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Immunochemical and Immunohistochemical Studies on Distribution of Elastin Fibres in Human Atherosclerotic Lesions using a Polyclonal Antibody to Elastin-derived Hexapeptide Repeat

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A polyclonal antibody to elastin-derived hexapeptide repeat, H–(Val–Gly–Val–Ala–Pro–Gly)$_3$–NH$_2$, was prepared in order to investigate the differences between elastin fibres in intimal hyperplasia and media in human atheroscleroic lesions. The hexapeptide repeat and α-elastin were recognized by this polyclonal antibody in enzyme-linked immunosorbent assay (ELISA), but other elastin-derived peptides such as tetrapeptide repeat, pentapeptide repeat and nonapeptide were not. In the series of hexapeptide repeats, H–(VGVAPG)$_n$–NH$_2$ where *n* is 1–7, the polyclonal antibody reacted strongly with oligomers (*n* = 3–7) and weakly with dimer (*n* = 2), but not with monomer (*n* = 1). CD measurements suggested that the β-turn structure is important for recognition by the polyclonal antibody. In an immunohistochemical study, elastin was stained more strongly in intimal hyperplasia than in media, suggesting that newly synthesized elastin in intimal hyperplasia is morphologically distinct from that in media.

Key words: circular dichroism, elastin, immunochemistry, immunohistochemistry, polyclonal antibody.

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; Fmoc, 9-fluorenylmethoxycarbonyl; ELISA, enzyme-linked immunosorbent assay; FT-IR, Fourier-transform infrared spectroscopy; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; NMR, nuclear magnetic resonance; TFE, trifluoroethanol.

Elastin is the major component of elastic tissues such as arterial walls, lungs and skin. Tropoelastin whose molecular weight is ~70 kDa is secreted as the precursor protein of elastin into extracellular space by such elastin-producing cells as smooth muscle cells, fibroblasts, etc. (*1*). This precursor protein self-assembles and is rapidly cross-linked by lysyl oxidase to form mature elastin (*2*). There are two types of functional regions in tropoelastin: one is potential cross-linking regions that are rich in alanine and lysine and the other is hydrophobic regions that are rich in valine, proline and glycine. The partial amino acid sequence of porcine tropoelastin (*3*) and the complete amino acid sequences of human, bovine, chicken, rat, sheep and mouse tropoelastins (*4–9*) indicate the existence of some repeating sequences, tetrapeptide Gly–Val–Pro–Gly (GVPG), pentapeptide Val–Gly–Val–Pro–Gly (VGVPG), hexapeptide Val–Gly–Val–Ala–Pro–Gly (VGVAPG) and nonapeptide Ala–Gly–Val–Pro–Gly–Leu–Gly–Val–Gly (AGVPGLGVG), in hydrophobic regions of tropoelastin. Among these peptide sequences, the pentapeptide sequence is considered to have an important role in the self-assembly of tropoelastin (*10*) and the hexapeptide sequence is known to show chemotactic activity against a variety of cell types (*11*,*12*).

In the microscopic examination of arterial walls, elastin exists physiologically as thick elastin fibres in media in normal tissues. Although it is reported that elastin is newly synthesized as thin or fine elastin fibrils in intimal hyperplasia in the development of atherosclerosis (*13–15*), the structural features of this elastin have not been elucidated in detail. It was demonstrated that VGVAPG sequence is a primary structural feature of tropoelastin in mammalian species and repeats seven plus times in human tropoelastin (*4*). In this study, a polyclonal antibody to an elastin-derived hexapeptide trimer, H–(VGVAPG)$_3$–NH$_2$, was prepared in order to explore the differences between medial and thickened intimal elastins. The characteristics of this antibody was performed by the use of a series of hexapeptide repeats, H–(VGVAPG)$_n$–NH$_2$ where *n* is 1–7, and other elastin-derived peptides such as α-elastin, tetrapeptide repeat, pentapeptide repeat and nonapeptide. Secondary

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structural analysis by CD measurement of hexapeptide repeats was also performed to elucidate structural features for recognition by this antibody. The immunohistochemical and light microscopical studies were performed to compare the morphological structures of these elastins.

MATERIALS AND METHODS

Peptide Synthesis—The series of hexapeptide repeats, H–$(VGVAPG)_n$–NH_2 where *n* is 1–7, and other elastin-derived peptides, tetrapeptide trimer Gly–$(X–Y–Pro–Gly)_3$–Gly where X is Gly or Ala and Y is Val or Ile, pentapeptide trimer H–$(Val–Pro–Gly–Val–Gly)_3$–NH_2 and nonapeptide AGVPGLGVG were synthesized by the solid-phase method with the 9-fluorenylmethoxycarbonyl (Fmoc) strategy. Fmoc amino acid derivatives and chemical reagents for peptide synthesis were purchased from Watanabe Chemical Industries Ltd (Hiroshima, Japan). All other chemicals used in this study were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Coupling reactions in the synthesis were carried out with 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxybenzotriazole (HOBt). Peptides were liberated from the resin by 95% trifluoroacetic acid. The crude peptides were purified by reversed-phase HPLC.

Preparation of the Polyclonal Antibody to the Hexapeptide Trimer—The hexapeptide trimer, H–$(VGVAPG)_3$–NH_2, was coupled with bovine serum albumin (BSA) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). The BSA-hexapeptide trimer conjugate was dialysed against water to remove the excess EDC and unbound peptide. New Zealand white rabbits (Kyudo Co., Ltd, Saga) were immunized initially with 200 μg of BSA–hexapeptide trimer conjugate solutions emulsified in 50% complete Freund's adjuvant and boosted at 2-week intervals with 200 μg of BSA–hexapeptide trimer conjugate solutions emulsified in incomplete Freund's adjuvant. After the anti-serum was detected by precipitation with the BSA–hexapeptide trimer, the animals were bled. AF-Tresyl TOYOPEARL 650 M gels (Tosoh Co., Tokyo) were coupled to BSA or hexapeptide trimer for preparation of an affinity column. The polyclonal antibody was purified by the following procedure. The anti-serum was precipitated with ammonium sulphate, dialysed against buffer A (0.02 M Tris–HCl, 0.15 M NaCl; pH 8.0), and loaded onto a BSA affinity column equilibrated with buffer A. The flow-through fractions were combined and then applied onto a hexapeptide trimer affinity column equilibrated with buffer A. After continuous washing with buffer A, buffer B (0.02 M Tris–HCl, 0.5 M NaCl; pH 8.0), and buffer A, column-bound antibodies were eluted with 3.0 M NaSCN containing 0.02 M Tris–HCl (pH 8.0). The eluates were pooled and dialysed against 0.02 M phosphate buffer (pH 7.4).

Enzyme-linked Immunosorbent Assay—The ability of the antibody to react with the series of hexapeptide repeats, H–$(VGVAPG)_n$–NH_2 where *n* is 1–7, other elastin-derived peptides and α-elastin was tested by enzyme-linked immunosorbent assay (ELISA) on microtitration polystyrene plates. The α-Elastin was purchased from Sigma-Aldrich, St. Louis, MO, USA. ELISA was performed at 25°C in a non-competitive ELISA system. Wells were incubated with peptides in 50 mM carbonate buffer (pH 9.7) and then blocked with 0.5% gelatin in carbonate buffer. The wells were washed and reacted with antibodies diluted stepwise with 0.1% BSA containing 0.05% Tween 20. After incubating with peroxidase-labelled anti-rabbit IgG antibody, the wells were reacted with *o*-phenylenediamine and determined by measurement of the absorbance at 492 nm.

CD Measurements—CD measurements were performed in a 0.1 mm cuvette using a JASCO J-725KI spectropolarimeter (JASCO Co., Tokyo). The series of hexapeptide repeats, H–$(VGVAPG)_n$–NH_2 where *n* is 1–7, were dissolved in trifluoroethanol (TFE) (1 mg/ml) and measurements were performed at 25°C. Spectra of solvent were obtained in the same manner and were subtracted from the sample spectra. Measurements were performed at a 190–280 nm wavelength.

Morphological Study—Aortic samples were obtained from autopsy cases within 2–6 h of death and stored at –40°C until use. For the immunohistochemical study, frozen sections (4 μm) were fixed with ethanol, blocked with 6% BSA, and reacted with the polyclonal antibody (10 μg/ml). The staining was performed by the labelled streptavidin–biotin method. For microscopic observation, aortic samples were fixed with 10% buffered formalin. After dehydration with ethanol, the tissues were embedded in paraffin. Paraffin sections (2 μm) were deparaffinized and stained with Elastica-van Gieson stain for elastin fibres, and stained with Masson's trichrome stain for collagen fibres (*16*). The frozen sections were stained with Oil red O stain (*17*) to evaluate lipid deposition in atherosclerotic lesions.

RESULTS AND DISCUSSION

Immunoreactivities of Polyclonal Antibody with Elastin Peptides—The polyclonal antibody against the hexapeptide trimer, H–$(VGVAPG)_3$–NH_2, was purified and screened for binding to several elastin-derived peptides by the ELISA method. Results are shown in Fig. 1. The hexapeptide sequence (VGVAPG) was strongly recognized by this polyclonal antibody and α-elastin was weakly recognized by it, but pentapeptide sequence (VPGVG), tetrapeptide sequence (XYPG where X = G or A and Y = V or I) and nonapeptide sequence (AGVPGLGVG) were not recognized. These results suggest that this polyclonal antibody is specific for the hexapeptide sequence in elastin.

In the series of the hexapeptide repeats, H–$(VGVAPG)_n$–NH_2 where *n* is 1–7, the polyclonal antibody reacted strongly with oligomers ($n = 3–7$) and weakly with dimer ($n = 2$), whereas it did not react with monomer ($n = 1$) (Fig. 2). The specificity of the antibody for hexapeptide repeats was also confirmed by competitive ELISA using each of hexapeptide repeats as competing inhibitors (data not shown). The results showed that only oligomers ($n = 2–7$) reacted with the polyclonal antibody similarly to the specificity with a non-competitive ELISA shown in Fig. 2, suggesting that the molecular size that is greater than two repetition of

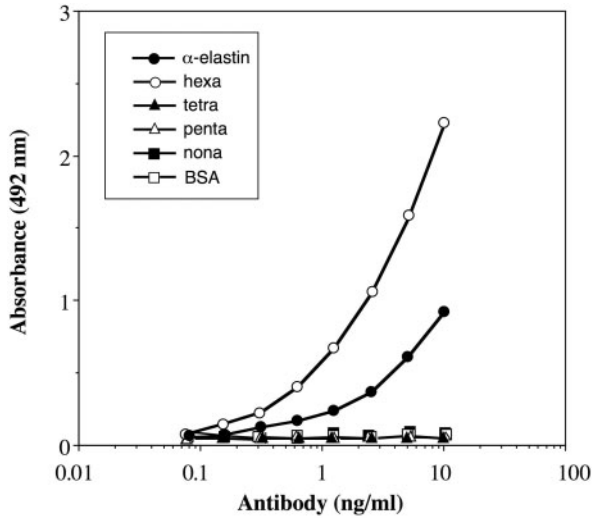

Fig. 1. **Immunoreactivities of polyclonal antibody with elastin-derived peptides.** The α-elastin (closed circle), hexapeptide (open circle), tetrapeptide (closed triangle), pentapeptide (open triangle), nonapeptide (closed square) and BSA (open square) as a control.

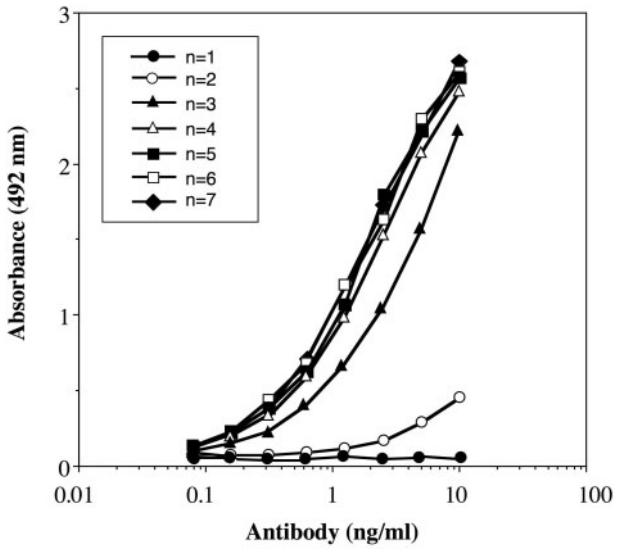

Fig. 2. **Immunoreactivities of polyclonal antibody with hexapeptide repeats.** H–(VGVAPG)$_n$–NH$_2$ where n is 1 (closed circle), 2 (open circle), 3 (closed triangle), 4 (open triangle), 5 (closed square), 6 (open square) and 7 (closed diamond).

hexapeptide is required for recognition by this polyclonal antibody against elastin-derived hexapeptide.

Secondary Structural Analysis of Hexapeptide Repeats by CD Measurements—The hexapeptide oligomers, H–(VGVAPG)$_n$–NH$_2$ where n is 3–7, have a β-turn characterized by a negative band between 230 and 200 nm in TFE (Fig. 3). The secondary structure of the hexapeptide monomer, H–(VGVAPG)$_1$–NH$_2$, is significantly different from those of oligomers, having been defined as an unordered conformation that is characterized by a spectrum with a strongly negative band around 200 nm. The secondary structure of the

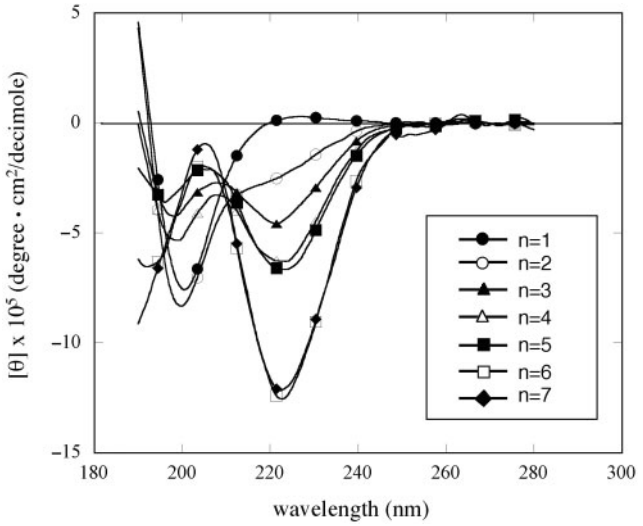

Fig. 3. **CD Spectra of hexapeptide repeats.** H–(VGVAPG)$_n$–NH$_2$ where n is 1 (closed circle), 2 (open circle), 3 (closed triangle), 4 (open triangle), 5 (closed square), 6 (open square) and 7 (closed diamond), in TFE.

hexapeptide dimer, H–(VGVAPG)$_2$–NH$_2$, contained a structure (β-turn) with a weakly negative band around 220 nm but seemed to be defined as an unordered structure with a negative band around 200 nm. The negative bands around 220 nm become deeper as the repetition of hexapeptide is increased, suggesting that the conformation of hexapeptide oligomers changes to an ordered structure. Urry *et al.* (*18*) have reported that the polyhexapeptide contains β-turns formed by Pro$_5$–Gly$_6$ at the corners to stabilize its secondary structure, and the secondary structure of the polyhexapeptide is stabilized by hydrogen bonds between Val$_3$–NH (in the unit of i) and Val$_3$–CO (in the unit of i+1). Since H–(VGVAPG)$_1$–NH$_2$ is composed of only one repetition of VGVAPG, it is incapable of having a stable secondary structure based on β-turn and intra-molecular hydrogen bond between units. From these data by CD measurements and the results of the ELISA shown in Fig. 2, it was suggested that the ordered structure of hexapeptide oligomers containing β-turn in TFE is related to recognition by the polyclonal antibody. It was also indicated that the secondary structure of H–(VGVAPG)$_1$–NH$_2$ in TFE is clearly different from other hexapeptide repeats in FT-IR measurement (data not shown).

The monoclonal antibody (BA-4) to bovine α-elastin reacted with VGVAPG and inhibited the chemotactic activity of VGVAPG (*19*). Some hydrophobic peptide sequences have been reported as epitopes on the elastin molecule that is recognized by an anti-κ-elastin antibody (*20*), and among them, VGVAPG weakly inhibited the reaction between κ-elastin and antibody. The fact that these antibodies recognize the hexapeptide monomer, VGVAPG, is distinct from that obtained in the present experiment. Probably, these antibodies may recognize the VGVAPG sequence. On the other hand, the polyclonal antibody prepared in this study may recognize the ordered structure of VGVAPG repeats.

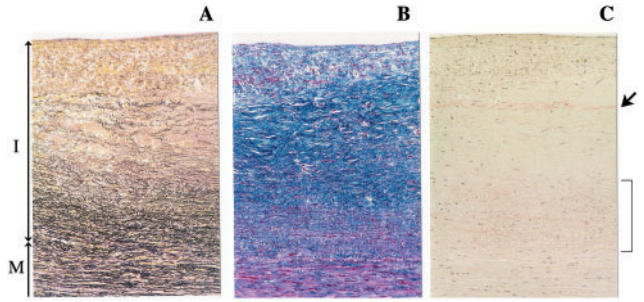

Fig. 4. **Light microscopic observation and immunostaining.** (A) Elastica van Gieson staining. (B) Masson's trichrome staining. (C) Immunohistochemical staining by polyclonal antibody against hexapeptide trimer, H–(VGVAPG)$_3$–NH$_2$. The polyclonal antibody showed a strong immunoreactivity with elastin in the mid-depths of thickened intima (denoted by arrow) and a weak immunoreactivity with elastin in the depths overlying the internal elastica (shown in bracket). I: tunica intima, M: tunica media.

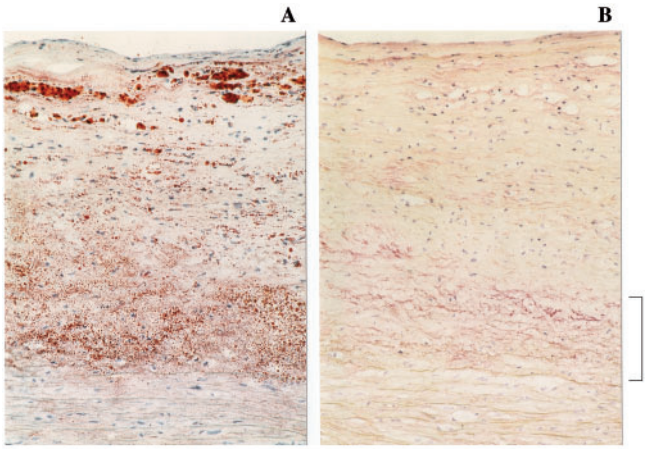

Fig. 5. **Comparison of oil red O staining for lipid droplets (A) and immunostaining for elastins (B) in the thickened intima.** Lipid deposits in the depths overlying the internal elastica accumulated on elastin synthesized newly as fine fibres (shown in bracket).

CD spectra of a series of hexapeptide repeats, H–(VGVAPG)$_n$–NH$_2$ where n is 1–7, did not show the ordered structure in H_2O (data not shown). Since it is known that the secondary structures of polypeptides and proteins can be controlled by solvent (*21*), the ordered or unordered structure of the hexapeptide oligomers in TFE or H_2O may be due to the solvent effect. More detailed structural analysis of hexapeptide oligomers in such conditions as different temperature, pH and salt concentration will elucidate the state of secondary structure of hexapeptide oligomers in aqueous solution system.

Histology and Immunohistochemistry of the Arterial Wall—Figure 4A–C show serial microscopic sections through one of the atherosclerotic lesions in human aorta. Elastin synthesized newly as fine fibres was sparsely observed in the mid-depths of the thickened intima and densely in the depths overlying an internal elastica, compared to that in media (Fig. 4A). Collagen was markedly accumulated in the thickened intima (Fig. 4B). The observation shown in Fig. 4B coincides with other reports that collagen were newly synthesized in the thickened intima with the development of atherosclerosis (*22–24*). In an immunohistochemical study, this polyclonal antibody showed a strong immunoreactivity with elastin in the mid-depths of thickened intima (denoted by arrow) and a weak immunoreactivity with elastin in the depths overlying the internal elastica (shown in bracket) but did not with elastin in media (Fig. 4C). These results suggest a structural difference between elastins in the thickened intima and those in media. It is well known that elastin exists physiologically in media. In the extracellular space in media, tropoelastin is assembled into or around microfibrils and cross-linked by the copper-requiring enzyme, lysyl oxidase, resulting in an insoluble elastic fibre. Since elastin in media is surrounded by microfibrils, it is reasonable that the polyclonal antibody against the hexapeptide repeat may fail to recognize it. It has been reported that elastin is newly synthesized in thickened intima in the development of atherosclerosis (*13–15*). It is indicated that elastin synthesized newly as fine fibres in the thickened intima may not be surrounded by microfibrils, indicating that the possibility of recognition of the exposed elastin by this polyclonal antibody.

Figure 5A and B show serial sections through one of the atherosclerotic plaques. Oil red O stain showed lipid deposits in the thickened intima (Fig. 5A). Deposition of lipids in this superficial intimal layer seemed to consist of lipid-loden macrophages or smooth muscle cells. Moreover, lipid deposits in the depths overlying the internal elastica appeared to accumulate on elastin synthesized newly as fine fibres shown in immunohistochemical staining (shown in bracket) (Fig. 5B). This accumulation on elastin supported the previous report that elastin is involved in lipid deposits (*25*). Since elastin is a hydrophobic (lypophilic) protein, it has a strong affinity for lipid.

In this study, the polyclonal antibody which recognizes the hexapeptide oligomers reacted specifically with newly synthesized elastin in thickened intima. A question whether the ordered structure of the hexapeptide oligomers is involved in recognition by the antibody is raised. The evidence that the structure of hexapeptide oligomers is ordered in ELISA will be required to solve this problem. In a previous report, it was shown that elastin-derived peptide in aqueous solution self-assembled with increasing temperature formed an ordered state and its CD spectrum was similar to that in TFE (*10*). This increase in structural order of polypeptide with an increase in temperature implies a decrease in mobility. In ELISA, the hexapeptide oligomers are immobilized by treatment of blocking agent on plates prior to reaction with antibody and this state may resemble the state in TFE. Furthermore, as it is not still clear whether the ordered and unordered states have any relevance to the states of elastin in intimal hyperplasia and in media, respectively, if so, in the immunohistochemical study the state of intimal elastin which can be recognized by antibody may reflect the

state in TFE. Further conformational analyses by spectroscopic techniques such as CD, FT-IR and NMR in buffer solution used on ELISA plates are in progress. The distinct and unique immunoreactivity of this antibody can be used for clarification of the structure of elastins in such atherosclerotic and emphysematous lesions by a combination with the technique of histological stain.

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