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Immunohistochemical localization of D-β-aspartic acid-containing proteins in pterygium

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ABSTRACT

Biologically uncommon D-β-aspartic acid (D-β-Asp) residues have been reported to accumulate in organs affected by age-related disorders. In the present study, we investigated the localization of D-β-Asp-containing proteins in cases of pterygium, one of the most prominent age-related ocular conditions. Immunohistochemical localization of D-β-Asp-containing proteins was investigated in surgical specimens of pterygium from 20 patients and control specimens from 10 patients. Strong immunoreactivity to D-β-Asp-containing proteins was observed in subepithelial elastotic lesions and surrounding collagenous lesions from all surgical specimens with pterygia. In contrast, no immunoreactivity to D-β-Asp-containing proteins was seen in pterygium-free specimens.

D-β-Asp-containing proteins are produced in organs as they are affected by the aging process. In addition, conversion of L- to D-aspartyl residues is accelerated by ultraviolet (UV) irradiation. Since pterygia can form due to aging or UV exposure, it is reasonable to find D-β-Asp-containing proteins in specimens with pterygia. Furthermore, since D-β-Asp is a non-native amino acid, D-β-Asp-containing proteins may be recognized as allogeneic antigens. Therefore, D-β-Asp-containing proteins in pterygia may responsible for the fibrovascular changes seen in the disorder.

1. Introduction

Occurrence of pterygia, one of the causes of vision-threatening disorders, has a high prevalence in equatorial regions [1,2]. Etiological studies have confirmed that ultraviolet irradiation, dry and dusty environments, and age are the major factors contributing to the development of pterygia [1,2]. Histologically, an activation of p53 [3], increased expression telomerase [4], infection with human papilloma virus and herpes simplex virus are detected in specimens containing pterygia [5]. However, the pathogenesis of the lesion, especially the association with ultraviolet irradiation and aging, remains unclear.

Recently, the racemization of amino acids and the presence of resultant D-amino acids in proteins have been investigated as potential biochemical markers of the aging process [6–15]. Previously, it was believed that proteins in all living organisms were composed only of L-amino acids, and that D-amino acids had been effectively eliminated on earth. However, recently, D-amino acids have been found in proteins from the lenses [6–9,13,16,17], drusen [18], teeth [12,14], bones [19,20], brains [15,21], skin [10], aortas [22], erythrocytes [23], lungs [24], and ligaments [14] of elderly people. The D-amino acids in these proteins are thought to be derived from the racemization of L-amino acids in proteins of various tissues. Thus, D-amino acids in the proteins of living organisms are considered markers of the aging process. In addition, racemization of amino acids is accelerated by ultraviolet irradiation. In fact, D-amino acid-containing proteins are detected in ultraviolet-induced conditions, including cataracts [13,16], pingueculae [11], climatic droplet keratopathy [25], and actinic keratosis of the skin [10]. Thus, D-amino acids in the proteins of living organisms are also considered markers of ultraviolet irradiation exposure.

The aim of the present study is to reveal the role of D-amino acids in proteins in the development of pterygia. Among various types of D-amino acids, we focused on the D-β-aspartic acid (D-β-Asp) in proteins because it is a major product that appears in the aging process and the molecular mechanisms of racemization is

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well analyzed [6,8,9]. We investigated the immunohistochemical localization of D-β-Asp-containing proteins in surgical specimens with or without pterygia. Based on the results, we would like to propose a hypothesis for the development of pterygia.

2. Materials and methods

2.1. Surgical specimens of conjunctivae with or without pterygia

Written informed consent concerning the use of surgical samples for the purpose of pathological experiments was obtained from every patient who underwent surgery in Miyata Eye Hospital. To investigate the pathogenesis of pterygia, the immunohistochemical localization of D-β-Asp-containing peptides in surgical specimens, with or without pterygia, was analyzed. Pterygium-containing samples from 20 patients (9 men and 11 women, 67.4 ± 18.1 years of age) were excised. As controls, surgical specimens of conjunctivae without pterygia, excised during conjunctivochalasis surgery, were analyzed from 10 individuals (four men and six women, 64.2 ± 12.7 years of age).

2.2. Hematoxylin–eosin staining

Sections were deparaffinized, soaked in Mayer's hematoxylin solution for 10 min, followed by washing in running water. Then the sections were treated with 0.3% hydrochloric acid in 70% ethanol for 2 s and washed in running water. Finally, the sections were treated with eosin solution for 3 min and dehydrated.

2.3. Antibody against D-β-Asp-containing peptides

The preparation and characterization of rabbit polyclonal antibody against D-β-Asp-containing peptides have been previously described [17]. Briefly, a polyclonal antibody against the peptide with the sequence Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr (peptide 3R), corresponding to three repeats of the amino acid sequence at positions 149–153 of human α-A-crystallin, was prepared and purified. The antibody clearly distinguished between different configurations of the Asp residue; it strongly reacted with the D-β-Asp-containing peptide but did not react with the analogous L-α-Asp, L-β-Asp, or D-α-Asp-containing peptides [17].

2.4. Immunohistochemistry

Localization of D-β-Asp in the surgical specimens of conjunctivae with or without pterygia was investigated immunohistochemically. Briefly, the deparaffinized sections were incubated overnight at 4 °C in the abovementioned polyclonal antibody, which was diluted 1:250 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. After washing with PBS, the sections were incubated for 30 min at room temperature in the reaction solution containing a secondary antibody against rabbit IgG labeled with polymerized horseradish peroxidase (Histofine Max-PO kit, Nichirei Biosciences, Tokyo, Japan). After washing (again) with PBS, the sections were incubated in 0.2 mg/mL of diaminobenzidine (DAB), dissolved in Tris buffer containing 0.005% H_2O_2. Finally, sections were counterstained with Mayer's hematoxylin solution for 10 s for staining of nuclei.

As a negative control, certain sections were incubated in normal rabbit serum IgG (1.0 μg/mL), diluted in PBS containing 1% bovine serum albumin, instead of primary antibody. All subsequent steps of processing for immunohistochemistry were conducted identically to those described above.

3. Results and discussion

3.1. Localization of D-β-Asp-containing proteins in pterygia

Fig. 1 shows an example of staining in specimens with pterygia. In specimens with pterygia, elastotic lesions, defined as aggregations of eosinophilic and amorphous materials, were detected in the subepithelial layer of the conjunctiva (Fig. 1A). In these pterygium-containing specimens, strong immunoreactivity to D-β-Asp-containing proteins was observed in the amorphous material in the subepithelial layer (Fig. 1B, arrows). In addition, D-β-Asp-containing proteins were also detected in the collagenous lesions surrounding the elastotic lesions in pterygia (Fig. 1B, arrowheads). In negative controls, no immunoreactivity was seen in the subepithelial layer in the surgical specimens with pterygia (Fig. 1C). The staining pattern was almost the same in all the 20 specimens with pterygia. Furthermore, we had no significant difference in the staining pattern between male and female, or the age of the patients.

3.2. Localization of D-β-Asp-containing proteins in control conjunctiva

Fig. 2 shows an example of the staining in the control conjunctiva without pterygia. In the control conjunctiva, no eosinophilic or amorphous materials were detected in the subepithelial layer of the specimens (Fig. 2A). No immunoreactivity to D-β-Asp was observed in the control conjunctiva (Fig. 2B), nor in the negative control without using primary antibody (Fig. 2C). The staining pattern was almost the same in the entire 10 normal conjunctiva.

3.3. Significance of D-β-Asp-containing proteins in the development of pterygia

The purpose of the present study was to investigate the localization of D-β-Asp-containing proteins in pterygia. In all of the specimens with pterygia, we found strong immunoreactivity to D-β-Asp-containing proteins elastotic degenerative lesions and in surrounding collagenous lesions in pterygia. The result indicated that D-amino acid-containing proteins may be involved in the development of pterygia.

A controversial hypothesis has been proposed that pingueculae are precursors to pterygia. Compared with the previous paper [11], the localization patterns of D-amino acid-containing proteins were different between pterygia and pingueculae. In surgical samples with pingueculae, D-amino acid-containing proteins were detected only in the elastotic degenerative lesions within the pingueculae. However, in pterygia, the elastotic degenerative lesions were detected in both the elastotic lesions and the surrounding collagenous lesions. Hence, there was a clear difference in the pattern of localization of D-β-Asp-containing proteins between pterygia and pingueculae.

As D-amino acids are non-native, racemization that results in D-amino acid-containing proteins may cause these proteins to be recognized as foreign bodies by the immune system and to be treated as antigens, eliciting an inflammatory response. Previous studies have reported the infiltration of lymphocytes and the accumulation of immunoglobulins in pterygia [26]; however, the cause of inflammation in pterygia has not been fully elucidated. We postulate that autoimmunity against non-native proteins, including D-amino acid-containing proteins, could cause inflammatory reactions in pterygia, leading to buildup of fibrovascular tissue.

In the present study, we used a primary antibody against peptide 3R, the synthetic peptide that corresponds to three repeats of the amino acid sequence at positions 149–153 of human α-A-crystallin. Our previous studies have confirmed that the antibody recognizes