Molekulare Einblicke in die Alterung elastischer Fasern

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Elastic Fibers

Skin

Aorta

Intervertebral disc

Tendon

Lung

Blood vessels
Core protein of elastic fibers → Elastin

- Very hydrophobic and highly cross-linked fiber protein, insoluble in any solvent
- Soluble precursor: tropoelastin (60 kDa – 70 kDa), undergoes extensive alternative splicing
- High reversible extensibility
- Half-life: ~70 years
Tropoelastin

```
1  MAGLTAAPRPGVLLLLLSLIHPSPGPGVPAGIPGPGPVPGVFYPGAGLGA
51  LGGGALPGPKPLKPVPGGLAGAGLGAGLGFAPAVTFPGALVPGGVADAA
101  AAYKAAGAGLGPGVPGVGLGVSAGAVVQPQGAGVPGVPVPGVLPGVY
151  PGGVLIPGAFPGVGLPQVGPTGAGVPGAIPGVPFGFPQP
201  GVPLGYPKAPKLPGYGYPYTGGLPYGYGPQPAGAAGYPTGTGV
251  GPQAAAAAAAKAKAFGAGAGVLPGVGGAGPGVPAGIPGIGIAGVGT
301  PAAAAAAAKAKAYGAAAGLVPGBPQPGFPQGVPAGDG
351  IPVVPAGIPQAAVPQVSPVEAAAKAAAKAYGARPGVGGAGPETYGVG
401  AGGFPGFGVGGVPQAGSVGGVPVGGVPVAVQPAQAAKAAKAA
451  KYVGTPPAAAAAKAAAKAAQFGLVPGVGPAGVPGVAPGVPAGVAPGVAIPG
501  VGVAPGVPAGVPGVAPGIPGGVAAAKSAAVAAAQTLRAAGGLG
551  PGLGPGVPGVPGVPGVPGVPGVPGVPGVPGVPGVPGVPGVPGVPGVPG
601  GVGLLGLGAGPGPVGAGPGAGPVPGVPGVPGVPGVPGVPGVPGVPG
651  GVGRLGVPVGGGLGGIPPAAAAAKAKAYGAAAGLGVGLGGAQFPLRPGVAA
701  PGFLSPIFPGGACLGLGACGR
```
Structure of elastic fibers

Cartilage

Skin

Aorta
Ageing

Solar elastosis

Williams syndrome

Cutis Laxa
Elastin - a mere structure protein?

**Cross-linked elastin**

**Elastases** (serine proteases, matrix metalloproteinases)

**Matrikines**
- Bioactive ligands that exist as part of an extracellular matrix protein

- Expression of MT-MMP-1, MMP-1 and MMP-2
- Chemotaxis
- Proliferation
- Induction of apoptosis
- Induction of angiogenesis
- Vasorelaxation and NO liberation
- Osteogenesis
- Th-1 polarization

**Interaction with elastin receptor complex**

**Elastin peptides**, some containing the **xGxxPG** motif

**Signal cascade**

**Cell types:** fibroblasts, endothelial cells, lymphocytes, monocytes, smooth muscle cells

**Cell surface**
Isolation of elastin
Isolation of elastin

→ Intact elastin free from contaminants and remnants of the ECM

- Hot alkali treatment (NaOH, 98 °C)
- Autoclave treatment
- Treatment with reducing and chaotrophic agents (mercaptoethanol, guanidine-HCl, urea)
- Treatment with enzymes (collagenase, trypsin)

Skin biopsy

Tissue biopsy

NaCl extraction

Organic solvents

CNBr digestion

Urea, 2-mercaptoethanol extraction

Trypsin digestion

NaCl extraction

Elastin
Morphological characterization

Human skin elastin isolated after Starcher and Galione

Human skin elastin isolated by a less destructive method
Molecular-level characterization

- Degradation of isolated elastin by porcine pancreas elastase
- Qualitative analysis by nanoHPLC-MS/MS and nanoLC/MALDI-TOF/TOF

<table>
<thead>
<tr>
<th>Identified precursors</th>
<th>InChorus Score</th>
<th>Identified precursors</th>
<th>InChorus Score</th>
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<tbody>
<tr>
<td>Tropoelastin</td>
<td>99 %</td>
<td>Tropoelastin</td>
<td>99 %</td>
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<tr>
<td>Collagen Type I α-1</td>
<td>99 %</td>
<td></td>
<td></td>
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<tr>
<td>Collagen Type I α-2</td>
<td>99 %</td>
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</tr>
<tr>
<td>Collagen Type III</td>
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</table>

The turnover of elastic fibers

~35 years

~90 years
Elastases

Matrix metalloproteinases

- Aortic stenosis
- Aortic aneurysm
- Photoaging of skin
- Tumor progression
- Pulmonary emphysema

MMP-7: epithelial cells
MMP-9: neutrophils, macrophages
MMP-12: macrophages

Connective tissue remodeling processes
Elastases

Serine proteases

Human leukocyte elastase (HLE)
- Stored in azurophilic granula of human neutrophils

Proteinase 3 (PR3)
- Released in response to inflammation processes, degradation of pathogens

Cathepsin G (CG)
- Chronic obstructive pulmonary disease
- Pulmonary emphysema
- Atherosclerosis
- Tumor progression
Enzymatic degradation of elastin

How do the enzymes act on elastin and tropoelastin?
- Cleavage site specificities
- Cleavage behavior

Are there any differences between elastin isolated by different methods?

Which peptides are released during degradation of elastin?
- Bioactive motifs?
### Cleavage behavior

#### Sequence coverages

- **MMP-7:** 71%
- **MMP-9:** 60%
- **MMP-12:** 81%

#### Peptide Counts

- **MMP-7:** 84 peptides
- **MMP-9:** 74 peptides
- **MMP-12:** 132 peptides

Tropoelastin (IF 2)
Cleavage site preferences

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>P₁ / %</th>
<th>P₂ / %</th>
<th>P₃ / %</th>
<th>P₄ / %</th>
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<tbody>
<tr>
<td>G</td>
<td>17</td>
<td>14</td>
<td>15</td>
<td>25</td>
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<tr>
<td>A</td>
<td>22</td>
<td>27</td>
<td>26</td>
<td>17</td>
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<tr>
<td>V</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>L</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>I</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
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<td>F</td>
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<td>2</td>
</tr>
<tr>
<td>Y</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>K</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>P</td>
<td>15</td>
<td>5</td>
<td>8</td>
<td>23</td>
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</table>

Occurrence of different amino acids at the substrate positions P₁ - P₄ and P₁' - P₄' after digestion with MMP-7, MMP-9, and MMP-12. Values are based on the number of cleavage sites identified upon mass spectrometric analysis of the digests.
Interaction of the natural substrate \textbf{LPYGYPG} (residues 226-233 from tropoelastin isoform 2) and the MMP-12 active site. The molecular surface of the binding pocket is colored according to electrostatic potential (red: negative electrostatic potential, blue: positive electrostatic potential).
Does HLE degrade elastin?
Digestion of tropoelastin by HLE
Cleavage behavior of NSPs - tropoelastin

Tropoelastin isoform 2

Sequence coverages

- HLE: 94 %
- PR3: 99 %
- CG: 96 %

- HLE: 408 peptides
- PR3: 305 peptides
- CG: 197 peptides
Digestion with HLE

Pancreas elastase

24 h incubation at 37 °C
enzyme-to-substrate ratio: 1:50

Characterization of elastin from different tissues
Challenges

... with elastin peptides

(1) Peptides mainly hydrophobic

(2) Proteolysis time consuming, not digestable with specific proteases

(3) Many of the peptides have equal or almost equal masses
Search for $M_r = 711.3915$

Tropoelastin
Challenges

... with elastin peptides

(4) Many repetitive regions

⇒ High similarity between peptides and thus between fragmentation patterns

(5) Cross-links and other PTMs

..GLGVPGVGG.. 711.39 Da

..LGGVPGVGG.. 711.39 Da

..GVPGVGG.. 711.39 Da
Initial situation

x 150 samples x 7 = 1050

- Skin
- Aorta
- Cartilage
- Intervertebral disc
- WBS skin
- WBS aorta
18-Sep-2011 Kontrollprobe WR, PE nach 24h, 1 : 8.75 verdünnt mit Wasser, 10 min beladen

WBS_WR_PEk2_3

TOF MS ES+ 31.73;598.3
BPI 1.06e3

ΔRT

Time

WBS_WR_PEk2_3

07:42:54
IonHunter

Preprocessing
- Smoothing
- Baseline correction
- Centroiding

Identification
- Deconvolution
- LC-Profiling

Combination
- Alignment
- Normalization
m/z 592.29; RT 42.08 min

m/z 939.44; RT 31.01 min

m/z 597.32; RT 29.38 min

Aorta (40 - 83 years)  Skin (4 - 13 years)  Skin (90 years)
Proline hydroxylation

<table>
<thead>
<tr>
<th>[M+H]$^+$</th>
<th>Sequence</th>
<th>Start/Stop residue (IF 9)</th>
<th>Pro residue (IF 9)</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>814.45 Da</td>
<td>F.AGIPGVGF.P.G</td>
<td>187 - 195</td>
<td>190</td>
<td>AUC$<em>{830.44}$/AUC$</em>{814.45}$ = HyP$<em>{190}$/P$</em>{190}$</td>
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<tr>
<td>830.44 Da</td>
<td>F.AGlpGVGF.P.G</td>
<td>187 - 195</td>
<td>190</td>
<td></td>
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<tr>
<td>939.53 Da</td>
<td>L.GGVGIPGGVVGA.G</td>
<td>678 - 689</td>
<td>683</td>
<td>AUC$<em>{955.52}$/AUC$</em>{939.53}$ = HyP$<em>{683}$/P$</em>{683}$</td>
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<tr>
<td>955.52 Da</td>
<td>L.GGVGIpGGVVGA.G</td>
<td>678 - 689</td>
<td>683</td>
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</tr>
</tbody>
</table>
Identified marker peptides

About 20 identified marker peptides that give insight into specific features of elastin such as:

- Age and/or degeneration status
- Type of tissue
- Existence and level of PTMs
- Presence of absence of certain domains
- Status of known point mutations
Characterization of cross-linking pattern of elastin
Recovered peptides from degradation with elastases
Cross-linking in elastin


Hydrophobicity/Hydrophilicity plot of human tropoelastin (Swiss-Prot accession number P15502, Isoform 9) calculated by the method of Hopp and Woods (1981).

Cross-linking of three tropoelastin molecules at domains 10, 19 and 25
Cross-linking in elastin

Bifunctional

Tetrafuctional

Trifunctional
Travelling wave ion mobility

z=1
z=2
z=3
Sum
Transfer-Coll. 50 eV, Mobility TOF, MS/MS 884.55
Potential b and y ions resulting from the fragmentation of a peptide cross-linked via dehydro-lysinosonorleucine.

Labeled TOF/TOF fragment spectrum of cross-linked species containing dehydro-lysinosonorleucine with a precursor mass of 1749.94 Da.
Scores for different cross-linked species containing dehydrolysinonorleucine. The score obtained for the correct peptide sequence is compared with scores obtained for its permutated decoy sequences.
Minimized structure of a desmosine-containing peptide after 20 ns of molecular dynamics simulations using the AMBER program. The central desmosine ring system is shown in white, the backbone of the peptide is shown as pink ribbon and hydrogen bonds are shown as green line.
Cross-linked tropoelastin

MALDI TOF/TOF tandem mass spectrum of propionylated demosine ([M+H]+ 750.39) detected in ANAO cross-linked tropoelastin.
Summary

• Isolation of intact and pure elastic fibers from single biopsies allows for elastolytic studies \textit{in vitro}.

• Intact elastin from young individuals (< 40 years) cannot be degraded by human leukocyte elastase.

• However, elastin from older patients is susceptible to HLE.

\textbf{With increasing age, elastic fibers lose their resistance against proteolytic degradation} \rightarrow \text{increased turnover}.

• Clear differences in the cleavage pattern of elastin from patients of different ages.

\textbf{Molecular clocks}

• Increased hydroxyproline levels for IVD elastin and decreased levels for elastin from WBS patients.
Summary

• Development of new algorithms for biomarker discovery taking into account common limitations and features of LC-MS measurements such as non-linear retention time shifts

• \textit{In vitro} formation of dehydrolysinonorleucine and desmosine containing peptides

\textbf{Scoring designed for the specific demands of the fragmentation pattern of cross-linked elastin peptides}

- Insights into the cross-linking of tropoelastin monomers and elastin-degrading processes

- Development of directed therapies against elastin-degrading diseases

- Biomaterials (stents, soft tissue repair matrix)
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