desmosine; DSSP, define secondary structure of proteins; EP, elastin polypeptide; ESI, electrospray ionization; FA, formic acid; GROMACS, Groningen machine for chemical simulations; HPLC, high performance liquid chromatography; IDES, isodesmosine; **k**, allysine residue; LC, liquid chromatography; LOX, lysyl oxidase; MALDI, matrix-assisted laser desorption/ionization; MD, molecular dynamics; MS, mass spectrometry; MS^2, tandem mass spectrometry; MS3D, studies of three-dimensional protein structure using mass spectrometry; NMR, nuclear magnetic resonance; OPLSAA, optimized potentials for liquid simulations all atoms; PE, pancreatic elastase; PQQ, pyrroloquinoline quinone; SEM, scanning electron microscopy; TE, tropoelastin; TFA, trifluoroacetic acid; TOF, time of flight; TR, trypsin; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol.

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mature elastin (Maquart et al., 2005). In this context, it is noteworthy that the destruction of elastic fibers and the release of elastokines have been shown to be associated with pathological conditions including emphysema, atherosclerosis and actinic elastosis (photoaging) (Vrhovski and Weiss, 1998; Saarialho-Kere et al., 1999; Mithieux and Weiss, 2005). Therefore, it is important to investigate the molecular-level structure of human elastin to better understand the biomechanical properties of elastin as well as elastic-tissue diseases and their functional consequences.

The diverse functions of elastin and its degradation products as well as its unique properties such as extensibility and recoil, chemical stability and capacity to self-assemble have made the protein of interest for biomedical applications including tissue engineering. In particular recombinant human tropoelastin, elastin polypeptides and elastin-like polypeptides have been increasingly used as versatile, soluble components for the preparation of a variety of biomaterials in recent years (Keeley et al., 2002; Wise and Weiss, 2009; Almine et al., 2010; MacEwan and Chilkoti, 2010). Among other methods, elastin-based biomaterials have been produced using chemical cross-linkers such as bis(sulfosuccinimidyl) suberate (Mithieux et al., 2004) or pyrroloquinoline quinone (PQQ) (Bellingham et al., 2003; Vieth et al., 2007). These materials have been mainly characterized through their rheological and mechanical properties, *e.g.* swelling behavior and shear modulus, and their structure using a variety of microscopic techniques such as atomic force microscopy, confocal laser scanning microscopy and electron microscopy (Bellingham et al., 2003; Mithieux et al., 2004; Li et al., 2005; Vieth et al., 2007; Lim et al., 2008). However, no study has provided significant insight into the cross-linking pattern of such materials. Structural analysis of elastin-based biomaterials could contribute to understanding not only the structure of the materials but also that of mature elastin.

Due to its low accessibility for structure analysis, investigations on elastin have mainly been carried out after total hydrolysis of the protein in a strongly acidic or basic environment or by treatment with elastases and subsequent analysis of the resulting hydrolytic products, in particular the cross-linked amino acids DES and IDES (Partridge et al., 1955; Foster et al., 1974; Gerber and Anwar, 1975; Baig et al., 1980; Spacek et al., 1998). Analytical methods to characterize the structures of elastin and elastin-derived peptides include circular dichroism, vibrational spectroscopy, NMR spectroscopy and different microscopic techniques (Keeley et al., 2002; Bochicchio et al., 2004; Kumashiro et al., 2006; Vieth et al., 2007; Dyksterhuis et al., 2009). In recent years, liquid chromatography–mass spectrometry (LC–MS) has become an important tool in protein analytics. Since available software only allows for the identification of chemically introduced and bifunctional cross-links from MS^2 data, in the scope of an earlier project the software PolyLinX (Heinz et al., 2013) was developed to facilitate the sequence elucidation of peptides containing polyfunctional elastin cross-links including DES/IDES based on LC/MALDI MS/MS data.

The present study deals with the structural characterization of two elastin-derived materials of different structural complexity and native human aortic elastin to establish analytical methods for the structural characterization of elastin, in particular with respect to its cross-linking pattern. Another aim was to investigate how similar the materials are as compared to native elastin with respect to their cross-linking degree and ultrastructure. One of the constructs was composed of cross-linked elastin-like polypeptide (EP) 20-24-24 (Bellingham et al., 2001), a 200 amino acid residue long recombinant elastin peptide and the other one was cross-linked tropoelastin, isoform 2 (McGrath et al., 2011), the natural precursor of mature elastin. First, the morphology of these samples was investigated using scanning electron microscopy. Then, the susceptibility of the three materials to enzymatic degradation was studied and the degree of cross-linking was analyzed using mass spectrometric and bioinformatics methods recently developed for the identification of cross-linked species (Heinz et al., 2013). Moreover, molecular dynamics (MD) simulations were used to better understand the formation of cross-links, *i.e.* typical conformations that occur in linear elastin peptides prior to cross-linking as well as conformations present in already cross-linked elastin peptides.

2. Materials and methods

2.1. Materials

Tropoelastin lacking domains encoded by exons 22, 24A and 26A (isoform 2, UniProt accession number P15502-2) and the elastin-like polypeptide EP20-24-24 were recombinantly produced as described by Martin et al. (1995) and Bellingham et al. (2001), respectively. Human aortic punch biopsies (5 mm in diameter) were taken from patients of both genders (aged between 66 and 72) during coronary artery bypass grafting. Pure elastin was isolated from these biopsies using a newly developed, gentle method, which removes all other extracellular matrix components and prevents damage to elastin (Schmelzer et al., 2012). The work on human samples was approved by the ethics committee of the Medical Faculty, Martin Luther University Halle-Wittenberg (Germany), and performed in compliance with the Helsinki Declaration. Porcine pancreatic elastase (PE), proteomics grade trypsin (TR) and sequencing grade chymotrypsin (CTR) were purchased from Elastin Products Company (Owensville, MO, USA), Sigma-Aldrich (Steinheim, Germany) and Roche Diagnostics (Mannheim, Germany), respectively. *Aspergillus nidulans* amine oxidase (ANAO) was purified from *A. niger* culture (McGrath et al., 2011). Pyrroloquinoline quinone (PQQ) and α-cyano-4-hydroxycinnamic acid were obtained from Sigma-Aldrich. HPLC-grade acetonitrile (ACN) (VWR Prolabo, Leuven, Belgium) was used. Analytical grade 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany).

2.2. In vitro cross-linking experiments

Tropoelastin was coacervated and subsequently cross-linked *in vitro* through addition of ANAO (McGrath et al., 2011). The resulting insoluble material was lyophilized prior to analysis and is referred to as ANAO cross-linked tropoelastin (ANAO-TE). The elastin-like polypeptide EP20-24-24 was dissolved at a concentration of 5 mg mL^{-1} in 50 mM Tris buffer, pH 7.5, containing 1.5 M NaCl and cross-linked upon addition of PQQ as described earlier (Heinz et al., 2013). The cross-linked material was washed three times with a solution of water and ethanol (7:3, V/V), dried at room temperature and stored at −26 °C prior to further analysis.

2.3. Scanning electron microscopy

Lyophilized cross-linked EP20-24-24, lyophilized ANAO-TE and human aortic elastin, dried at room temperature after isolation from human aortic tissue biopsies, were analyzed by scanning electron microscopy (SEM) using an environmental scanning electron microscope ESEM XL 30 FEG (Philips, Amsterdam, Netherlands) as described previously (Heinz et al., 2013).

2.4. Proteolysis of tropoelastin, human aortic elastin and the cross-linked materials

Cross-linked EP20-24-24, ANAO-TE and human aortic elastin were dispersed in 50 mM Tris buffer, pH 7.5, at a concentration of 1 mg mL^{-1}, respectively. All samples were digested with PE, TR and CTR for 24 h at 37 °C using enzyme-to-substrate ratios of 1:100 (w/w), respectively. All digestions were stopped by adding TFA to a final concentration of 0.5% (V/V), and the samples were stored at −26 °C prior to further analysis.