Alteration of Elastin, Collagen and their Cross-links in Abdominal Aortic Aneurysms

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Objectives: although the mechanism of arterial dilation and aneurysm development has not been clarified, the degradation of elastin and collagen plays undoubtedly a critical role. We evaluated the elastin and collagen content through the detection of their cross-links in aneurysmal and non-aneurysmal abdominal aortic walls.

Materials and methods: in 26 human abdominal aortic aneurysm specimens obtained during surgery and in 24 autopsy control samples of non-aneurysmal abdominal aorta the tissue content of elastin and collagen cross-links were measured by HPLC. Collagen was also detected by evaluating two characteristic amino acids, 4-hydroxyproline (4-hypro) with a colorimetric method and 5-hydroxylysine (5-hylys) by gas chromatography.

Results: significantly fewer elastin cross-links were found in aneurysm samples compared to controls (desmosines and isodesmosines: 90% reduction; p < 0.01). The opposite was true for pyridinoline collagen cross-links (350% increase) and deoxypyridinolines (100% increase, p < 0.01). Tissue content of 5-hylys, 4-hypro and total amino acids were reduced significantly by 50% in aneurysmal samples.

Conclusions: beside confirming decreased elastin content in aneurysmal walls, these results show a concurrent increase of collagen cross-links. Since total collagen markers were decreased (decreased 4-hypro and 5-hylys) it is reasonable to suggest that in aneurysmal aortic walls old collagen accumulates cross-links while new collagen biosynthesis is somehow defective.

Key Words: Abdominal aortic aneurysm; etiology; Collagen; Elastin; Cross-links; HPLC.

Introduction

Collagen and elastin are the most abundant fibrous proteins of the arterial wall and they are responsible for its characteristic mechanical resistance, tensile strength and elasticity.¹ Changes in their content and/or quality are likely to play a key role in the development of abdominal aortic aneurysm (AAA).

Elastic fibres are made of elastin molecules organised in long cross-linked filaments: their cross-links are desmosine (DES) and isodesmosine (isoDES). Organs containing elastin, such as aorta, lungs, blood vessels and ligamentum nuchae, can stretch out and recoil.

Collagen fibres represent the predominant connective element in arterial vessel as well as the major component of the aneurysm wall. Mature collagen is stabilised mainly by two form of stable, non-reducible cross-links: pyridinoline (PYR) and deoxypyridinoline (DPD), the former being mostly represented.

Studies on AAA suggested that elastin degradation may be important in the development of aneurysmal dilatation, while changes in collagen structure may predispose to aneurysm rupture.² This process is characterised by elevated activity of both collagenase and elastase.

We investigated quantitative alterations of collagen and elastin and of their cross-links in order to define the biochemical characteristics of the AAA wall relative to the normal aorta. Other biochemical markers such as 4-Hydroxyproline (4-hypro) and 5-Hydroxylysine (5-hylys) for collagen and total amino acid content of the samples were also evaluated together with histopathological features.
Materials and Methods

Patients and tissue sampling

Samples of aneurysm walls from 26 patients (23 men, 3 women aged 69 years) operated on for non-ruptured AAA were compared with samples obtained at autopsy from 24 non-aneurysmal patients (18 men and 6 women; aged 74 years). None of the patients undergoing surgery reported a family history of connective tissue disorders. The average aneurysm size was 5.6 cm.

A full thickness strip of the anterior aneurysmal wall was excised along the infrarenal aorta. All patients gave written consent to the procedure and the protocol was approved by the Ethics Committee of the S. Carlo Borromeo Hospital. Control samples were collected following the same procedure. Each aneurysmal and control sample was divided longitudinally in two parts. The first was fixed in 10% neutral buffered formalin, the second was washed with saline and stored at −80°C until analysis.

Tissue analysis

Biochemical analysis was carried out in the Laboratory of Pharmacological Biochemistry at the “Mario Negri” Institute of Pharmacological Research, Milan. Aneurysmal and control specimens were cleared from fat, blood, foreign tissue and areas of definite, hard calcification were discarded. Samples obtained using a circular cutter 8 mm in diameter were immediately weighed and hydrolysed with HCl 6N for 72 h at 110°C and evaporated to dryness under vacuum. All analyses were measured on the same punch biopsy, by drawing an amount of hydrolysed as necessary for each evaluation. All amounts were referred to mg of dry weight.

All methods were validated by adding to the first sample, before evaporation to dryness, a known amount of analyte. All analytes had a recovery between 90–95%.

Measurement of total pyridinolines and deoxypyridinolines

According to the original method of Eyre et al. with some modifications, 500 μl of hydrolysate, equivalent to 10 mg of tissue, was diluted with glacial acetic acid and n-butanol (1:1:4) and then applied to 200 mg of purified Whatman CF1 cellulose column to extract the analytes. PYR and DPD were eluted with 5 ml of bi-distilled water and the elute was evaporated to dryness. The dried samples were redissolved in 200 μl of 1% aqueous HFBA solution and a 20 μl aliquot of the sample was injected into a reversed-phase Spherisorb ODS2C18 (25 cm × 4.6 mm) column. After an isocratic separation, the analytes were detected fluorimetrically (295 nm Ex, 400 nm Em). The eluent was 0.02 M HFBA-acetonitrile (77:23, v/v). The flow-rate was 0.5 ml/min. The detection limit, for both analytes was 60 fmole for both cross-links.

Measurement of total desmosines and isodesmosines

DES and isoDES were measured by the techniques described elsewhere. Briefly, DES and isoDES, separated with the same method used for collagen cross-links, were evaporated to dryness and then derivatised with the reagent naphthalendialdehyde. A 20 μl aliquot of the sample was then injected into a 3 μm reversed-phase Supelcosil LC-18-DB (15 cm × 4.6 mm) column. The solvent system consisted of two eluents: (a) 0.005 M sodium citrate and 0.05 M sodium perchlorate monohydrate in water; and (b) 0.05 M sodium perchlorate monohydrate in methanol. Separation was done with isocratic elution at 55% (b) for 15 min, a nonlinear gradient [55–72% (b) for 22 min] was than used, followed by another isocratic step at 72% (b) for 13 min. The flow rate was 1 ml/min. DES and isoDES were detected at 420 nm. According to Starcher, elastin content was calculated from DES, assuming that 3.2 μg of DES is contained in 400 μg of elastin.

Measurement of 4-hydroxyproline

Hydrolisate samples containing 0.2 mg of tissue were evaporated to dryness under vacuum and 4-hypro was measured according to the colorimetric method described by Prockop and Kivirikko. This method involves the oxidation of 4-hypro to pyrole and formation of a chromophore with p-dimethylaminobenzaldehyde or Ehrlich’s reagent (Merck, Darmstadt, Germany) and the samples were read at 550 nm. Collagen content was calculated converting 4-hypro values in collagen as described by Laurent et al.

Measurement of 5-hydroxylysine

Hydrolisate samples containing 2.0 mg of tissue were evaporated to dryness under vacuum and 5-hyllys was measured by a technique described elsewhere. The dried sample was redissolved in bi-distilled water and applied to a column (2.5 × 1 cm I. D.) packed with Bio-Rex 70 (100–200 mesh, NH4+) washed with bi-distilled water, eluted with acetic acid and dried under vacuum. The carboxy group of 5-hyllys was esterified with dry acetyl chloride in n-propanol, the sample was then dried under nitrogen flow and trifluoroacetic anhydride was added and the sample was
dried again. A suitable volume of the internal standard solution was added and 1 μl of the sample was injected in the gas chromatograph.

**Measurement of total amino acid**
Hydrolysate samples containing 0.1 mg of tissue were evaporated to dryness under vacuum. Dried samples were redissolved in 1 ml of bi-distilled water and then 0.5 ml of a cyanide-acetate solution (2 ml of sodium cyanide solution 10 mM is diluted to 100 ml with an acetate buffer 250 mM, pH 5.3) and 0.5 ml of a 3% of ninhydrine solution in methyl cellosolve was added. Samples were heated at 100 °C for 15 min, then removed from water and a 5 ml of iso-propanol/water (1:1) was added. They were shaken and cooled at room temperature. Samples were read at spectrophotometer at 570 nm.

**Histological analysis**
Specimens were fixed in 10% buffered formalin and processed for routine embedding in paraffin for conventional light microscopy. Eight adjacent sections were cut at 5 mm intervals. Sequential tissue sections were stained with Hematoxylin and Eosin, Heidenhain’s connective tissue stain (Azan), Hart’s method for elastic fibres and Novelli’s stain.

**Statistical analysis**
Results were expressed as mean ± standard deviation (s.d.). Student’s t-test, Fisher’s exact test and logistic regression analysis were used for comparisons between variables. Differences were considered significant at the 95% confidence level (p < 0.05). Statistica for Windows (Statsoft Inc. 1995) was used for the statistical analysis.

### Results

#### Biochemical assays

**Collagen analysis**
Aneurysmal specimens contained significantly less 4-hypro (50% reduction) and 5-hyls (60% reduction) as compared to autopic controls (Table 1). Thus, also collagen content, as calculated converting 4-hypro values in collagen according to Laurent et al., was significantly lower in AAA samples (p < 0.01).

**Collagen cross-links**
We observed a significant increase of PYR (by 350%) and DPD (by 100%) in AAA specimens (pmoles/mg dry weight) (Table 1).

**Elastin cross-links**
DES and isoDES in AAA were reduced significantly by 90% as compared to autopsy control samples. The difference between the mean DES/isoDES ratios of AAA and controls were not significant. Elastin was correspondingly depleted in aneurysm tissue by 90% (Table 1).

**Amino acid analysis**
We observed a 50% decrease of amino acid content in AAA samples (p < 0.01, Table 1).

**Histological assay**
Routine histologic study for elastin and collagen demonstrated characteristic patterns for AAA, normal aorta and aortic occlusive disease (AOD).

In AAA the arterial wall was characterised by intimal atherosclerosis, virtual absence of elastin in the media, and dense inflammatory infiltrates in the outer aortic wall, as well as in the plaque. The inflammatory infiltrate was mainly mononuclear (T cells, B cells and

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 24)</th>
<th>AAA (n = 26)</th>
<th>% decrease</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Hydroxylysine</td>
<td>4.9 ± 1.8</td>
<td>1.8 ± 0.8</td>
<td>−62%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4-Hydroxyproline</td>
<td>21 ± 7</td>
<td>11 ± 4</td>
<td>−47%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pyridinolines</td>
<td>53 ± 7</td>
<td>242 ± 197</td>
<td>+353%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Desmosines</td>
<td>613 ± 221</td>
<td>51 ± 24</td>
<td>−91%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IsoDesmosines</td>
<td>552 ± 194</td>
<td>48 ± 25</td>
<td>−91%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Amino acids</td>
<td>311 ± 85</td>
<td>161 ± 36</td>
<td>−48%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Collagen</td>
<td>174 ± 53</td>
<td>92 ± 34</td>
<td>−47%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Elastin</td>
<td>146 ± 52</td>
<td>12 ± 6</td>
<td>−91%</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
macrophages) in variable number and commonly located at the junction of the media and adventitia. By Hart staining for elastic fibres, the elastic media was completely disrupted, and marked deposition of collagen was present. By Novelli staining for collagen, old collagen was abundant and prevalent in the elastic media and in the periphery of the plaque, while new collagen was mostly in the fibrous cup.

**Analysis of other variables**

Age did not significantly influence biochemical and histologic variables ($p > 0.05$). Smoking history was not significantly associated with most of the biochemical and histologic variables as well. Only 4-hypro content was significantly associated with smoking history. In fact, smokers had a mean 4-hypro value of 15 compared to 8 in non-smokers ($p = 0.05$).

Female gender was significantly associated with higher values of 5-hyls ($p = 0.02$), 4-hypro ($p = 0.04$), and total aminoacids ($p = 0.02$), whereas PYR, DPD, DES, isoDES were not significantly different between male and female cases.

**Logistic regression analysis**

We included in different logistic regression models for the prediction of biochemical and histologic variables the following factors: sex, smoking history, and presence or absence of aneurysms. We observed that the presence of aneurysm was the only independent characteristic that significantly influenced the levels of all the different biochemical variables (i.e. 5-hyls, 4-hypro, PYR, DPD, DES, isoDES, total aminoacids) ($p < 0.05$).

**Discussion**

Elastin and collagen content of the aortic wall has been the subject of extensive study with conflicting reports (Table 2). While most investigators showed a substantial reduction of elastin, the same does not apply to collagen. In addition, the content in collagen cross-links (PYR and DPD) has never been directly studied previously. Reported discrepancies in the results might be partly due to the wide variability of assays employed.

We investigated biochemical changes of the aneurysmal aortic wall involving elastin by HPLC determination of DES and isoDES and by calculating conversion ratios according to Starcher (3.2 µg of desmosine correspond to 400 µg of elastin). Most studies carried out to date have evaluated the amount of elastin after removing lipids and calcium from tissue samples by centrifugation of the NaOH-insoluble fraction. Moreover, when this fraction is evaluated by cross-link analysis the method does not allow one to discriminate the two cross-linking polyaminoacids DES and isoDES. Only one reported study has assayed elastin by alternative methodologies, i.e. HPLC dosage of the characteristic peptide VAPG which, however, includes an unreliable gravimetric step. This study confirmed the concordance between the reduction of the typical cross-links and of elastin itself.

Our results confirm most of previous literature reports, even if the degree of depletion that we observed is higher. Only Minion et al. observed an absolute increase in elastin content, which however, when expressed relatively to total protein content, was actually reduced.

DES and IsoDES have been previously determined separately in only one study, in which elastin content and elastase activity were assessed in aneurysmal and non-aneurysmal aortas. The two cross-links showed different ratios in AAA and controls (7 to 1 and 2 to 1 respectively). The method employed (amino-acid analyser), however does not seem to generate data sufficiently reliable due to the difficulty in discriminating the two aminoacid peaks. We were not able to show any significant difference in DES and isoDES ratios between aneurysms and control samples suggesting that elastin is altered as a whole, keeping the ratio of the two components unaltered.

An indirect confirm of our results comes from two recent studies. In the first Lindholt et al. determined the serum elastin peptides (SEP) concentration among 83 patients during follow-up for small AAA. Their results showed an increase in SEP (markers of elastin degradation) as the aneurysm enlarged. In the second, Wilson et al. related inversely SEP concentration with pressure strain elastic modulus and stiffness.
Fig. 1. Novelli’s stain – 200× – abdominal aortic aneurysm: the deep red coloration shows the abundant presence of the old collagen.

Fig. 2. Novelli’s stain – 200× – normal aorta: newborn collagen is colored in blue; the old collagen is almost absent.
(evaluated by ultrasound) in AAA, thus confirming that elastin degradation is correlated with increased aortic wall distensibility (and aneurysm formation). Collagen data are more conflicting. To date, all reports use 4-HyPro dosing for collagen determination in aneurysmal tissue; some authors\textsuperscript{11,12} observed a modest increase while others showed a reduction\textsuperscript{14,17} or no change\textsuperscript{15,16}.

We also employed the classical method based on 4-Hydroxyproline determinations which showed about a 50% reduction. To confirm this data, we also carried out the analysis of an another collagen marker: 5-HyLys. This was performed by using an original and innovative gas-chromatographic method\textsuperscript{13} (conceived and improved in our laboratory) which is more accurate than the colorimetric one generally used for 4-HyPro, showing a 60% 5-hylys reduction.

The behaviour of PYR and DPD in aortic walls was studied by Watanabe,\textsuperscript{20} who assessed its content relatively to age in autoptic controls; he found a direct correlation between their content and age. Whittle\textsuperscript{11} assessed the difference in cross-links between desicated and normal aortas and observed a progressive decrease going from aortic root towards descending aorta.

Our determinations show a marked increase in PYR of approximately 350\% (p < 0.01) and also DPD raised up of more than 100\% (p = 0.01).

Any previous paper directly compared collagen cross-links concentration in aneurysmal aortic specimens and normal aorta. The histological examination seems to support our hypothesis: in fact Novelli’s stain showed a prevalence of aged collagen in aneurysmal aorta rather than in non-aneurysmal one (Figs 1 and 2).

A possible explanation of increased collagen cross-links (in absence of concomitant collagen increase) may come from the observation that collagen molecules continue to form cross-links throughout their lifespan.\textsuperscript{20} This relative excess of cross-links might thus be due to their over-representation on aged collagen.

Indirect confirms to our results come from a series of studies which used immunnoassay methods\textsuperscript{22-25} (developed by the University of Oulu, Finland) to evaluate type I and III collagen metabolism.

Satta \textit{et al.}\textsuperscript{26} compared through a quantitative radio-immunoassay a group of aneurysmal patients to second group with peripheral arterial disease and a third aorto-iliac diseased group. They found a significant increase of serum aminoterminal propeptide of type III collagen (s-PIIINP) in aneurysmal patients. s-PIIINP is a marker of type III collagen synthesis or degradation, thus confirming an increased type III collagen turn-over.

Treska \textit{et al.}\textsuperscript{27} detected serum carboxyterminal propeptide of type I collagen (s-PICP) and s-PIIINP through a quantitative radioimmunoassay in aneurysmal and non-aneurysmal patients. They also collected aneurysmal aortic walls to perform a tissue analysis of both analytes. Their results showed no significant difference for s-PICP (a marker of type I collagen biosynthesis) and an increase in aneurysmal s-PIIINP. Tissue analysis found direct correlation between aneurysm diameter and t-PIIINP concentration, while t-PICP (also increased with aneurysm enlargement) did not reach statistical significance.

Bode \textit{et al.}\textsuperscript{28} immunohistochemically stained specimens from healthy and aneurysmal aortas to evaluate tissue aminoterminal propeptide of type I collagen (t-PINP) content, aminoterminal propeptide of type III collagen (t-PIIINP) content and aminoterminal telopeptide of type III collagen (IIINTP) (which recognises old and fully cross-linked collagen). No difference was discovered between the groups for t-PINP and mild increase in aneurysmal for t-PIIINP, especially in the media layer. IIINTP was strongly prevalent in AAA, located in all the three layers, but especially in the media. t-PINP and t-PIIINP indicate collagen neo-synthesis because their antibodies bind to collagen (free PIIINP molecules are washed out during sample processing) whereas these propeptides are still attached and cross-links are under construction. The same molecule is much more susceptible to proteolytic attack because of the delay in cross-links formation.

These results give evidence of a low biosynthesis rate of type I collagen (which is prevalent in arterial walls) in aneurysmal aortas. This may be the explanation of the decreased total collagen content that we observed, as our analysis is not able to distinguish between the different forms. We also received a confirm in our increased cross-links findings.

Our data cannot support any hypothesis about type III collagen metabolism results.

Conclusions

Our data confirm the decrease of elastin cross-links, collagen and total protein content in aneurysmal walls. Surprisingly, while collagen content seems to be reduced, its cross-links increase. These data suggest that in aneurysmal aortic walls the synthesis of new collagen could be somehow stopped while the aged one accumulates cross-links. Histopathological analysis seems to confirm this hypothesis.
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