Age-Related Alteration of Cross-Linking Amino Acids of Elastin in Human Aorta

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¹Department of Pathology, Tohoku University Hospital, ²Department of Pathology, Tohoku University School of Medicine, Sendai 980-77, and ³Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Sendai 981

Watanabe, M., Sawai, T., Nagura, H. and Suyama, K. Age-Related Alteration of Cross-Linking Amino Acids of Elastin in Human Aorta. Tohoku J. Exp. Med., 1996, 180 (2), 115-130 —— It is well known that the elastic property of human aorta decreases gradually with age. Since the cross-linking structures are responsible for this elasticity, age-related changes of cross-linking amino acids in human aorta were studied using a high-performance liquid chromatography (HPLC). Non-atherosclerotic areas of thoracic aorta of 27 autopsy cases which had no particular aortic disease were obtained. After acid hydrolysis, SEPPAK™ silica-gel column and Fe³⁺/activated charcoal column pretreatment were carried out for analysis of desmosine (DES), isodesmosine (ISDES), neodesmosine (NEO), oxodesmosine (OXO) and iso-oxodesmosine (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 cross-linking amino acids of elastin rapidly increased in infancy and then gradually decreased with age. In the middle- and old-age, the amount of OXO showed marked variety. PYR was little detected at 0 year-old, and then gradually increased with age. The crosslinks of elastin were rapidly formed in childhood and then decreased with age. These findings suggest that the relative increase of NEO, OXO or ISOXO to DES and ISDES is associated with age-related weakening and/or damage of elastin, and that the gradual shift from elastin- to collagen-dominant state is a possible cause of the loss of elasticity and the gain of stiffness in the aging aorta. ———— cross-linking amino acids; elastin; high-performance liquid chromatography (HPLC); aging

Elastic fiber is mainly composed of elastin, a highly hydrophobic insoluble protein, which provides the characteristic rubber-like properties produced by extensive intra- and inter-molecular crosslinks (Eyre et al. 1984; Reiser et al. 1992; Rosenbloom et al. 1993). Elastin is secreted from the cells as a soluble precursor protein, tropoelastin. Then the tropoelastin molecules are highly cross-linked into a rubber-like network in the extracellular matrix (Eyre et al. 1984; Reiser et
The principal step in elastin biosynthesis is well characterized; 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 steps to form more stable polyfunctional crosslinks (Eyre et al. 1984; Reiser et al. 1992; Rosenbloom et al. 1993). Recently several new cross-linking amino acids have been isolated from the hydrolysate of elastin, which are desmosine (DES) and isodesmosine (ISDES) (Thomas et al. 1963), neodesmosine (NEO) (Nagai 1983), allodesmosine (Suyama and Nakamura 1990), oxodesmosine (OXO) (Nakamura and Suyama 1992; Suyama and Nakamura 1992a), isooxodesmosine (ISOXO) (Nakamura and Suyama 1992; Suyama and Nakamura 1992a). Furthermore we have isolated a novel amino acid named aldosine (ALD), which is derived from aldol-crosslink (Suyama and Nakamura 1992b; Nakamura and Suyama 1993, 1994, 1996). The structure of these cross-linking amino acids is represented in Fig. 1.

It is well known that the elastic property of human aorta decreases gradually with age (Fujimoto 1982). This could be caused by a decrease of elastin content.
or the structural change of elastin itself. Elastin concentration of the aortic wall has been reported to decrease with increasing age, while other report demonstrated constant concentration (Halme et al. 1985). Since the cross-linking structures are responsible for the elastic property and insolubility of elastin, the changes of cross-linking amino acids in aging process are especially interesting. There are some reports about the concentration of DES and ISDES, which have shown the lower contents in senile subjects than young persons (John and Thomas 1972; Fujimoto 1982; Halme et al. 1982, 1985). Since both DES and ISDES contents in tissues reflect the content of elastin (Starcher 1977; Yamaguchi et al. 1987), these results might indicate not the change of crosslinks but that of elastin itself.

In the present study, we applied the high-performance liquid chromatographic (HPLC) method for the simultaneous analysis of elastin crosslinks, including O XO, ISOXO, NEO, and ALD, in addition to DES and ISDES (Nakamura and Suyama 1991a; Suyama and Nakamura 1992c), and report age-related changes of these elastin crosslinks.

**Materials and Methods**

Aortic samples were obtained from 27 autopsy cases which had no particular aortic diseases but had sclerotic changes corresponding with their ages. The range of age was from 0 to 85 year-old (mean ± s.e.: 45.3 ± 4.67). The materials were taken from the non-atherosclerotic areas of thoracic aorta, and were preserved in methanol at −20°C until analysis.

The standard sample for every cross-linking amino acid of elastin analysed were prepared from the bovine ligaments (Suyama and Nakamura 1990, 1992a,b; Nakamura and Suyama 1991b, 1992, 1993, 1994). Pyridinoline (PYR) was prepared from bone collagen as described previously (Fujimoto et al. 1977, 1978).

All chemicals and solvents were of HPLC grade purchased from Nacalai Tesque (Kyoto). Phosphate buffer (0.1M) solutions were prepared by using 0.1M sodium dihydrogenphosphate dihydrate and 0.1M phosphoric acid, adjusted to various pH. A SEP-PAK™ (silica cartridge) for preliminary purification of crosslinks was purchased from Waters Associates (Milford, MA, USA). Activated charcoal (660-150 mesh) for column chromatography was obtained from Nacalai Tesque.

**Sample preparation**

The flow diagram for sample preparation is shown in Fig. 2.

After removing periaortic connective tissue as completely as possible, the aortic tissues were cut into small segments and washed twice with 1M NaCl for 24 hr. After the supernatant was discarded, the insoluble residue was delipidated with chloroform/methanol (2:1, v/v) for more than 24 hr. The delipidated samples, composed of polymeric collagen and insoluble elastin (PCIE), were dried over phosphorus petaoxide in vacuo. Approximately 20 mg of dried material was
Fig. 2. Flow diagram showing the process of preliminary treatment.

PCIE indicates polymeric collagen and insoluble elastin.

precisely weighed and then hydrolyzed under nitrogen reflux with 6 N HCl for 48 hr at 110°C. At the hydrolysis, 3% phenol was added in the sample of the SEP-PAK™ treatment (Nakamura et al. submitted). The acid solution containing hydrolysate was evaporated at 50°C under reduced pressure and the residual syrup thus obtained was dissolved in water.

**Preliminary treatments of hydrolysate**

Before HPLC analysis, two preliminary treatments were carried out on the hydrolysate of samples.

One was SEP-PAK™ silica gel column for the partial purification of cross-linking amino acids. The syrup obtained from aortic hydrolysate was dissolved in 1 ml of mobile phase solvent (ethyl acetate/acetic acid/water, 2:1:1, v/v/v) and charged on the SEP-PAK™ column. The column was eluted with 5 ml of mobile phase, then cross-linking amino acids remained were eluted from the column with 5 ml each of 100% methanol, 50% methanol, distilled water and 2% HCl/methanol successively. The methanol/water fraction was evaporated to a syrup at 50°C, then the syrup was diluted with 500 μl of distilled water. Twenty microliters of the solution was injected onto the HPLC column.

Another was Fe³⁺/activated charcoal mini column for detection of ALD as described in our previous reports (Suyama and Nakamura 1992b; Nakamura and Suyama 1993, 1994, 1996). The mini column was made by a disposable glass Pasteur pipette (146 mm long, Becton Dickinson, Lincoln Park, NJ, USA) which
was plugged with glass wool and packed with charcoal of 50 mm height (10 ml in volume). The charcoal packed column was then treated with 3 ml of 10% iron (III) sulfate aqueous solution, and then washed successively with excess water, methanol and then rewashed with water. The syrup obtained from aortic hydrolysate was dissolved in 500 μl of distilled water and charged on the column. The column was washed with 3 ml water to remove most of neutral amino acids and then cross-linking amino acids were eluted with 3 ml each of 50% aqueous methanol and 100% methanol successively (Suyama and Nakamura 1992b; Nakamura and Suyama 1993, 1994, 1996). The methanol fraction was 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

The HPLC system consisted of an L-600 pump, an L-4000 UV spectrophotometric detector, and a D-2500 chromato-integrator (Hitachi, Tokyo). A reversed-phase LiChrospher RP-18 column (125 × 4 mm; Merck, Darmstadt, Germany) was used for analytical purposes.

An ion-pair reversed phase HPLC system was used for the analysis of the samples. The solvent was 0.1M 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 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

About 3 × 20 mm portions were cut from aortic tissues and fixed by formalin. Embedded in paraffin and sliced in 4 μm thickness, Elastica-Masson staining was performed.

Statistical analysis

Data of crosslinks was grouped in every 20 years, and compared using the Mann-Whitney U-test.

Result

Analytical HPLC

Fig. 3 shows a typical chromatogram of human aortic hydrolysate that was partially purified by SEP-PAK™ pretreatment (a) and Fe³⁺/activated charcoal column pretreatment (b), respectively. In Fig. 3-b, 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.

Age-related changes of cross-linking amino acids

Fig. 4 shows typical chromatograms of human aortic hydrolysate in 2 years-old (a) and 85 years-old (b) with SEP-PAK™ pretreatment detected at 265 nm for general detection. Although the amount of crosslinks is different between the infant and senile subjects, the pattern of each peak is not changed with age, and unusual peaks were not detected in senile subject.

Fig. 5 shows the age-related quantitative changes of each crosslinking amino acid against PCIE in human aorta. The left diagrams show scattergrams and the right diagrams revealed mean (columns) ± s.e. (lines) of each age group represented by the horizontal axis. All crosslinks rapidly increased in infancy and then reduced gradually with age. ALD, DES and ISDES showed continuous decrease in the middle and advanced ages, although the rate of decrease in the adult is more remarkable in ALD than those of DES and ISDES. DES and ISDES rapidly decreased in young ages, and the mean value of 20–39 age group was significantly less than 0–19 age group (p < 0.05 in DES, p < 0.01 in ISDES) (Fig. 5). The
Fig. 4. HPLC of human aorta of various ages after SEP-PAK™ minicolum pretreatment detected at \( \lambda = 265 \) nm. (a) 2-years-old, and (b) 85-years-old. The symbols of each peak are the same as in the legend to Fig. 3.

decrease of ALD from childhood to young adult was slower than that of DES and ISDES. The age-related difference was not statistically significant between 20–39 age group and 0–19 age group, and became significant in 40–59 age group as compared with 0–19 age group \( (p < 0.01) \). The content of OXO after the adolescence varied widely. The differences of OXO among all age groups were not significant (Fig. 5), which would be due to the wide variance of OXO. ISOXO decreased in young age, and the difference of the mean value became significant between 0–19 age group and 40–59 age group \( (p < 0.05) \). NEO also decreased between the age of 0–19 and 20–39, however the difference was not statistically significant \( (p = 0.0931) \). The value of ISOXO and NEO after middle age remained almost unchanged.

The ratio of elastin crosslinks to \((\text{DES} + \text{ISDES})\) is shown in Fig. 6. From the scattergram, the value of \(\text{OXO}/(\text{DES} + \text{ISDES})\) was a wide variance. Comparing the mean value in each two decades’ age group, the ratio had a tendency to increase until the middle age and then slightly decrease, however the differences were not statistically significant. \(\text{ISOXO}/(\text{DES} + \text{ISDES})\) also increased after middle age, although the mean value of 20–39 age group revealed slightly higher value than 40–59 age group. \(\text{OXO} + \text{ISOXO})/(\text{DES} + \text{ISDES})\), which means the total oxidative state of elastin crosslinks, tended to increase slightly with age as a whole. \(\text{NEO}/(\text{DES} + \text{ISDES})\) was slightly high in the infancy, and after adolescence it tended to increase toward senescence.
Fig. 5–1.
Fig. 5-2.

Fig. 5. Age-related changes of crosslinks in human aorta. The left diagrams show scattergrams, and the right diagrams show mean (columns) ± SEM (lines) of each age group represented by the horizontal axis. *p < 0.05, **p < 0.01. PCIE indicates polymeric collagen and insoluble elastin.

PYR was little detected in newborn, and then increased rapidly until adolescence and gradually over the middle and senile periods (Fig. 5). Since PYR is a collagen-specific crosslink while DES and ISDES are elastin-specific crosslinks, it could be speculated that the collagen/elastin ratio is represented by PYR/(DES + ISDES). As shown in Fig. 6, PYR/(DES + ISDES) increased linearly with age.

Histological findings

Fig. 7 shows the microscopical findings of human aorta in newborn (a), 2 years-old (b), 46 years-old (c) and 85 years-old (d). The elastic fibers of newborn were rather thin and arranged sparsely with small amounts of collagen and other extracellular matrix substances. In the aorta of 2 years-old, the elastic fibers were thick and showed a close arrangement. The thickness and length of elastic fibers appeared to become more diverse in the 46 years-old aorta, with decrease of elastic fibers and increase of collagen and other matrix substances. The elastic fibers decreased still more in 85 years-old aorta, and they appeared to show fragmentation. The increase of collagen and matrix substances was marked in the senile aortic wall.

Discussion

It has been known that the age-related change of aorta is the fragmentation and progressive lysis of elastic fibers (Hornebeck et al. 1978). Powell et al. (1992)
Fig. 6. Age-related changes of crosslinks represented by the ratio to (DES + ISDES). The procedure and symbols are the same as in the legend to Fig. 5.
indicated that cross-linked elastin resides during a lifetime and mature cross-linked elastin is not synthesized in the adult aorta. On the other hand, Hornenbeck et al. (1978) showed an exponential increase of elastinolytic activity and a linear decrease of the crosslinked elastin content in aging aorta. From these studies, the elastin of human aorta shifts from the anabolic state to the catabolic state with aging. Several studies described an accumulation of polar amino acids in human elastin with age (Keely and Partridge 1974; Spina and Garbin 1976; Fujimoto 1982; Najjar et al. 1990), although the association between these biochemical changes and age-related weakening of elastin have not been elucidated.

As mentioned above, cross-links between polypeptide chains play an important role for elastic recoil, however we have little information concerning the age-related changes of elastin cross-links. Several reports described DES and ISDES decreased with increasing age (John and Thomas 1972; Spina and Garbin
1976; Fujimoto 1982). Age-related changes of crosslinking amino acids other than these two major amino acids had not been studied, and this is the first report about precise analysis of elastin crosslinks.

Our method of cross-linking analysis using HPLC is useful for the simultaneous detection of several crosslinks by a simple procedure (Suyama and Nakamura 1992c). Until now, there have been some reports of HPLC methods to detect DES and ISDES (Starcher 1977; Stoskel 1987; Yamaguchi et al. 1987). Our procedure can detect not only these two major crosslinks but also other more dynamic crosslinks, such as ALD, OXO, and ISOXO, therefore, we believe this method can express more detailed state of elastin crosslinkage. Although the standard method of elastin purification is a hot diluted alkaline treatment (Lassing et al. 1952), aldol-crosslinks and oxopyridine crosslinks such as OXO and ISOXO are destroyed by this procedure (Nakamura and Suyama 1994). Furthermore ALD is related not only to elastin crosslink but also to collagen and elastin-collagen crosslinks (Nakamura and Suyama 1994), therefore, it would not be suitable to remove collagen completely. Thus, we used a milder method using 1M NaCl to prepare a fraction consisting of both polymeric collagen and insoluble elastin (PCIE).

From our observation, all cross-linking amino acids of elastin was highest in the early childhood, and the crosslinks markedly increased during the infancy (Fig. 5). The same result was also observed in short-lived species such as rat (Nakamura and Suyama 1996). Although our result suggests that the maturation of elastin crosslink takes place after birth and rapidly proceeds in this short period, more study is needed to clarify the changes in the infancy. After adolescence, the contents of crosslinks tend to decrease with age (Fig. 5). These HPLC findings were well consistent with the histological findings (Fig. 7). The elastic fibers of aorta were thick and dense in the young childhood, and they became thin and sparse with increasing of age.

DES and ISDES are two major cross-linking amino acids of elastin, and the contents of these amino acids in aorta of aged subjects were shown to be lower than those of younger subjects (John and Thomas 1972; Spina and Garbin 1976; Fujimoto 1982). Our findings support those previous reports. The rate of decrease has become slower in the middle and senile subjects (more than 40 years-old) (Fig. 5). This reflects the rather stable character of these two cross-links.

As mentioned above, aldol crosslink is the first intermediate step for the formation of stable quaternary pyridinium crosslinks in elastin and collagen, and it can be a reflection of the turnover of the crosslinks. The content of ALD in newborn is low, and it increases rapidly in childhood to reach peak at 2–5 years old, then decreases gradually in the adult life (Fig. 5). The similar result has been found in rat (Nakamura and Suyama 1996). This indicates that the turnover of crosslinks occur most actively in childhood. The decrease of ALD was
slower in the young adult than that of DES and ISDES, and continued gradually in the middle and senile periods. This reflects the gradual decrease of crosslinking turnover in elastin. Interestingly, some amount of ALD is persisting even in the 9th decade. This indicates the presence of lifetime turnover of elastin crosslinks even in those ages. It is possible that elastin is synthesized in aging aorta as an immature form, but not deposited into mature cross-linked elastin (Powell et al. 1992).

OXO and ISOXO are unique substances that have dihydroxyoxypyrindine skeleton. It is possible that these amino acids are formed by oxidation of DES and ISDES as an intermediate for further decomposition of these major pyridinium crosslinks, and that this is related with the degradation process of crosslinks (Suyama and Nakamura 1992a). These are unstable amino acids that are easy to break down. From our data, the contents of these oxypyrindine crosslinks showed highest content in childhood, like DES and ISDES. This indicates increased catabolism of elastin in accord with active metabolism during this period. The content of OXO in the adult life varied widely. This indicates the fact that they are prone to be influenced by not merely aging process but also various physiological and pathological conditions. The oxidation ratio of crosslinks represented by \((OXO + ISOXO) / (DES + ISDES)\) tended to increase with age. This result suggests an age-related increase of oxidative damage to elastin crosslinks.

NEO is also pyridinium cross-linking amino acid whose structure is shown in Fig. 1 (Nagai 1983). NEO is supposed to be synthesized by dealkylation of ISDES, however, it is possible that formaldehyde could be associated with its generation in conjunction with two allysine molecules and one lysine residue. This indicates the possibility of NEO as a formaldehyde-associated abnormal crosslink. The values of NEO after 40 years-old age groups were rather constant. The ratio of NEO / (DES + ISDES) tended to increase gradually after the adolescence (Fig. 6). This suggests the increase of this abnormal crosslink in senile subjects. Since NEO is a trifunctional crosslink in contrast to DES and ISDES, which are tetrafuctional crosslinks, the increase of NEO / (DES + ISDES) may result in conformational changes and weakening of elastin polypeptide.

Tinker et al. (1990) demonstrated that inhibition of elastin crosslinking induces the enhancement of elastin degradation. Partially or abnormally cross-linked elastin can be subject to elastolysis, and decreased crosslinking may alter permeability to increase of serum proteinase infiltration into vessels (Tinker et al. 1990). It is possible that the qualitative change of elastin crosslinks, i.e., relative increase of oxypyrindinium crosslinks and NEO, can promote the elastin degradation.

PYR, a collagen-specific crosslink with trifunctional 3-hydroxy-pyridinium ring, is now 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).
In our study, PYR was little detected in the newborn and then increased with age. The rate of increase is more rapid in the youth than in the adult life. It is possible that the former increase is due to the maturation process of collagen crosslinks and the latter is derived from the progress of aortic fibrosis. The increase of collagen content and PYR content in the aged aorta has been reported in several morphological and biochemical studies (Fujimoto 1982; Andretti et al. 1985). It is considered that fibrous tissue replaces myocytes and elastic fibers which have undergone degenerative change (Andretti et al. 1985; Powell et al. 1992), since the turnover of fibrillar collagen appears to continue throughout life (Powell et al. 1992). Our data show a linear increase of the ratio of PYR/(DES + ISDES) with age. This seems to indicate the gradual shifts from elastin-dominant to collagen-dominant condition of aorta, and thus corresponds to the elastin degradation and collagen replacement. This result of HPLC well reflected in histological findings, which revealed marked decrease of elastic fibers and increase of collagen in the senile aorta. This is one of the possible causes of the loss of elasticity and the gain of stiffness in the aging aorta.

In conclusion, the crosslinks of elastin gradually decreased with age. The changes of stable cross-links, DES and ISDES, were lesser and slower than those of more unstable, dynamic crosslinkks (ALD, OXO, ISOXO). ALD rapidly decreased with increasing age in parallel with the decline of metabolism, however a certain amount remained even in the senile subjects. This suggests that turnover of elastin closslinks lasts till the senescence. Relative increase of oxopyridinium crosslinks and NEO may indicate the degenerative change of crosslinkage, and this may lead to the enhanced degradation of elastin. The linear increase of PYR/(DES+ISDES) is suggested to be associated with elastolysis and subsequent collagen replacement, resulting in the age-related arterial sclerosis.

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References


