Localization of D-\(\beta\)-Aspartic Acid–Containing Proteins in Human Eyes

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PURPOSE. Biologically uncommon D-\(\beta\)-aspartic acid (D-\(\beta\)-Asp) has been detected in proteins from various human tissues in elderly donors. Previous studies have identified D-\(\beta\)-Asp residues at four different specific sites in \(\alpha\)-crystallin from aged human lenses and an increased amount of D-\(\beta\)-Asp residues with age. D-\(\beta\)-Asp is formed as a result of racemization and accumulates with age; therefore, it is thought to be a potential marker of aging. To reveal the role of the D-\(\beta\)-Asp formation in the aging process of eyes, immunohistochemical localization of D-\(\beta\)-Asp was investigated in ocular samples of various ages.

METHODS. Polyclonal antibody to the D-\(\beta\)-Asp–containing peptide was prepared. To confirm the specificity of the antibody, SDS-PAGE and Western blotting analyses of lens were performed. To detect the locality of the D-\(\beta\)-Asp–containing protein, immunohistochemical staining using the antibody was carried out in ocular samples obtained from nine donors 18 to 88 years of age and two fetuses.

RESULTS. The antibody to the D-\(\beta\)-Asp–containing peptide reacted with the lens peptide of the aged donors around 20 kDa that was compatible with \(\alpha\)-A crystallin. In addition, the binding of the antibody to the \(\alpha\)-A crystallin was almost completely blocked with the addition of the excess D-\(\beta\)-Asp–containing peptide. The antibody showed a negative reaction with any of the tissues in the eye of human fetuses and in donors younger than 18 years. In contrast, relatively strong immunoreactivity to the D-\(\beta\)-Asp–containing peptides was seen in the nuclei of the lens, in nonpigmented ciliary epithelial cells, in drusen, and in the sclera of elderly donors. In addition, moderate to weak immunoreactivity was seen in the cortex of the lens, in the blood vessels of the retina, in the optic nerve head, and in the lamina cribrosa of elderly donors. Furthermore, the immunoreactions were almost completely blocked with the addition of the excess D-\(\beta\)-Asp–containing peptide in the reaction mixture.

CONCLUSIONS. The D-\(\beta\)-Asp–containing proteins appeared in various ocular tissues with age. This study clearly demonstrated that the D-\(\beta\)-Asp–containing proteins are more widespread in aged tissues than previously thought. The formation of D-\(\beta\)-Asp in protein can cause major changes in its structure because different side chain orientations can induce an abnormal peptide backbone, and the main chain of the peptide can then become elongated by the \(\beta\) linkage. Therefore, this modification can be the result of the partial unfolding of protein, leading to various age-related ocular diseases. In particular, D-\(\beta\)-Asp would provide a new aspect of the molecular mechanisms of age-related macular degeneration because drusen is positive for D-\(\beta\)-Asp. (Invest Ophthalmol Vis Sci. 2007;48:3923–3927) DOI:10.1167/iovs.06-1284

Amino acids contain one (or more) asymmetric tetrahedral carbon atoms that make these molecules two nonsuperimposable mirror images—that is, they are right-handed (D-enantiomer) and left-handed (L-enantiomer) structures. Although the chemical and physical properties of the L-amino acids and D-amino acids are the same, differing only in their optical character, the L-amino acids were selected for the polymerization and formation of peptides and proteins during the chemical evolution step before the emergence of life. Therefore, all living organisms are now composed exclusively of L-amino acids.

Recently, D-Asp was found in the lenses,1–4 teeth,5 bones,6 brains,7 skin,8 aortas,9 erythrocytes,10 lungs,11 and ligaments12 of elderly donors. The presence of D-Asp in aged tissues of the living body is considered the result of racemization of L-Asp in proteins in such metabolically inert tissues. It is well known that aspartic acid is the amino acid most amenable to racemization. For this reason, D-Asp acid in living organisms is considered a useful marker of the aging process.1,4,5,8,9,12

We previously reported the presence of D-isomers at Asp-58 and Asp-151 in \(\alpha\)-A-crystallin13 and at Asp-56 and Asp-62 in \(\beta\)-crystallin14 from aged human lenses. The D-Asp formation was accompanied by isomerization from the natural L-Asp to the abnormal \(\beta\)-Asp through a succinimide.1 This leads to the formation of four isomers, namely, the normal L-\(\alpha\)-Asp plus the biologically rare L-\(\beta\)-Asp, D-\(\alpha\)-Asp, and D-\(\beta\)-Asp in proteins. Among the uncommon isomers, D-\(\beta\)-Asp is the major isomer found in elderly tissues.1,4

In a previous study, we prepared a polyclonal antibody against the peptide Gly-Leu-D-Asp-Ala-Thr-Gly-Leu-D-Asp-Ala-Thr-Gly-Leu-D-Asp-Ala-Thr-Gly-Leu-D-Asp-Ala-Thr (anti-peptide 3R antibody), which corresponds to three repeats of positions 149 to 153 of the \(\alpha\)-A-crystallin. This antibody can distinguish the configuration of the Asp-residue. As such, the antibody reacts strongly against the D-\(\beta\)-Asp–containing peptide, but not with the L-\(\alpha\)-Asp, L-\(\beta\)-Asp, or D-\(\alpha\)-Asp–containing peptides.3 The D-\(\beta\)-Asp–containing protein in sun-damaged skin was found in elderly donors using the antipeptide 3R antibody.3 The abnormal protein was localized in the elastic fiberlike structures of skin samples from elderly donors with actinic elastosis.15 However, no immunoreactivity was found in the sun-exposed skin from young donors. Recently, we discovered that many proteins from the NN 1003A cell line, which is derived from rabbit lens, contain D-\(\beta\)-Asp residues.16 Results showed that these abnormal proteins are present in the living body more extensively than previously thought.

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We now report the detection of the D-β-Asp-containing protein in ocular samples, such as those obtained from donors of various ages, using the anti-peptide 3R antibody.

**Materials and Methods**

**Eye Samples**

Nine eyes from nine donors 18 to 88 years of age and two eyes from two fetuses were obtained at the time of necropsy from the Division of Pathology at the University of Tokyo Hospital. None of the subjects had any history of ocular disease. The present study adhered to the tenets of the Declaration of Helsinki.

**Antibody against D-β-Asp–Containing Peptides**

The preparation and characterization of the polyclonal antibody against D-β-Asp-containing peptides were the same as those described in a previous paper. The polyclonal antibody against the peptide Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr (anti-peptide 3R antibody), which corresponds to three repeats of positions 149 to 153 of the human α-A-crystallin, was prepared and purified as previously described. The antibody clearly distinguished the configuration of the Asp residue—that is, it reacted strongly with the D-β-Asp-containing peptides but did not react with the L-α-Asp-, L-β-Asp-, or D-α-Asp-containing peptides.

**SDS-PAGE and Western Blot Analysis of Crystallin**

Human lenses with cataracts from a 50-year-old donor were obtained from the National Disease Research Interchange (Philadelphia, PA). High molecular-weight and low molecular-weight forms of α-A-crystallin were prepared as previously described. High and low molecular-weight α-A-crystallin fractions from the 50-year-old samples were electrophoresed by polyacrylamide gel electrophoresis. The proteins in the gels were transferred onto polyvinylidene difluoride (PVDF) membranes, and the blotted proteins were then incubated with the primary antibody diluted to 1:250 with or without 1, 10, and 100 μg/mL peptide 3R, followed by incubation with the peroxidase-conjugated antibody polypeptides at a dilution of 1:500, dissolved with phosphate-buffered saline (PBS) containing 1% normal bovine serum albumin, and kept at 4°C overnight. After the section was washed with PBS, it was treated with the reaction solution containing a secondary antibody labeled with an amino acid polymer and horseradish peroxidase (Histofine Max-PO kit; Nichirei Co. Ltd., Tokyo, Japan) and kept for 30 minutes at room temperature. The sections were then incubated with diaminobenzidine (DAB) in PBS and were counterstained with hematoxylin.

**Immunohistochemistry**

Immunohistochemical localization of the D-β-Asp-containing peptides was investigated using the antibody mentioned earlier. After fixation with 10% formalin solution, 4-μm-thick sections of the paraffin-embedded eye samples were prepared. After deparaffinization, the sections were treated with a polyclonal antibody to the D-β-Asp-containing peptides at a dilution of 1:500, dissolved with phosphate-buffered saline (PBS) containing 1% normal bovine serum albumin, and kept at 4°C overnight. After the section was washed with PBS, it was treated with the reaction solution containing a secondary antibody labeled with an amino acid polymer and horseradish peroxidase (Histofine Max-PO kit; Nichirei Co. Ltd., Tokyo, Japan) and kept for 30 minutes at room temperature. The sections were then incubated with diaminobenzidine (DAB) in PBS and were counterstained with hematoxylin. As the negative control, the primary antibody was replaced with normal rabbit serum IgG (1.0 μg/mL) diluted in PBS containing 1% bovine serum albumin.

To evaluate the specificity of the binding of the primary antibody to the D-β-Asp-containing peptides, the sections were incubated with the mixture of the primary antibody and 10 μg/mL peptide 3R instead of only the primary antibody. Immunohistochemistry results were checked and graded in a double-masked manner.

**RESULTS**

**Characterization of the Primary Antibody Using Western Blot Analysis of Lens**

Figure 1 shows the results of the SDS-PAGE and Western blot analysis of lens using the primary antibody to peptide 3R. The antibody reacted with the lens peptide at around 20 kDa, compatible with α-A-crystallin (lane 3). Incubation with 1, 10, and 100 μg/mL peptide 3R containing D-β-Asp acid, together with the primary antibody, resulted in almost complete inhibition of the reaction (lanes 4, 5, and 6, respectively). Lane 1, molecular marker; lane 2, blank.

**Cornea-Conjunctiva**

In the cornea or conjunctiva, no immunoreactivity to the D-β-Asp-containing peptides was seen in any of the specimens (data not shown).

**Lens**

No immunoreactivity to the D-β-Asp-containing peptides was seen in the lenses of fetuses or donors younger than 18 (Fig. 2A). In contrast, a relatively strong immunoreactivity in the nuclei (Fig. 2B, arrowhead) and moderate immunoreactivity in the cortex of the lens (Fig. 2B, arrow) were detected in the 74-year-old male donor (Fig. 2B). No immunoreactivity to the D-β-Asp-containing peptides was found in the lens epithelium or lens capsule in the elderly. Incubation with D-β-Asp-containing peptides with a reaction mixture completely blocked the immunoreaction of the lenses (Fig. 2C).

**Iris–Ciliary Body**

Immunoreactivity to the D-β-Asp-containing peptides was not seen in the irises or ciliary bodies of fetuses or donors younger than 18 (Fig. 2D). In contrast, relatively strong immunoreactivity of the D-β-Asp-containing peptides was observed on the surfaces of the nonpigmented ciliary epithelia, corresponding
to the basement membrane of the nonpigmented ciliary epithelium in a 74-year-old male donor (Fig. 2E). Incubation with the D-ß-H9252-Asp– containing peptides with the reaction mixture completely blocked the immunoreaction of the ciliary body (Fig. 2F).

**Retina–Choroid–Sclera**

No immunoreactivity to the D-ß-Asp– containing peptides was seen in the retinas, choroids, or scleras of fetuses or donors younger than 18 (Figures 2G, 2J, 2M). In contrast, relatively strong immunoreactivity to the D-ß-Asp– containing peptides was detected in the sclera (Fig. 2H, arrow), and moderate immunoreactivity of the D-ß-Asp– containing peptides was seen in the internal limiting membrane (ILM; Fig. 2K, arrow), retinal vessels (Fig. 2N, arrows), Bruch membranes, and choriocapillaris. In addition, drusen were seen in all the specimens of persons older than 70. Relatively intense immunoreactivity to the D-ß-Asp– containing peptides was seen in all the drusen (Fig. 2N, arrow). Incubation with the D-ß-Asp– containing pep-
### Table 1. Summary of Immunohistochemistry

<table>
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<tr>
<th>Donor</th>
<th>Age</th>
<th>Sex</th>
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<th>Lamina Cribr.</th>
<th>Bruch Membrane</th>
<th>Choriocapillaris</th>
<th>Sclera</th>
<th>Cribrosa Cortex</th>
<th>Nuclei Capsule</th>
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Age-dependent accumulation of the D-Asp–containing peptides is clearly shown. Intensity of the immunoreaction was classified as follows: - weak; +, very weak; ++, strong; ND, not detected. Donor age is in years unless otherwise specified.

#### DISCUSSION

In the present study, the immunohistochemical localization of the D-Asp–containing protein was investigated in human eyes that ranged from 0 to 80 years of age. Immunoreactivity to the D-Asp–containing protein was observed in the lens, sclera, nonpigmented ciliary epithelium, internal limiting membranes, lamina cribrosa, Bruch membranes, and vessels in the retina of the elderly donors, but not in the tissues of the donors younger than 18 years of age. Racemization and isomerization of the Asp residues in protein are thought to be the result of ultraviolet irradiation. In addition, metabolic changes in the body with age may affect racemization because the process of advanced glycation end products is known to accelerate racemization. This may be why D-Asp is detected not only in aged tissue but in cultured cells derived from rabbit lens.

Cataract formation is one of the most prominent age-related changes in the lens. Proteins of the lens have long half-lives; therefore, various posttranslational modifications, such as the formation of disulfide bridges,18 deamidation of asparaginyl and glutaminyl residues,18,19 racemization of aspartyl residues,1,3 and advanced glycation, have been reported.20 These modifications may cause the formation of high molecular-weight protein aggregates that can lead to the formation of cataracts. Among them, the racemization and isomerization of aspartyl residues may affect the aggregation of proteins because the side chain orientation of the D-Asp residues turns upside down, and the main chain of the peptide bond–containing β-linkage is elongated. In the present study, we revealed that relatively strong immunoreactivity to D-Asp–containing peptides, especially in the nuclei of the lens of elderly donors, was detected, whereas in young lenses, no immunostaining was observed. It is well known that old proteins accumulate in nuclei; therefore, the localization of the D-Asp–containing protein in the nuclei seems appropriate. This result is consistent with the results from a previous study.7

Increased thickness of the basement membrane with age is a prominent histologic change in the body. For example, the increased thicknesses of the basement membrane of the nonpigmented ciliary epithelium, retinal vessels, choriocapillaris, Descemet membrane, inner limiting membrane, and Bruch membrane are observed with age or in animal models of aging.18,19 However, the thickening mechanism of the basement membranes is still unclear. The present study shows the presence of the D-Asp–containing protein in the basement membranes with the exception of Descemet membrane. Generally, the appearance of D-Asp in protein tends to form the protein aggregate and deposit in the tissue. For this reason, the deposition of the D-Asp–containing protein may increase the
thickening of the ocular basement membranes. The present study did not detect the D-β-Asp–containing peptides in Descemet membrane. Further studies are needed to show whether the lack of the D-β-Asp–containing peptides in Descemet membrane results from different thickening mechanisms or from a sensitivity to the immunohistochemistry.

The sclera and lamina cribrosa are also the target tissues of the aging process. In addition, in aged tissues, the amount of D-β-Asp in the elastin increases and accumulates in aged tissues. Previous studies showed that elastin from sun-exposed skin, ligaments, and the aorta contain D-Asp residues. The elastin molecule has three aspartic acids. We synthesized three different Asp-containing peptides corresponding to the elastin sequences and studied the kinetics of the Asp racemization using these model peptides. Results indicated that the Asp residues in the elastin mimic peptides are susceptible to racemization during a normal human lifetime. The development of drusen and the increased thickness of Bruch membrane are primary histologic changes during age-related macular degeneration. In three preparations, we observed relatively strong immunoreactivity to the D-β-Asp–containing peptides in the retina and hard drusen and in the thickened Bruch membrane. In addition, in one preparation, moderate immunoreactivity to the D-β-Asp–containing peptides in the drusen near the optic nerve head was seen. These results lead to the hypothesis that the D-β-Asp–containing peptides are among the major components of the hard and soft drusen. This hypothesis is supported by the fact that the irradiation of ultraviolet rays is an accelerator of racemization and the risk factor of drusen formation. To reveal the role of the D-amino acids in the development of age-related macular degeneration, further studies, including mass spectrometry of drusen and in vivo, are needed regarding the biological effect of D-amino acids in relation to angiogenesis. Immunohistochemistry results may be affected by various factors, including morphologic changes in the eyes with age and causes of death, especially in the donors of the elderly. Aging changes play important roles in the development of various ocular diseases including cataract, glaucoma, and age-related macular degeneration. For this reason, the investigation of the molecular mechanism of the aging process is needed for further prevention and treatment of age-related ocular diseases. D-β-Asp will provide important new insights into the molecular mechanism of various ocular diseases.

References