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Change in elastin structure in human aortic connective tissue diseases

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Summary. Biochemical pathogenesis of the aortic connective tissue diseases (such as, Marfan's syndrome, dissecting aneurysm or aortic aneurysm) was examined by estimating glycoprotein, collagen and elastin contents in the aorta and the intramolecular cross-linking component (isodesmosine) and the intermolecular cross-linking components (cystine, histidinoalanine) in comparison with the control samples obtained from subjects with aortic regurgitation. The elastin content in the aorta and isodesmosine content obtained from the extract of the aortic sample found to be decreased. Ratio of cysteine residues (Cys/Cys-Cys) in the elastin fraction in disease increased. Content of histidinoalanine was found to be decreased. It may be suggested that elastin is maintained in its native nature and shape by intra- and inter-molecular cross-linking bridges, and they are readily denatured by various disease conditions. After elastin was solubilized by elastase, immunoreactive elastin content in those aortic diseases was found to be increased in the human connective tissue. Serum elastase and elastase-like activities tend to increase more than those in the control. These findings may suggest that the change in the structure of elastin would make more susceptible to elastase and other proteolytic enzymes. The reasonable hypothesis may be that molecular defect of fibillin or other constitutional structural glycoproteins produce deficient and functionally incompetent elastin associated microfibrils, and the defect of microfibrils cause to insufficient intra- and inter-molecular cross-links in elastin.

Keywords: Amino acids – Elastin – Cross-link – Connective tissue – Marfan's syndrome – Aneurysm

Introduction

Marfan's syndrome and aneurysm were histologically defined as connective tissue desease accompanying aortic media distruption and systemic medial necrosis of aorta. Diseases of the connective tissue can result either from alterations in the quantity of normal connective tissue or from abnormalities affecting

the molecular structure and organization. Previously, biochemical abnormality has been demonstrated in elastin content and its cross-links in those patient's aortae (Abraham et al., 1982; Campa et al., 1987). Recently, fibrillin, one of the structural glycoproteins, was characterized and proved immunologically to be elastin-associated microfibrils in human tissues (Hollister et al., 1990). The ocular and cardiovascular manifestations of Marfan's syndrome are consistent with a defect in the gene coding for a structural constituent of these connective tissues (Lee et al., 1991; Maslen et al., 1991). Mature elastic fibers are composed of two distinct structural components of amorphous elastin, surrounded by microfibriles. It may be suggested that disorder of elastin and elastin-associated microfibriles (containing fibrillin) interaction were one of pathogeneses of connective diseases, Marfan's syndrome and aneurysm. In this study, we have examined the change of aortic connective tissue components and elastin related intra- or inter-molecular cross-links to estimate biochemical pathogenesis of these aortic connective tissue diseases (Marfan's syndrome, dissecting aneurysm or aortic aneurysm).

Material and methods

The abdominal aortae and sera with Marfan's syndrome ($n = 8$), dissecting aneurysm ($n = 6$) and aortic aneurysm ($n = 6$) were obtained from the human adults (Age: 20–50) at surgical operation at Tokyo Womens Medical University. For comparison, aortae were obtained from age-matched control patients with aortic regurgitation ($n = 5$). All samples of them were analyzed for the enzyme activity in serum, and several samples of them were estimated from content of connective tissue or content of cross-linking components, because aortic sample was not sufficient, since it was obtained from small pieces at surgical operation. The intima-media of the aortic samples were separated from the adventitia, washed with 0.9% NaCl and then water, and the aortae were freeze-dried, defatted and dried with a mixture of $CHCl_3$-MeOH (2 : 1) and then with acetone (dry tissue). Each connective tissue was separated as described in the previous report (Seyama et al., 1990), that is, the sample was left in 0.9% NaCl for 24 h. The extract was then decanted and the residue was extracted with 5.1 M guanidium chloride for 24 h. The supernatant solution (A: glycoprotein fr.) was decanted and then the precipitate was suspended in water and autoclaved according to the method of Fisher and Liaurado (1966). The supernatant (B: collagen fr.) and residue (C: elastin fr.) were separated by the autoclaving. Each fraction (A, B and C) were dialyzed against water, then freeze-dried (freeze dry weight of each fraction), and then elastin fr. was purified by extraction with CNBr-HCOOH solution as described by Rasmussen et al. (1975). The purified elastin fr. was neutralized with dil.NH_4OH and then freeze-dried. The elastin content in the aorta was expressed as the amount of (C) fr./tissue dry weight (% dry weight). The amount of purified elastin fr. was also expressed as the amount of purified elastin fr./(C) fr. (% dry weight). The measurement of isodesmosine, which is the specific component in elastin, in the acid hydrolysate of tissue have been analyzed by HPLC as described in the previous report (Seyama et al., 1987). We have applied on enzyme-linked immuno sorbent assay (ELISA) for the quantitative determination of elastin in the purified elastin fr. solubilized by porcine pancreatic elastase (Eisai Co.), by using the method described by Rennard et al. (1980). For ELISA, the antigen and antibody were prepared by the method of Mecham and Lange (1982). Insoluble purified elastin (antigen) was prepared from human aorta by the above described method of purification method. The proportion of the elastin as judged by isodesmosine content was analyzed by HPLC. The human insoluble elastin solubilized by porcine pancratic elastase (an enzyme: substrate mg ratio of 1 : 100), and antiserum was prepared using the above mentioned elastase solubilized insoluble elastin. The antiserum (dilution 1 : 200) can be obtained and maintained rabbits immunized with the above antigen

in the complet Freund's adjuvant injected twice weekly for 6 months. A useful property of thus obtained antiserum to human elastin is the ability to specifically react with elastase solubilized elastin and to cross-react with human α-elastin, and the antiserum showed similar properties to commercially available antihuman α-elastin. For the determination of cysteine and cystine residues in elastin fr., the cysteine residue in purified elastin fr. was derivatized with a fluorogenic reagent, ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) with or without tributylphosphine (reductive disulfide bonds dissociating reagent) according to the method of Sueyoshi et al. (1985). The reaction product was hydrolyzed with 6N HCl for 24 h at 110°C and then the cyteine in hydrolyzate was separated on HPLC as described by Toyooka and Imai (1983), and the ratio (Cys/Cys-Cys) was calculated by results of HPLC. On the other hand, collagen fr. was hydrolyzed to estimate hydroxyproline (Hyp) content as described by Jamall et al. (1981), and the collagen content in the aorta was expressed as μg Hyp/mg freeze-dried weight of collagen fr. and the glycoprotein fr. also hydrolyzed to estimate sialic acid content by acid ninhydrine reaction (Yao et al., 1989). To obtain the glycoprotein content, the sialic acid content (pmol sialic acid) was divided by amount of glycoprotein fr. Another aliquot of dried aortic sample was weighed, and hydrolyzed with 6N HCl for 24 h at 110°C. The histidinoalanine, new cross-links of connective tissue proteins, was analyzed according to the method described by Fujimoto (1982). Elastase [EC: 3.4.21.11] and elastase-like (N-succinyl-Ala_3-p-nitroanilide; Suc-Ala_3 pNA hydrolytic) activities in the serum were measured according to the previous reports (Seyama et al., 1982; Katagiri et al., 1979). For the comparison with other enzymatic activities, β-glucuronidase [EC: 3.2.1.31] and collagen related peptidase (X-prolyl dipeptidyl-aminopeptidase; Gly-pro-pNA hydrolytic) activities were measured according to the previous reports (Inoue et al., 1982 ; Hino et al., 1976).

Results and discussion

In this study, we examined various connective tissue contents in the aortae of patients with Marfan's syndrome, dissecting aneurysm or aortic aneurysm in comparison with the control samples obtained from subject with aortic regurgitation (Table 1). Collagen, glycoprotein and elastin were fractionated by the method of previous report (Seyama et al., 1990), and amount of each connective

Table 1. Comparison of aortic glycoprotein, collagen and elastin contents obtained from different patients

	AR	M	DA	AA
Glycoprotein content (p mol sialic acid/ mg glycoprotein fr.)	34.1 ± 3.62 (5)	39.3 ± 4.26 (5)	30.8 ± 7.07 (3)	24.5 ± 5.00 (3)
Collagen content (μg Hyp/mg collagen fr.)	41.9 ± 2.92 (5)	39.2 ± 3.18 (7)	39.3 ± 7.46 (4)	39.6 ± 3.10 (4)
Elastin content elastin fr./tissue dry weight (% dry weight)	43.2 ± 5.23 (5)	18.4 ± 2.38** (8)	29.6 ± 4.71 (6)	19.9 ± 3.20** (6)
Purified elastin content purified elastin fr./elastin fr. (% dry weight)	32.2 ± 3.13 (2)	8.47 ± 3.12* (3)	24.0 ± 6.06 (4)	27.7 ± 11.0 (4)

AR Aortic regurgitation, *M* Marfan's syndrome, *DA* Dissecting aneurysm, *AA* Aortic aneurysm. $*P < 0.05$, $**P < 0.01$. Significantly from the control (Aortic regurgitation). ( ): Nos. of assay sample. Results are given as mean ± SE.

tissue was estimated by specific component of each fraction. Because, extraction of the composition of each fraction revealed that the glycoprotein fraction contained mostly sialic acid, the collagen fraction and elastin fraction have Hyp and the elastin fraction have only isodesmosine, and the amount of Hyp in the collagen fr. accounted for 80–90% of the sum of the Hyp content of each connective tissue, as described by previous report (Seyama et al., 1990). Collagen content (estimated from Hyp) and glycoprotein content (estimated from sialic acid) are similar to those in the control sample. While, the elastin content in the aortae obtained from those patients decreased in comparison with the control sample. The relative amount of purified elastin fr. from those patients, especially Marfan's syndrome, decreased in comparison with the control sample. We suggest that the reduction of elastin is due either to altered properties of elastin or to negative balance in its turnover, and then the content of intramolecular cross-linking component (isodesmosine) and intermolecular cross-linking components (cystine, histidinoalanine)in the aortae was examined (Table 2). Elastin specific cross-linking component, isodesmosine obtained from the extract of aortic sample greatly decreased in comparison with control. Recently, Indik reported that tropoelastin containes two Cys residues and basic amino acid residues located in the near C-terminus, and tropoelastin may interact, possibly through disulfide bonds, with other acidic matrix protein, such as microfibrill. Ratio of cysteine residues (Cys/Cyc-Cys) in the elastin fr. in these diseases increased more than those in the control. The content of histidinoalanine, formed between acid structure protein residues and elastin or collagen, was found to be decreased in comparison with control. Such abnormally intra- or inter- molecular cross-linked elastic fibers can also be expected to be solubilized more easily by the extraction. These alterations can make the elastin fibers more susceptible to elastolytic processes, e.q., to proteases of the circulating blood or to endogeneous aortic elastase. These metabolic change is similar to experimental lathyrism (treatment with inhibitor of cross-linking required enzyme), and the possibility was supported by previous reports (Dubick et al., 1988) in case of

Table 2. Contents of inter- and intra-molecular cross-linking components and immunoreactive elastin obtained from different patients

	AR	M	DA	AA
Isodesmosine content (nmol isodesmosine/mg elastin fr.)	13.8 ± 3.43 (4)	3.49 ± 1.68* (5)	3.83 ± 1.66* (6)	3.38 ± 1.22* (6)
Ratio of Cys/Cys-Cys in the elastin fr. (%)	8.82 (2)	19.8 (2)	22.7 (2)	12.7 (2)
Histidinoalanine content (nmol histidinoalanine/mg dry tissue)	14.6 ± 4.27 (3)	3.17 ± 1.24 (3)	3.73 ± 0.602 (3)	4.85 ± 2.18 (3)
Immuno-reactive elastin content (μg elastin/mg elastin fr.)	265 ± 30.4 (4)	572 ± 111 (6)	354 ± 57.4 (3)	629 ± 156 (3)

Refer to Table 1 for various abbreviations, etc.

Table 3. Comparison of various serum enzymatic activities obtained from different patients

	AR (5)	M (8)	DA (6)	AA (6)
Elastase activity	0.0465 ± 0.0165	0.0689 ± 0.0245	0.179 ± 0.0807*	0.0770 ± 0.362
Elastase-like activity	0.115 ± 0.0389	0.240 ± 0.0683	0.306 ± 0.0765*	0.195 ± 0.0778
β-glucuronidase activity	26.4 ± 8.48	13.3 ± 2.37	25.8 ± 6.50	19.6 ± 3.68
X-prolyl dipeptidyl-amino peptidase activity	43.3 ± 2.42	46.8 ± 4.67	42.0 ± 5.23	50.8 ± 2.33

Elastase activity (substrate; elastin, unit; Leu μmol/2 h/0.1 ml serum), Elastase-like activity (substrate; Suc-Ala_3 pNA, unit; pNA μmol/min/l serum), β-glucuronidase activity (substrate; 4-methylumbelliferyl-sulfate, unit; 4-methylumbelliferone nmol/h/l serum), x-prolyl dipeptidyl-aminopeptidase activity (substrate; Gly-Pro pNA, unit; pNA μmol/min/l serum).
Refer to Table 1 for various abbreviations, etc.

aortic aneurysm. These findings may suggest that elastin lost its specific elastomer due to its hydrophobic nature and to a series of lysine-derived or cysteine-derived covalent cross-links. After elastin was solubilized by elastase, immuno-reactive elastin content in those aortic diseases were found to be increased in comparison with the control sample as demonstrated by ELISA (Table 2). The data would suggest that the denatured elastin due to various disease conditions caused to increase solubilization by elastase treatment, and consequently caused to increase immuno-reactivity, and in addition to serum elastase and elastase-like activity tend to increase especially in dessecting aneurysm more than that in control (Table 3). These findings may suggest that the change in the structure of elastin fiber due to various disease conditions would make more susceptible to elastase and other proteolytic enzymes. These findings well correlate with the histopathogenic changes observed in the aorta, demonstrating fragmentation and loss of elastic fibers in disease states. A reasonable hypothesis may be that molecular defect of fibillin or other constitutional stractural glycoproteins produce deficient and functionally incompetent elastin associated microfibrils, and the defect of microfibrils cause to insufficient intra- and inter-molecular cross-links in elastin, these structual changes make more susceptible to elastase and other proteolytic enzymes.

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