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Immunohistochemical localisation of D-β-aspartic acid in pingueculae

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ABSTRACT

Background: D-β-Aspartic acid residues, which are biologically uncommon, have been reported to accumulate in various proteins of the living body with age. In the present study, D-β-aspartic acid-containing proteins were found to be localised in pingueculae, which represent one of the most prominent age-related ocular changes.

Methods: Surgical specimens of conjunctivae with or without pingueculae were obtained from eight patients. Immunohistochemical localisation of D-β-aspartic acid-containing proteins was performed using a polyclonal antibody against D-β-aspartic acid-containing peptides.

Results: Strong immunoreactivity to D-β-aspartic acid-containing peptides was detected in the subepithelial amorphous materials of all surgical specimens with pingueculae. In contrast, no immunoreactivity to D-β-aspartic acid-containing peptides was detected in the specimens without pingueculae.

Conclusions: Pingueculae are thought to be aggregates of proteins that contain D-β-aspartic acid residues. It is known that the conversion of L- to D-aspartyl residues is accelerated by ultraviolet irradiation. In addition, D-β-aspartic acid-containing proteins, in general, tend to aggregate with each other and accumulate in the tissues. These facts indicate that ultraviolet irradiation-induced racemisation of aspartic acid is closely related to the development of pingueculae.

The ageing process is known to be related to various ocular lesions and diseases, including pingueculae. Epidemiological studies have revealed that ultraviolet irradiation as well as the ageing process is involved in the development of pingueculae.¹ However, the pathogenesis of these lesions remains unclear. Recently, racemisation of amino acids and the resultant D-amino acids in proteins have been studied as biochemical markers of the ageing process.²⁻⁷ It was previously believed that living organisms contained only L-amino acids and that organisms containing D-amino acids had become extinct. However, D-amino acids have been found in proteins obtained from the lenses,²⁻⁵ drusen,⁷ teeth,¹² bones,¹³ brains,¹⁴ skin,⁶ aortas,¹⁵ erythrocytes,¹⁶ lungs¹⁷ and ligaments¹⁸ of elderly people. These D-amino acids in proteins are considered to be derived from the racemisation of L-amino acids in various tissues at body temperature. Thus, D-amino acids in the proteins of living organisms are considered to be markers of the ageing process.

In the present study, we investigated the immunohistochemical localisation of D-β-aspartic acid-containing proteins in surgical specimens with and without pingueculae.

MATERIALS AND METHODS

Surgical specimens of conjunctivae with or without pingueculae

In the present study, informed consent was obtained from all patients who were undergoing surgery. To investigate the pathogenesis of pingueculae, the immunohistochemical localisation of D-β-aspartic acid-containing peptides in surgical specimens with and without pingueculae was performed. A total of eight pingueculae from eight patients (four men and four women; ages, 57.1 (SD 13.0) years) were excised. As controls, specimens of conjunctivae that did not contain pingueculae were obtained from the temporal or nasal part of the cornea; the specimens were excised during strabismus surgery and then analysed (four men and four women; ages, 59.4 (11.3) years). All men were outdoor workers, and the women were housewives or indoor workers. Haematoxylin-eosin staining and immunohistochemistry were performed on all surgical specimens.

Antibody against D-β-aspartic acid-containing peptides

The preparation and characterisation of a polyclonal antibody against D-β-aspartic acid-containing peptides have been previously described.^{5 6 10 19} In brief, a 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, designated peptide 3R, was prepared and purified. This peptide corresponds to three repeats of the residues at positions 149–153 of human α-A-crystalline. The antibody clearly distinguished between aspartic acid residues of different configurations; that is, it reacted strongly with the D-β-aspartic acid-containing peptides but did not react with the L-α-Asp-, L-β-Asp- or D-α-Asp-containing peptides.

Immunohistochemistry

D-β-Aspartic acid was immunohistochemically localised in the surgical specimens of conjunctivae with or without pingueculae. In brief, 4-μm-thick formalin-fixed paraffin-embedded sections were prepared. After deparaffinisation, the sections were treated with a polyclonal antibody (dilution, 1:500) against D-β-aspartic acid-containing peptides; the antibody was dissolved in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, and the sections were maintained overnight at 4°C. After the sections were washed with PBS, they were treated with a reaction solution containing the secondary antibody labelled with an amino acid polymer and horseradish peroxidase (Histofine Max-PO kit; Nichirei Co., Tokyo) and maintained at room temperature for 30 min. The sections were

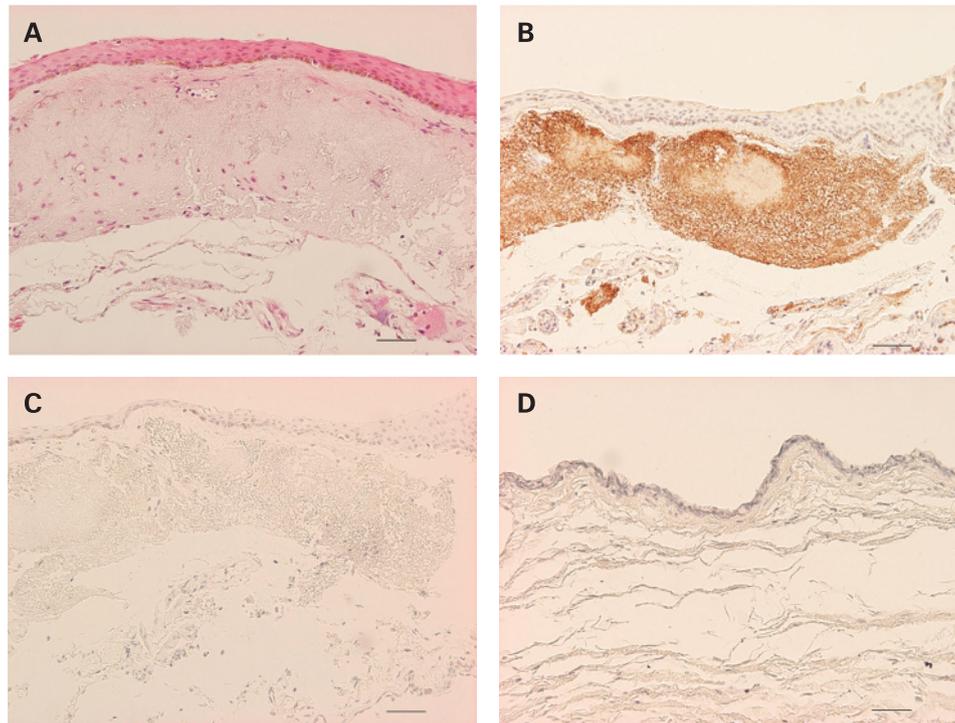


Figure 1 Immunohistochemical localisation of D- β -aspartic acid in pingueculae. Eosinophilic amorphous materials were detected in the subepithelial layer of the conjunctiva in specimens with pingueculae (A). Strong immunoreactivity to D- β -aspartic acid is seen in the subepithelial amorphous materials (B). No immunoreactivity to D- β -aspartic acid is seen in specimens after the absorption test (C). No immunoreactivity to D- β -aspartic acid is seen in surgical specimens without pingueculae (D). Bar = 100 μ m.

then washed with PBS again and incubated with diaminobenzidine (DAB) in PBS. Finally, the sections were counterstained with haematoxylin.

An absorption test was used to show the specific affinity of the antibody for D- β -aspartic acid-containing peptides. In brief, instead of using the polyclonal antibody to D- β -aspartic acid-containing peptides, a mixture of the polyclonal antibody (dilution 1:500) and peptide 3R (final concentration 1 μ g/ml) was used for immunohistochemistry.

As a negative control, the primary antibody was replaced with normal rabbit serum immunoglobulin (Ig)G (1.0 μ g/ml) diluted with PBS containing 1% bovine serum albumin. After these preparatory steps, the sections were processed for immunohistochemistry as described above.

RESULTS

Figure 1 shows the typical features observed on haematoxylin-eosin and immunohistochemical staining of conjunctival specimens obtained from donors in their 50 s. In the specimens with pingueculae, eosinophilic and amorphous materials were detected in the subepithelial layer of the conjunctiva (fig 1A). Strong immunoreactivity to D- β -aspartic acid-containing proteins was detected in the amorphous materials in the subepithelial layer of the samples with pingueculae (fig 1B). After the absorption test, the immunoreactivity to D- β -aspartic acid was greatly reduced in the conjunctival specimens containing pingueculae (fig 1C). In the negative controls, non-specific immunoreactivity was not detected in the subepithelial layer of the surgical specimens with pingueculae (data not shown). Further, eosinophilic and amorphous materials were not detected in the subepithelial layer of the surgical specimens without pingueculae. No immunoreactivity to D- β -aspartic acid was observed in the control conjunctivae (fig 1D), the

conjunctival specimens after the absorption test (data not shown) or the negative controls (data not shown).

DISCUSSION

The purpose of the present study is to reveal role of D- β -aspartic acid-containing proteins in the development of pingueculae. Previous studies have shown that pingueculae are aggregations of hyaline materials and arise because of elastotic degeneration.^{20 21} However, the concept of hyalinisation or degeneration is too vague to explain the exact nature and pathogenesis of pingueculae. Recently, it has been reported that pingueculae contain advanced glycation end products (AGEs) such as N^ε-(carboxymethyl)-L-lysine, pentosidine, imidazolone and pyrrole.²² We demonstrated for the first time, to the best of our knowledge, that pingueculae are accumulations of D- β -aspartic acid-containing proteins. The appearance of these proteins is likely to induce a major change in the higher-order protein structure because D-aspartic acid residues are oriented against the direction of the peptide plane. In addition to the formation of D-aspartic acid residues, beta-linkage of these residues affects the quaternary structure of proteins because the main chain of the proteins is elongated. Thus, these uncommon configurations of aspartic acid are considered to be one of triggers for the abnormal aggregation of proteins. D- β -Aspartic acid in proteins is formed via a succinimide intermediate, as described in our previous papers.³ Racemisation was previously shown to rapidly proceed when the carboxyl side group of the aspartic acid residue is small, such as glycine, alanine and serine; in this case, L-aspartic acid residues of proteins are easily converted to D-aspartic acid residues under physiological conditions. For this reason, D-amino acids are regarded as one of the biological markers of the ageing process.^{5 6 9 12 15 18} In fact, we have found that D- β -aspartic acid tends to accumulate with age in various ocular tissues, including

the lens, sclera, lamina cribrosa, Bruch membrane, inner limiting membrane, basement membrane of non-pigment ciliary epithelial cells and drusen.⁷ These results indicate that racemisation and the resultant D-amino acids are involved in the aggregation of proteins, which leads to the appearance of age-related changes in the eyes. The results of the present study show that pingueculae are aggregations of D-β-aspartic acid-containing proteins, and this finding supports the hypothesis that D-amino acids are biological markers of the ageing process.

Both the present and recent studies have shown that pingueculae are aggregations of proteins containing D-amino acids and AGEs, both of which are similar in various aspects. First, ultraviolet irradiation accelerates the formation of both D-amino acids and AGEs.^{6 19 23} Second, D-amino acids and AGEs tend to accumulate in the body with age.^{3 6 9 12 15 18 23} Third, the molecular structure and the function of proteins are altered because of D-amino acid and AGE formation.^{24 25} For these reasons, D-amino acids and AGEs are regarded not only as markers but also as the molecular basis of the ageing process. Furthermore, AGE formation is known to accelerate the racemisation of amino acids. These facts indicate that the accumulation of D-β-aspartic acid in pingueculae is accelerated not only by topical irradiation by ultraviolet rays but also by the formation of AGEs.

Diabetes is known to accelerate AGE formation in tissues, including corneal epithelial cells, endothelial cells and the basement membrane of the cornea. Neither AGEs nor D-β-aspartic acid was observed in the conjunctival cells of non-diabetic patients. However, the localisation of AGEs and D-β-aspartic acid in patients with diabetes was not assessed. Further studies are required to reveal the role of AGEs and D-β-aspartic in diabetes.

The racemisation of amino acids in proteins progresses very slowly, with a half-life of thousands of years. This means that aggregation of D-β-aspartic acid-containing proteins in pingueculae cannot be attributed to a low turnover rate of subconjunctival tissue. Some molecular mechanisms must underlie the development of pingueculae and involve the acceleration of the racemisation of amino acids in proteins.

Pingueculae themselves do not deteriorate visual acuity; they are primarily cosmetic problems. The molecular mechanism underlying the development of pingueculae would facilitate further understanding the ageing process of various organs. Further studies that aim to reveal the role of D-amino acids in the ocular and systemic ageing process are ongoing.

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