Increased Amount of Type III pN-collagen in AAA when Compared with AOD

M. K. Bode¹, M. Mosorin², J. Satta², L. Risteli¹, T. Juvonen² and J. Risteli∗¹

Departments of ¹Clinical Chemistry and ²Surgery, P.O. Box 5000, FIN-90014, University of Oulu, Finland

Objective: the extent of the processing of type III procollagen to type III collagen was determined in nine human abdominal aortic aneurysms (AAA), and compared with ten samples of aortoiliac occlusive disease (AOD).

Methods: the aminoterminal propeptide (PIIINP) and telopeptide (IIINTP) of type III procollagen and collagen, respectively, were immunologically measured in the soluble and insoluble fractions of the extracellular matrix. The assay for PIIINP in the insoluble matrix was further validated.

Results: the insoluble matrices of AAAs contained at least 12 times more incompletely processed type III pN-collagen than AOD specimens (0.74% and 0.061%, respectively). Also, the soluble extracts of AAAs tended to contain more non-processed type III pN-collagen than free, properly cleaved aminoterminal propeptide.

Conclusions: the larger amount of type III pN-collagen suggests an alteration in the metabolism of type III collagen in AAAs. This may partially explain the decreased tensile strength of the aortic tissue.

Key Words: Aneurysm; Atherosclerosis; Collagen; Extracellular matrix.

Introduction

Abdominal aortic aneurysms (AAAs) were traditionally thought to be caused simply by atherosclerosis, but epidemiologic, genetic and biochemical information has shown that other etiologic factors are involved as well. Several studies have indicated a familial component, and type III collagen gene mutations have, indeed, been shown to contribute to the development of AAAs. The expansion of infrarenal AAAs is accompanied by an increase in the content of total proteins and collagens in the diseased vessel wall, together with a dilutional decrease in the elastin content and the number of the smooth muscle cells. There is also evidence of increased collagenolytic and elastolytic activity.

Collagens (including at least types I, III, IV, V, VI and VIII) are, together with elastin, the most abundant extracellular components of the aortic vessel wall. Type III collagen in particular has been suggested to provide the aortic tissue with tensile strength. Type III collagen is synthesised as procollagen, with extra propeptide domains at both ends (Fig. 1). Part of type III collagen is known to be present in tissues as type III pN-collagen, with a retained aminoterminal propeptide.

The aim of this study was to assess the processing of type III collagen and the proportion of type III pN-collagen in AAAs using atherosclerotic, but non-aneurysmal, aorta tissue as control. The presence of type III pN-collagen prevents the further lateral growth of type III collagen fibres and may also affect collagen cross-linking at the aminoterminus. Here, we wanted to characterise the degree of processing of type III collagen in AAAs.

Materials and Methods

Subjects and tissue samples

Fresh longitudinal strips were cut from the anterior wall of the full-thickness aneurysm sacs of 9 consecutive patients (6 men and 3 women, mean age 72 years, range 67 to 84 years). Seven were from patients undergoing elective repair of asymptomatic infrarenal AAA, and two samples had been taken during emergency operations after rupture. All the aneurysms were degenerative and fusiform, and their maximum diameters (mean 6.1 cm, range 3.0 to 13.0 cm) had been measured preoperatively by ultrasonography. All the
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Preparation of samples

The tissue specimens of 0.27–5.95 g were cut into small pieces, and phosphate-buffered saline containing 0.04% Tween 20 was added up to 1 ml/100 mg of wet tissue weight. The samples were homogenised and centrifuged, and the supernatants (soluble fractions) were collected for analyses as described earlier. The pellets (insoluble fractions) were treated with sodium borohydride (50 mg NaBH₄/g tissue weight) to stabilise the possibly reducible collagen cross-links. Fats were removed by washing briefly with acetone/methanol (1:2, v/v) and finally with ethanol, followed by centrifugation and collection of the pellet. The pellets were freeze-dried, weighed and suspended in 0.2 mol/l NaHCO₃ (1 ml/10 mg dry weight), denatured at +70°C for 1 h and treated with 1 mg of trypsin per 100 mg of sample. The digestions were performed for 4 h at +37°C, after which the samples were again denatured, homogenised and retreated overnight with a similar amount of fresh trypsin. The residual trypsin activity was destroyed at +70°C, and the samples were centrifuged at 10 000 × g for 30 min. The supernatants were collected for immunochemical and gel filtration analysis.

Immunoassays for type III collagen metabolites

The concentration of the aminoterminal propeptide of type III procollagen, PIINP (Orion Diagnostica), was measured under equilibrium conditions in 1:10 dilutions of the tissue extracts and in dilutions varying from 1:1 to 1:100 of the tissue digests, the former for the AODs and the latter for the AAAs. The intra- and interassay coefficients of variation were 4 and 5%. The concentrations of PIINP observed in the trypsin digests were multiplied by two to obtain the real values, because this enzyme digestion decreases the immunoreactivity of PIINP by 50%. The concentration of IIINTP, which represents the aminoterminal telopeptide of type III collagen (in-house method), was analysed in undiluted tissue extracts and in tissue digests diluted from 1:100 to 1:1000. The intra- and interassay coefficients of variation were 6 and 10%.

Trypsin digestion of PIINP

The intact aminoterminal propeptide of type III procollagen, PIINP, was purified from human ascitic fluid as described earlier and digested with trypsin at +37°C after heat denaturation at +70°C. The digestion products were purified by Sephacyrl S-100 gel filtration and chromatographies on a reverse-phase C₁₈, a DEAE, and finally a reverse-phase C₅₈ column.
A smaller trypsin-generated fragment of PIIINP was similarly purified from the tissue type III pN-collagen of human uterine leiomyoma. The purification was followed with the PIIINP assay. The purities and apparent sizes of the different forms of PIIINP were determined in 18% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing and reducing conditions. The true molecular weights were determined by MALDI-TOF mass spectrometry (Biflex™, Bruker-Franzen Analytic).

**Statistical analyses**

Statistical analyses were carried out with the SPSS statistical analysis tool. The significances of the differences between two groups were calculated with the non-parametric Mann–Whitney U-test. The data are expressed as means with 95% confidence intervals (CI). Spearman’s rank correlation was used for correlation analysis.

**Results**

**Sizes of the cross-linked type III collagen telopeptide antigens in digested aorta tissue**

The cross-linked type III collagen telopeptide antigens in the trypsin digests of aortic tissue were analysed by gel filtration (Fig. 2). In both the AAA and the AOD tissue samples, the majority of the PIIINP antigen eluted in one peak corresponding to the trypsin-generated standard antigen that has been isolated previously and is known to be this aminoterminal part of the type III collagen molecule in a trivalently cross-linked form. In addition, minor amounts of smaller molecular species representing divalentely cross-linked and non-cross-linked type III collagen telopeptides were seen, especially in the AOD samples (Fig. 2).

**Aminoterminal propeptide antigens of type III procollagen in aorta tissue**

78% (CI 69–86%) of the PIIINP antigen was easily extractable from the tissue in the atherosclerotic aortas, as expected, but in the aneurysms most of it was surprisingly associated with the insoluble matrix and only 6% (CI 4–8%) found in the soluble fraction. In Sephacryl S-300 gel filtration a major part of the antigenicity of the AAA samples eluted far ahead of the free propeptide, in a position corresponding to type III pN-collagen molecules (Fig. 3A). In the AODs most of the antigenicity was due to the free propeptide, indicating that type III procollagen had been completely processed (Fig. 3B).

The PIIINP antigens in the insoluble fraction were obtained into solution by trypsin digestion and similarly analysed by gel filtration on Sephacryl S-100 (Fig. 2). The proteolytic digestion releases the propeptide domain from the pN-collagen molecule. Detectable amounts of PIIINP were seen in the AAAs (Figs. 2A–C), whereas practically no such antigen was seen in the AOD samples (Figs 2D–F), again suggesting differences in the processing of the biosynthetic precursors of type III collagen between the two situations. Most of the PIIINP antigen solubilised by trypsin from the insoluble aortic matrix eluted in one major peak (fractions 43–49), although some antigen, representing a clearly smaller molecule, was found in the fractions 61–64 (Fig. 2).

**Characterization of trypsin digestion peptides of PIIINP**

To elucidate the nature of the PIIINP antigens released from aortic tissue by trypsin digestion, we compared them with the tryptic digestion products of soluble intact PIIINP from ascitic fluid (larger antigen) and of insoluble type III pN-collagen from leiomyoma (small antigen). In SDS-PAGE, the latter was found not to contain disulphide bonds and without reduction; Fig. 4. The reduced individual chains of the standard propeptide were clearly larger than this.

As expected, the intact standard PIIINP that had been isolated without proteolytic treatment contained only disulphide-bound polypeptide chains in trimeric structures. Trypsin treatment of this protein produced a larger PIIINP-related antigen that resolved into three different components in SDS-PAGE without reduction. Upon reduction, these gave rise only to two polypeptide chains, one corresponding to the monomer of standard PIIINP and the other to the smaller trypsin-generated form (Fig. 4).

The real molecular sizes were assessed with MALDI-TOF analysis. The intact PIIINP gave a molecular mass of 43 258, as expected on the basis of known sequence data. The size of the small antigen produced from pN-collagen was 10 604, indicating that the trypsin cleavage most probably has occurred after the amino acid residue Arg-95 and removed the most carboxyterminal part of the propeptide that contains the disulphide bonds (Fig. 1). The molecular weight distribution of the larger trypsin-treated PIIINP was...
Fig. 2. Gel filtration (Sephacryl S-100 column) profiles of the antigenicities of the cross-linked aminoterminal telopeptide of type III collagen (IIINTP; open symbols) and the aminoterminal propeptide of type III procollagen (PIIINP; closed symbols) in trypsin-digested insoluble matrices isolated from (A–C) AAAs; and (D–F) AODs. Note the marked difference between the AAAs and AODs in the amounts of trypsin-digested PIIINP antigen, the major and minor elution positions of which are indicated by the arrows. The arrows indicate the verified elution positions of the larger and smaller forms of trypsin-generated PIIINP.

heterogeneous, as expected from the SDS-PAGE analysis. Here the main form had a size of 11 054, suggesting some difference either in the trypsin cleavage site(s) or in the post-translational modifications between this and the small antigen from pN-collagen. Our conclusion is that trypsin had cut one or two of the three component chains in soluble, intact PIIINP (Fig. 1B) and that such cleavage products still partially adhered to the rest of the propeptide.

In Sephacryl S-100 gel exclusion chromatography the elution position of the larger trypsin-generated PIIINP antigen corresponded to the major PIIINP peak found for the trypsin-digested AAA samples (Fig. 2). The minor component of fractions 61–64 corresponded to the elution position of the small antigen.

There was also a major difference between the two trypsin-generated antigen forms in their behaviour in the PIIINP radioimmunoassay, the small antigen being 30 times less effective as inhibitor than the larger (Fig. 5).

**Increased amounts of biochemical precursor forms of type III collagen**

In order to exclude a decreased rate of synthesis of type III collagen as the reason for the difference in the proportions of fibrillar collagens between AAA and AOD, we quantified the aminoterminal propeptide of type III procollagen in both the soluble and the insoluble fractions of tissues (Fig. 6). Although the soluble fraction only contains about one per cent of total collagen, the molecules that are in transit from
biosynthesis to collagen fibres as well as the free propeptides released during synthesis are normally recovered in this fraction.

In the soluble extracts, the amount of type III pN-collagen (as a percentage of the sum of type III pN-collagen and type III collagen, which were calculated from the molar amounts of PIINP and IIINTP, respectively) accounted for 69% and 41% of the total type III collagen in AAAs and AOD samples, respectively ($p<0.001$) (Table 1). As mentioned above, there were, unexpectedly, significant amounts of the propeptide antigen also in the insoluble matrix in the AAAs, where the proportion of type III pN-collagen was 0.74%, whereas the corresponding proportion in the AOD specimens was 0.06% (Table 1, Figs 2 and 6).

**Discussion**

Aneurysm formation is a complex remodelling process that involves both synthesis and degradation of extracellular matrix proteins. In recent immunohistochemical studies we have found abundant type III pN-collagen staining in the media of aneurysms, which suggested a role of type III collagen in aneurysm formation. The present study gives direct biochemical evidence that the proportion of incompletely processed type III pN-collagen is, indeed, high in AAAs (Table 1). However, the overall rate of type III collagen synthesis did not seem to be increased, since there was no increase in the amount of free propeptide derived from the complete processing of type III procollagen in the soluble extract (Fig. 3). These findings suggest a possibly delayed maturation of type III collagen.

The analysis of the soluble extracts gives information on the rate of turnover of extracellular matrix proteins,
Fig. 5. Antigenicities of the two trypsin-generated forms of PIIINP and an intact PIIINP standard. Serial dilutions (starting from 500 (μg/L) of the trypsin-generated larger (□-□) and smaller (○-○) PIIINP antigens are compared as inhibitors in the radioimmuno inhibition assay for intact PIIINP (●-●). The concentrations of the larger and smaller trypsin-digested peptides giving 50% inhibition are 12.5 μg/l and 375 μg/l, respectively. There is also a small difference between the slopes of the linear parts of the inhibition curves\textsuperscript{5} with intact PIIINP having a slope of −1.070, the larger trypsin-generated PIIINP antigen −0.951 and the smaller trypsin-generated PIIINP antigen −0.800.

since their biosynthetic precursors are normally recovered in this fraction. Although the extract only represents a quantitatively small part of the total amount of collagens in tissue, it is relevant for assessing the amounts of procollagens, partially processed procollagens and non-cross-linked collagens (Fig. 1). We compared AAAs with AODs, since these are two anatomically distinct disease processes that are both associated with atherosclerosis.\textsuperscript{16} This was also appropriate because of the controversy concerning the role of atherosclerosis in the pathogenesis of AAAs, as the aneurysms in this study were also associated with atherosclerosis.

In this study, we further validated our method for estimating the amount of tissue type III pN-collagen, which involves trypsin digestion. The end product of such digestion of PIIINP was found to be the most aminoterminal part of the propeptide, and the size of this digestion product was confirmed by MALDI-TOF analysis. The intermolecular disulphide bridges at the carboxyterminal end of the intact PIIINP had thus been digested away (Fig. 1B), and the immunoreactivity was also significantly reduced (Fig. 5). However, our gel filtration analyses (Fig. 2) confirmed that most of the

PIIINP that was released from insoluble aortic matrix by trypsin was still in the larger form, instead of this small end product. Thus, we could use the same correction factor 2 as in our previous study on ath-

erosclerotic plaques\textsuperscript{14} for calculating the real concentration of type III pN-collagen in the tissue.

Collagen continues to be synthesised throughout the human life span, and the collagen contents of tissues reflect the net effects of synthesis and degradation. Recently we provided evidence for increased type III collagen turnover in AAA by demonstrating increased concentrations of PIIINP in AAA patients' blood.\textsuperscript{18} This suggests that the remodelling of type III collagen is enhanced in AAA, even within the stable aneurysm wall. Our follow-up study, in which the growth of the aneurysm led to an accelerated turnover of type III collagen, also pointed out that aortic tissue has a capacity to react to demands for repair.\textsuperscript{19} This assumption is also supported by the reported positive correlation between the increasing aneurysm size and circumferential collagen content.”
Our present results demonstrated that there is much more type III pN-collagen in AAAs than in the vessel walls of patients with AODs (Fig. 6). As pN-collagen can normally only occur on the surface of collagen fibres, its accumulation in tissue suggests impaired formation of the fibrillar structure and secondary interference with collagen cross-linking. As proper cross-linking, however, is necessary to maintain normal tensile strength of the tissue, this could result in weaker mechanical properties. Thus, incomplete processing of type III collagen, even to a seemingly minor degree, is likely to affect aneurysm stability.

Even though there are clear differences between AAA and AOD samples in their contents of type III pN-collagen (Table 1), the absolute amount of type III pN-collagen is still quite small in both. This leads to a conclusion that collagen turnover in AAAs is a relatively balanced process. In a more advanced state, however, the increased rate of type III collagen metabolism may exceed the rate of proper processing of collagens, impair tensile strength and thus predispose the vessel wall to rupture. In addition, the incompletely processed type III pN-collagen may be more susceptible to proteolytic attack than completely processed collagen. This is supported by the increased solubility of collagens in AAA tissue reported. Increased activities of specific matrix metalloproteinases that can degrade collagens have been reported in aortic tissue and in blood in the patients with AAA.

For technical reasons, our samples from both AAAs and AODs were from the anterior aortic wall. Most aneurysms are known to rupture in their posterior parts, however. For the time being, there is nothing to indicate that the structure or production of type III collagen would be different in the different parts of the aneurysms.

The integrity of the vessel wall depends on collagen rather than elastin. Mutations in the gene encoding type III procollagen cause the Ehlers–Danlos syndrome type IV (EDS IV), where AAAs and spontaneous arterial ruptures are typical features. These mutations can lead to structurally altered type III collagen, e.g. a truncated protein that cannot be incorporated properly into type III collagen fibrils. Unstable mRNA due to frameshift mutations, in turn, leads to decreased production of type III collagen. Furthermore, the phenotype of mice lacking type III collagen resembles the clinical manifestations of the EDS IV. There is evidence that the proportion of type III collagen is lower in some cases of AAAs than in controls. In addition, it has been reported that type III collagen production is decreased in cultured skin fibroblasts from familial AAA patients. Furthermore, unusual fragments of type III collagen have been found after pepsin digestion of atherosclerotic AAAs. In the present study, the proportion of type III pN-collagen in AAA tissue was higher than in corresponding AOD without aneurysmal changes. In principle we cannot exclude a genetic variation in the type III procollagen gene as the reason for this, although our patients had no family history of the disease. However, the synthesis of type III procollagen did not seem to be significantly decreased when analysed quantitatively (Fig. 6).

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