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Alteration of Cross-Linking Amino Acids of Elastin in Human Aorta in Association with Dissecting Aneurysm: Analysis Using High Performance Liquid Chromatography

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WATANABE, M. and SAWAI, T. *Alteration of Cross-Linking Amino Acids of Elastin in Human Aorta in Association with Dissecting Aneurysm: Analysis Using High Performance Liquid Chromatography.* Tohoku J. Exp. Med., 1999, **187**(4), 291-303 —— Elastic fiber is one of the major component of the extracellular matrix, which provides the resilience to many tissues. Elasticity is an important property of human aorta, and this elastic property decreases in various pathological conditions such as dissecting aneurysm (DA). Since the cross-linking structures in elastin are responsible for this elasticity, we studied the alteration of various cross-linking amino acids in human aorta associated with DA by a new method using high-performance liquid chromatography (HPLC). Materials were obtained from non-atherosclerotic areas of thoracic aorta of 27 autopsy cases which had no particular aortic disease and 19 cases of DA at replacement operation. After acid hydrolysis, SEP-PAK™ silica-gel column and Fe^{3+}/activated charcoal column pretreatment were carried out for analysis of desmosine (DES), isodesmosine (ISDES), neodesmosine (NEO), oxodesmosine (OXO) and isooxodesmosine (ISOXO), and for analysis of aldosine (ALD), respectively. These prepared samples were applied to the reversed-phase HPLC column. We also analyzed pyridinoline (PYR), a major cross-linking amino acid of collagen as an index of fibrosis. All crosslinks of elastin were decreased in DA as compared to the age-matched control. The decrease of ISOXO was marked. The increase of PYR and PYR/(DES+ISDES) were not statistically significant. It is suggested oxidative degradation on elastin crosslinks occur in DA, and the ratio of collagen to elastin didn't contribute to the pathogenesis of DA. ———— cross-linking amino acids; elastin; high-performance liquid chromatography (HPLC); dissecting aneurysm © 1999 Tohoku University Medical Press

Elastic recoil is a critical property of human aorta in the maintenance of blood pressure and in the continuous perfusion of the tissue (Paz et al. 1976; Rosenbloom et al. 1993). This elastic property is provided by elastin, which is

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the major component of the elastic fiber in the extracellular matrix. Elastmeric force results from entropy-driven mechanism like a true rubber (Rosenbloom et al. 1993). Elastin exists predominately in kinetically free largely random coil network. Upon stretching, displacement from this position of the highest entropy occurs, and thus provides the restoring elastic force (Rosenbloom et al. 1993). This characteristic rubber-like property of elastin is produced by extensive intra- and inter-molecular crosslinks (Eyre et al. 1984; Reiser et al. 1992; Rosenbloom et al. 1993). After secreted from the cells, such as fibroblasts and smooth muscle cells, as a soluble precursor protein tropoelastin, the tropoelastin molecules are highly cross-linked to become a hydrophobic insoluble elastin in the extracellular matrix (Eyre et al. 1984; Reiser et al. 1992; Rosenbloom et al. 1993). Desmosine (DES) and isodesmosine (ISDES) (Thomas et al. 1963) are two major cross-linking amino acids, and recently several new cross-linking amino acids, which are neodesmosine (NEO) (Nagai 1983), allodesmosine (Suyama and Nakamura 1990), oxodesmosine (OXO) (Nakamura and Suyama 1992; Suyama and Nakamura 1992b), and isooxodesmosine (ISOXO) (Nakamura and Suyama 1992; Suyama and Nakamura 1992b), have been isolated from the hydrolysate of elastin. Furthermore we have isolated a novel amino acid named aldosine(ALD), which is derived from aldol-crosslink (Suyama and Nakamura 1992c; Nakamura and Suyama 1993, 1994, 1996). The structure of these cross-linking amino acids is

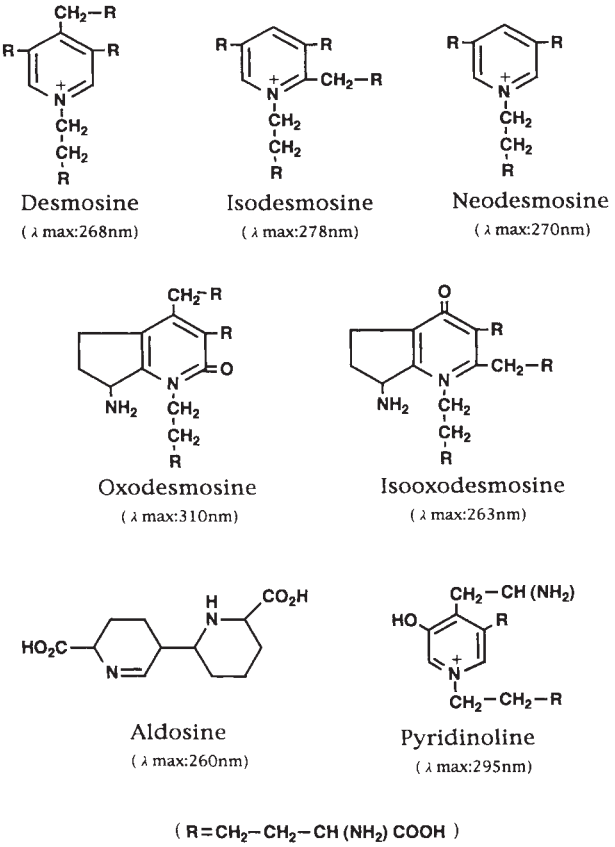

Fig. 1. Structure of cross-linking amino acids isolated from polymeric collagen and insoluble elastin (PCIE).

represented in Fig. 1.

It has been reported that many diseases were associated with abnormal elastin, such as cutis laxa, pseudoxanthoma elasticum, senile elastosis, emphysema, and Marfan syndrome (Fukuda 1992). Dissecting aneurysm (DA) of aorta, which is one of the serious complication of Marfan syndrome, is characterized by the split of aortic wall, usually at the portion between the middle and outer thirds of the tunica media, forming blood filled channel within the aortic media (Roberts 1981). As for Marfan syndrome, many studies have suggested collagen aberrations (Laitinen et al. 1968; Priest et al. 1973; Boucek et al. 1981) and elastin abnormalities (Abraham et al. 1982; Halme et al. 1982, 1985; Perejda et al. 1985; Parsons et al. 1992), but recently mutations in fibrillin-1, the major constituent of microfibrils, have been reported. However the pathogenesis of non-Marfan DA has not been elucidated. It is conceivable that weakening of the media is causative factor of DA, but specific histological lesions cannot be detected in most cases of aortic dissection (Wilson and Hutchins 1982; Nakashima et al. 1990b; Cattell et al. 1994). It had been believed that cystic medial necrosis and laminar medial necrosis are the characteristic lesions, but recent histopathologic studies have shown that cystic medial necrosis is also found in normal aging aorta (Schlatmann and Becker 1977; Wilson and Hutchins 1982; Larson and Edwards 1984; Nakashima et al. 1990a) and laminar medial necrosis can be caused by ischemic change associated with aortic dissection itself (Wilson and Hutchins 1982; Kita et al. 1990; Reiser et al. 1992). Kita et al. (1990) reported that the aortic media in dissecting aneurysm showed a higher grade of elastin fragmentation and less fibrosis, so the association between elastin damage and aortic dissection is suggested. Nakashima et al. (1990b) studied the three-dimensional architecture of the elastic laminae in the media by a scanning electron microscope, and detected the irregular arrangement and decreased number of interlaminar fibers. The same scanning electronmicroscopical findings were also detected in the β-aminopropionitrile fumarate (BAPN) treated lathyritic rat, which is the most successful model of dissecting aneurysm (Nakashima and Sueishi 1992). Since BAPN is biochemically reported to be an inhibitor of lysyl oxidase (Tang et al. 1983) which is a key enzyme for the crosslinking formation in elastin, it is suggested that the disturbance of elastin crosslink is associated with aortic dissection.

Previously we have reported age-related changes of elastin crosslinks and suggested the association with aging of aorta (Watanabe et al. 1996). Here we described the changes of elastin crosslinking of human aorta in association with the aortic dissection using a high-performance liquid chromatographic (HPLC) method (Nakamura and Suyama 1991a; Suyama and Nakamura 1992a; Watanabe et al. 1996).

Materials and Methods

Dissecting aneurysm samples

Aortic samples of 19 cases of DA were obtained at replacement operation. All of them were thoracic aortic dissection cases with hypertensive histories. Age range of DA was between 41 and 73 year old (Mean±SEM: 59.0±2.2, male : female=16 : 3). None of DA patients were suspected as Marfan syndrome. Aortic tissues were preserved in methanol at −20°C until analysis.

Control samples

Aortic samples for the control against dissecting aneurysm were 17 age-matched cases picked from autopsy cases which had no particular aortic diseases but had sclerotic changes corresponding with their ages. We omitted the severe atherosclerotic cases. The range of age was from 38 to 75 year old (Mean±SEM: 58.4±2.7). The control materials were taken from the non-atherosclerotic areas of thoracic aorta, at the beginning portion of descending aorta just below the aortic arch, and were preserved in methanol at −20°C until analysis.

Instrumentation

The solvents used for HPLC analysis were of HPLC grade, all purchased from Nacalai Tesque (Kyoto). Activated charcoal (660–150 mesh) for column chromatography was obtained from Nacalai Tesque (Kyoto), and a SEP-PAK™ silica gel column for preliminary purification of crosslinking amino acids was obtained from Waters Associates (Milford, MA, USA). The standard sample for every cross-linking amino acids of elastin analyzed are prepared from the bovine ligaments (Stoskel 1987; Nakamura and Suyama 1991b, 1992, 1993, 1994; Suyama and Nakamura 1990, 1992b). Pyridinoline (PYR) is prepared from bone collagen as described previously (Fujimoto et al. 1977, 1978)

Sample preparation

The method of HPLC analysis for elastin crosslinking amino acids was previously reported (Watanabe et al. 1996). Briefly, the aortic samples was cut into small segments, then washed twice with 1 M NaCl for 24 hour and delipidated with chloroform/methanol (2 : 1, v/v) for more than 24 hour. These delipidated samples were composed of polymeric collagen and insoluble elastin (PCIE). After being dried over phosphorus pentaoxide in vacuo, approximately 20 mg of dried material (PCIE) was precisely weighed and then hydrolyzed under nitrogen reflux with 6 N HCl for 48 hour at 110°C. At the hydrolysis, 3% phenol was added in the sample of the SEP-PAK™ treatment (Nakamura et al. 1999, personal communication). Following evaporation of the acid at 50°C under reduced pressure, the residual syrup was reconstituted in 1 ml of mobile phase solvent (ethyl acetate/acetic acid/water, 2 : 1 : 1, v/v/v) for SEP-PAK™ pretreatment,

and in 500 μl of distilled water for Fe^{3+}/activated charcoal column pretreatment. These two preliminary treatments, SEP-PAK™ silica gel column for the partial purification of cross-linking amino acids and Fe^{3+}/activated charcoal column for detection of ALD (Suyama and Nakamura 1992c; Nakamura and Suyama 1993, 1994, 1996), were described in our previous report (Watanabe et al. 1996). The fractions obtained after these treatment were evaporated to a syrup at 50°C under reduced pressure, the syrup was diluted with 500 μl of distilled water, then 20 μl portion of the solution was injected onto the HPLC column.

Chromatography

HPLC was performed with a reversed-phase LiChrospher RP-18 column (4 × 125 mm; Merck, Darmstadt, Germany). The eluent was 0.1 M phosphate buffer-acetonitrile (5 : 1, v/v) containing 20 mM sodium dodecyl sulfate (SDS) at pH 3.95. The flow rate was 1.0 ml/min. The HPLC system consisted of an L-600 pump, an L-4000 UV spectrophotometric detector, and a D-2500 chromato-integrator (Hitachi, Tokyo). The absorbance was monitored at 265 nm for general detection. For their quantitation, cross-linking amino acids of elastin and PYR were detected at the following absorption wavelength: DES, NEO, and ISOXO at 265 nm, ISDES at 278 nm, OXO and PYR at 310 nm. ALD was detected at 260 nm as a peak of 6-(3-pyridyl) piperidine-2-carboxylic acid (PPCA), which was formed from ALD by oxidative decarboxylation.

Histological study

Histological examination on 11 cases out of 19 DA could be performed. After fixed by formalin and embedded in paraffin, tissue slices in 4 μm thickness were stained with hematoxylin-eosin and Elastica-Masson staining.

Statistical analysis

Data of each crosslinking amino acid of DA group and the age-matched control group were compared using the Mann-Whitney's test.

Results

Alteration of crosslinks in DA

Fig. 2 shows the HPLC of human aortic hydrolysate that was partially purified by SEP-PAK™ pretreatment in dissecting aneurysm (b) and age-matched control (a). The peaks of all crosslinking amino acids of elastin decreased in compared with control, although the pattern of peaks was similar and abnormal peaks of crosslink were not detected. Fig. 3 shows the HPLC after Fe^{3+}/activated charcoal column pretreatment, and the peak of 6-(3-pyridyl) piperidine-2-carboxylic acid (PPCA), which is synthesized from ALD by oxidative decarboxylation using Fe^{3+} on activated charcoal, appeared before ISDES peak. The peak of PPCA as compared with DES and/or ISDES peak in DA (b) was more

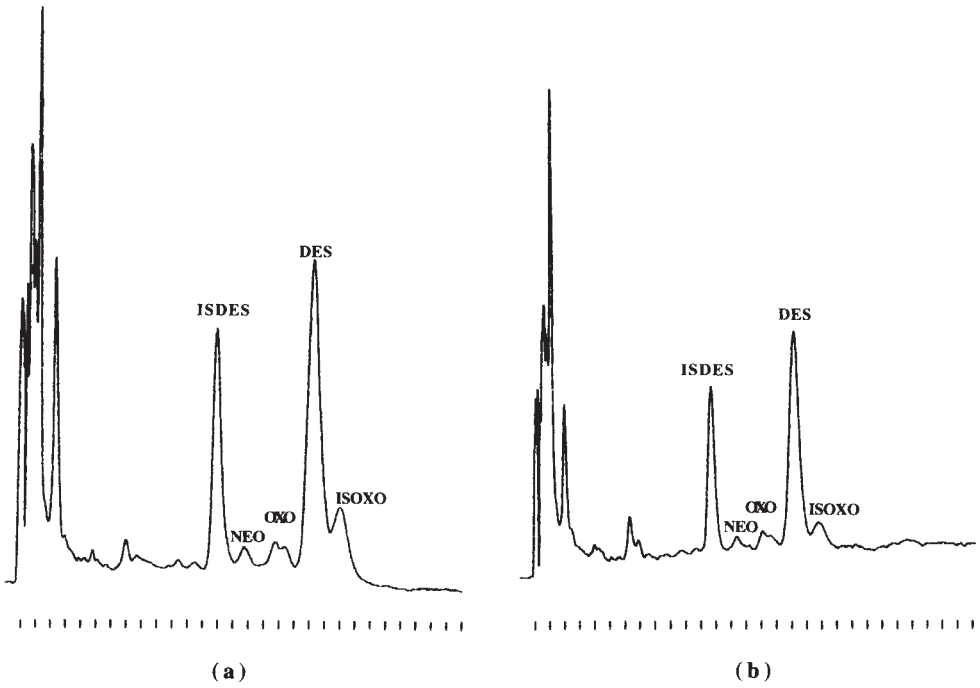

Fig. 2. HPLC of dissecting aneurysm (b) and control aorta (a) after SEP-PAK™ silica gel column pretreatment ($\lambda = 265$ nm). All peaks of crosslinks, especially that of isooxodesmosine, decrease in DA.
The symbols indicating the peak of cross-linking amino acids.
(ISDES, Isodesmosine; NEO, Neodesmosine; OXO, Oxodesmosine; DES, Desmosine; ISOXO, Isooxodesmosine)

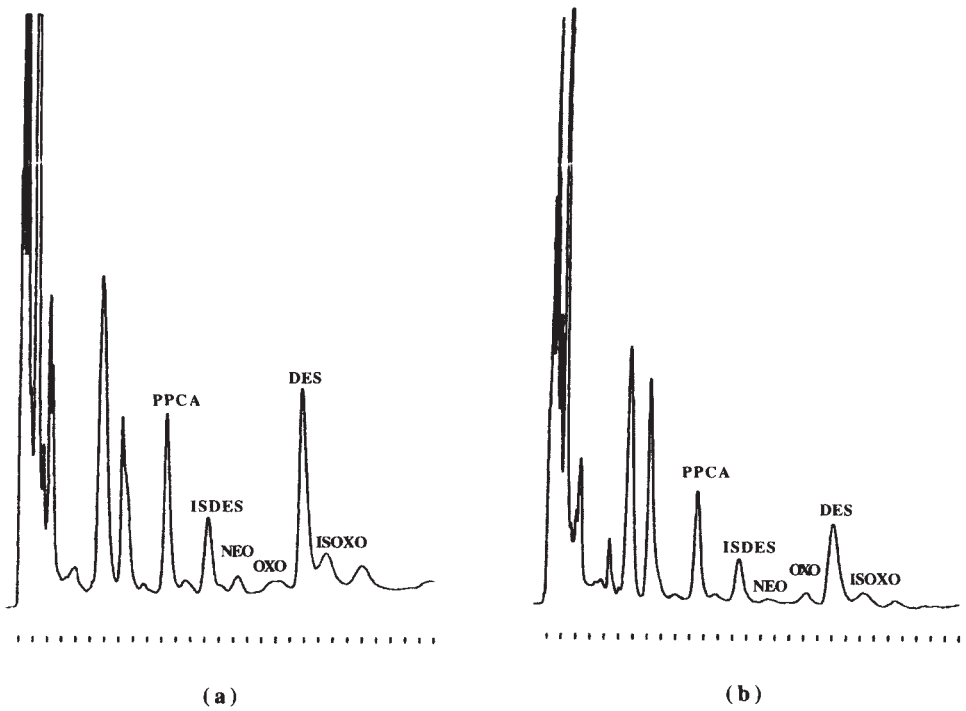

Fig. 3. HPLC of dissecting aneurysm (b) and control aorta (a) after activated charcoal column treatment ($\lambda = 260$ nm).
The symbols indicating the peak of cross-linking amino acids.
(ISDES, Isodesmosine; NEO, Neodesmosine; OXO, Oxodesmosine; DES, Desmosine; ISOXO, Isooxodesmosine; PPCA, 6-(3-pyridyl) piperidine-2-carboxylic acid)

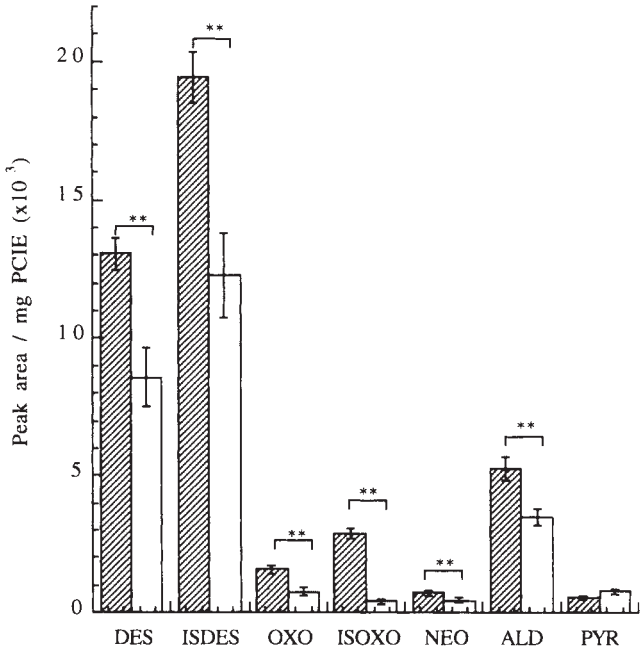

Fig. 4. The changes of cross-linking amino acids between DA and control.
The histograms represent mean (columns) ± S.E.M. (lines). Significant difference from control was shown by $^{**}p<0.01$. ▨, Control; □, DA.
All of the elastin crosslinks significantly decrease in dissecting aneurysm.

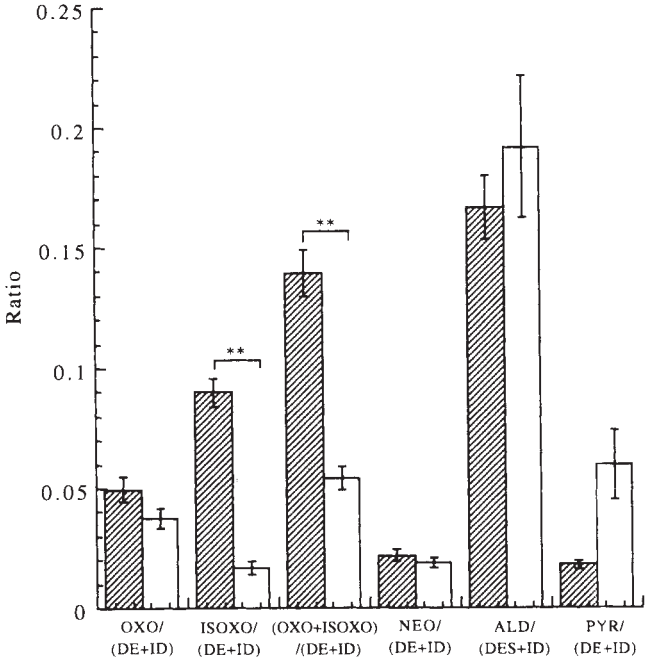

Fig. 5. The changes of crosslinks between DA and control represented by the ratio to (desmosine + isodesmosine).
The histograms represent mean (columns) ± S.E.M. (lines). Significant difference from control was shown by $^{**}p<0.01$. ▨, Control; □, DA.

increased than control (a).

The mean and standard error of mean (S.E.M.) of each crosslink was shown in Fig. 4. All crosslinks of elastin decreased in DA, which were statistically significant. The decrease of ISOXO was most remarkable. The amount of PYR

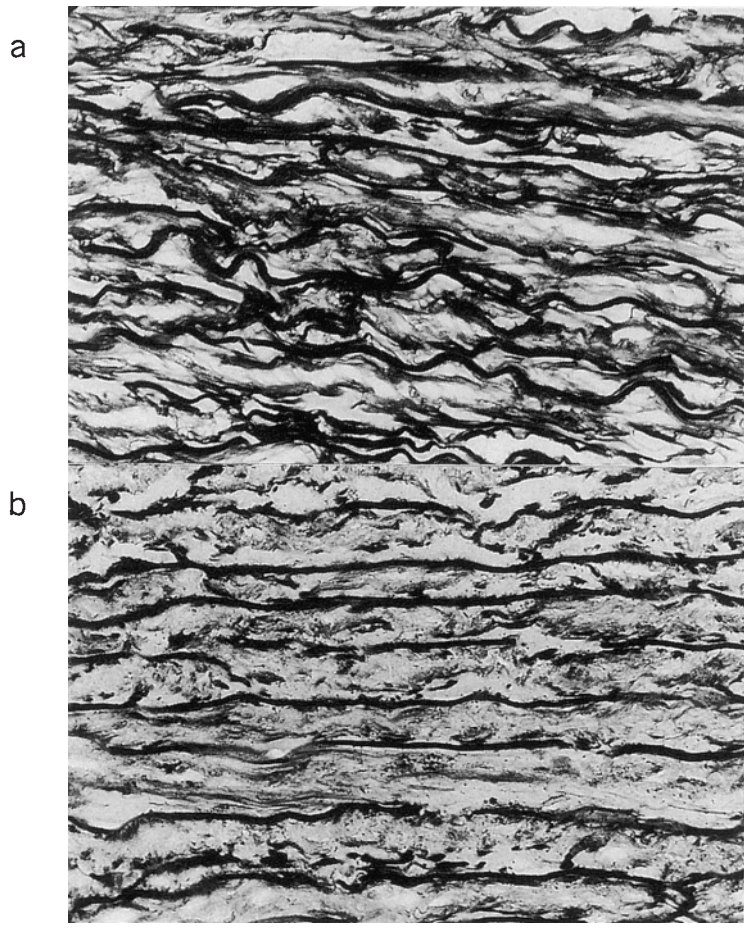

Fig. 6. Histological findings of human aortic media of DA (b) and control (a). (×1600, Elastica-Masson stain)

was not significantly different between DA and control. The ratio of elastin crosslinks to (DES+ISDES) showed in Fig. 5. ISOXO/(DES+ISDES) and (OXO+ISOXO)/(DES+ISDES) decreased significantly in DA, and OXO/(DES+ISDES) was also low, although it was not statistically significant. NEO/(DES+ISDES) was not different between control and DA. ALD/(DES+ISDES) and PYR/(DES+ISDES) revealed to increase in DA, however both were not statistically significant.

Histological findings

Fig. 6 shows the aortic media of DA (b) and control (a). In DA, there were rarefaction and fragmentation of elastic fibers, and each elastic fiber became thinner and shorter than that of control. Cystic medial necrosis was detected in two cases (18.2%), and laminar medial necrosis was observed four cases (36.4%).

Discussion

Elastin is a highly hydrophobic, insoluble protein with rubber-like characteristic, and these properties are associated with crosslinks between elastin peptides. The principal step of this cross-linking process is as follows; first the lysine

residues of tropoelastin react with lysyl oxidase to form α-amino adipic acid δ-semialdehyde (allysine), a reactive aldehyde. Then allysine molecules react with each other to yield aldol condensation product, aldol cross-link, which is one of the first step to form more stable polyfunctional crosslinks (Rucker and Tom 1976; Eyre et al. 1984; Reiser et al. 1992; Rosenbloom et al. 1993). Elastin is a long-lived protein, which has been consider to be last throughout life once a mature elastic fiber is produced (Tinker et al. 1990; Rosenbloom et al. 1993). During an average lifetime, the elastic fibers in human aorta undergo more than a billion stretch/relaxation cycles (Rosenbloom et al. 1993), therefore, it is conceivable that elastin crosslinks suffer some physiological and pathological influences through life.

In DA, all elastin crosslinks were decreased as compared with age-matched control. As mentioned above, we used polymeric collagen and insoluble elastin (PCIE) in our study and didn't strictly purify elastin, therefore these decrease might be due to the decrease of elastin itself. Cattell et al. (1993) reported the DES and ISDES crosslinks per elastin were not changed in dissected tissue relative to control. The decreases of crosslinks in our study might be a reflection of decreased elastin concentration in aortic tissues of DA.

As mentioned in the previous report (Watanabe et al. 1996), we didnot take the standard method of elastin purification, a hot diluted alkaline treatment (Lansing et al. 1952), because of the influence to the aldol-crosslinks and oxopyridine crosslinks (Nakamura and Suyama 1994). By our current method the absolute contents per elastin couldnot be mentioned. Both DES and ISDES contents in tissues reflect the content of elastin (Starcher 1977; Yamaguchi et al. 1987), we substitute the ratio to DES+ISDES for the ratio to elastin. ISOXO/(DES+ISDES) and (OXO+ISOXO)/(DES+ISDES) significantly decreased in DA, and OXO/(DES+ISDES) also showed slight decrease, although this was not statistically significant. OXO and ISOXO are unique substance that have dihydrooxopyridine skeleton (Fig. 2), which are suggestive of the intermediate substances during the oxidative degradation of DES and ISDES, and they are unstable amino acids which are prone to be easily degraded. The decrease of these crosslinks might indicate that the oxidative degradation of elastin crosslink progress in DA, although the degradation pathway of crosslinks is not still elucidated. NEO/(DES+ISDES) was not different between DA and control, which means the decrease of NEO in DA was associated with elastin decline itself. NEO (Nagai 1983) is supposed to be synthesized by dealkilation of ISDES, however it is possible that NEO is a formaldehyde-associated abnormal crosslink which is generated by the conjugation of formaldehyde with two allysine molecules and one lysine residue. In our previous report about the age-related change, the ratio of NEO/(DES+ISDES) tended to increase gradually with ageing (Watanabe et al. 1996). This relative increase of the trifunctional crosslink against tetrafunctional crosslinks might be associated with conformational

changes and weakening of elastin polypeptide in aged aorta. In our result, this abnormal crosslink might not be associated with the pathogenesis of DA.

ALD/(DES+ISDES) and PYR/(DES+ISDES) increased in DA, however, these differences were not statistically significant. This might be due to a wide variance of these two values in DA. The increase of ALD/(DES+ISDES) means relative increase of aldol crosslink, which is an intermediate substance in the formation of stable quaternary pyridinium crosslinks and can be a reflection of the turnover of the crosslinks. Thus, the accelerated metabolism of elastin crosslink is suggested in DA. This may be a reaction against the elastin degradation. The large variation of this value may reflect the differences of metabolism among the cases.

Since the stable pyridinium crosslinks decreased in DA, the crosslink regeneration after its degradation might be incomplete and be easily destroyed. The increase of PYR/(DES+ISDES) is mainly due to the decrease of elastin, because the amount of PYR was not so different between DA and control. PYR is a collagen-specific crosslink with trifunctional 3-hydroxy-pyridinium ring, which is known to be a predominant crosslinking residue in fibrous collagen of most mature connective tissues except skin (Eyre et al. 1984; Whittle et al. 1987). It is suggested that the degree of collagen crosslinks was not associated with the pathogenesis of aortic dissection. The same observation as to PYR crosslink in DA was also reported by Whittle et al. (1987).

In conclusion, the decrease of all elastin crosslinks in DA indicates some damages may occur on elastin, and it may be associated with aortic dissection. The remarkable decrease of oxopyridinium cross-linking amino acids suggests the progress of oxidative degradation of crosslinks in DA. The relative increase of ALD indicates that accelerated metabolism of crosslinks takes place in DA as a reaction against the degradation. However, its regeneration may be incomplete and be easily destroyed, following the decrease of crosslinks and resulting in the solubilization of elastin.

Acknowledgments

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