Collagen type IX and HNK-1 epitope in tears of patients with pseudoxfolliation syndrome


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Abstract

Pseudoexfoliation syndrome (PEX) is an age-related condition, which may cause open-angle glaucoma and has increasing interest since it seems to affect additional human tissues, i.e., cardiovascular tissue, skin, and still lacks elucidated pathogenesis. Collagen type IX and HNK-1 epitope have been considered characteristic constituents of the aqueous humour of PEX patients, since their amounts were increased in PEX aqueous humour compared to normal eyes. Since it has been proposed that the initial manifestations of PEX syndrome occur in conjunctiva, the present study was undertaken to investigate the presence of the same antigens in tears of PEX patients and their possible use as the biochemical markers for early diagnosis. Tears of PEX patients and healthy individuals were subjected to western blotting analysis for various basement membrane components identified in aqueous humour. It was found that collagen type IX and HNK-1 epitope were present in tears, the amount of the former being increased 2.7 times compared to normal \((P < 0.05)\), surprisingly high as compared with total protein or lysozyme activity in tears, which were found to be increased in PEX patients about 25% with no statistical differences \((P = 0.4)\). The results suggest the possible use of tears’ collagen type IX for the diagnosis of PEX syndrome.

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1. Introduction

Pseudoexfoliation syndrome (PEX) is an age-related condition of increasing interest since it affects many human tissues, mainly the ocular ones, and still lacks elucidated pathogenesis. The predominant feature of PEX is an abnormal fibrillar extracellular material (exfoliation material, XFM), the ocular production of which results to its deposition mostly at the pupillary border, the anterior lens capsule, the surface of the iris, the ciliary body and the corneal endothelium [1]. This syndrome is frequently associated with increased intraocular pressure and secondary open-angle glaucoma, as well as with an impairment of the blood-aqueous barrier [2].

It has been proposed that the irregular metabolism of basement membrane components such as glycosaminoglycans and proteoglycans may result in the formation of XFM [3,4]. Although the actual pathogenetic mechanism is not clear, numerous immunohistochemical investigations of the XFM have shown a variety of basement membrane epitopes, such as heparan sulphate proteoglycans, chondroitin sulphate, laminin, entactin/nidogen, hyaluronan, fibronecint and HNK-1 epitope [5–11]. In addition, based on the structural association of the XFM fibrils with elastic system components, it has been suggested that the exfoliation material is in fact degenerative elastic microfibrils, which may result from abnormal enzymatic activities [3]. Previous results from our laboratory concerning increased concentration of metalloproteinases in PEX aqueous humour compared to normal aqueous humour seem to be in accordance with this concept [12].

Exfoliation material may arise independently in different locations and all the involved intraocular sites are in close contact with the aqueous humour. It seems that the secondary distribution of XFM by aqueous humour is responsible for deposition of the material on the central anterior lens capsule, the zonules and the anterior hyaloid surface, in addition to its
production by the ciliary epithelium, the iris pigment epithelium, and the preequatorial lens epithelium [2]. In extra-ocular tissues, deposits of XFM have been demonstrated by electron microscopy in the conjunctiva of affected eyes and of fellow eyes in unilateral cases [13]. Interestingly, there are reports that show the occurrence of exfoliation material in the conjunctiva of patients with suspected PEX but without any evidence of ocular XFM [14–16]. It has also been suggested that the conjunctiva may be an additional source of XFM, which seems to precede the clinical recognition of XFM on anterior segment structures. The conjunctiva contains accessory lacrimal glands bearing goblet cells, which secrete the mucin layer, the inner layer of tear film [17]. Therefore, it seems that any alterations in the macromolecular mucous products of these cells would appear in the tears of patients.

In a recent study, we have demonstrated an increase in the amounts of HNK-1 epitope and collagen type IX in PEX aqueous humour compared to normal eyes [18]. HNK-1 presence was expected, since this component is present in most ophthalmic tissues and it has been linked with many ocular diseases [2,19], whereas collagen type IX is identified only in vitreous humour and cornea [20]. We also showed that the two components co-migrated upon SDS-electrophoresis and western blotting and it was proposed that they might represent components of the same macromolecules. The observation that collagen type IX was heard HNK-1 epitope suggested that both or one of these components might be used for diagnostic purposes. The present study was therefore undertaken to investigate the presence of the same antigens in tears of PEX patients and their possible use as biochemical markers for early diagnosis.

2. Materials and methods

2.1. Materials

Monoclonal antibodies against collagen IX (B3-1 and D1-9, recognising its LMW fragment) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA). The polyclonal antibody against collagen type IX was obtained from Chemicon (USA). As a source of HNK-1 monoclonal antibody (recognising 3-sulphoglucuronic acid) the culture supernatant of hybridomas, A.T.T.C. TIB 200 (American Type Culture Bank (University of Iowa, IA, USA). The polyclonal antibody against collagen type IX was obtained from Chemicon (USA). As a source of HNK-1 monoclonal antibody (recognising 3-sulphoglucuronic acid) the culture supernatant of hybridomas, A.T.T.C. TIB 200 (American Type Culture Collection, Rockville, MD, USA), was used. SDS (sodium dodecyl sulphate), acrylamide (twice crystallised), N,N'-methylene-bis-acrylamide and PAGE blue stained with 0.25% Coomassie Blue R 250 in methanol/acetic acid/water (50:10:40) was performed according to standard protocols [25].

2.2. Human samples

Aqueous humour samples were obtained during extracapsular cataract surgery from 37 patients (mean age 78.1 ± 6.1; range 64–90 years) with PEX syndrome and 52 normal controls (non-PEX specimens), (mean age 74.8 ± 9.5; range 56–90 years).

Thirty to 40 µl of aqueous humour was collected by limbal paracentesis; aqueous humour was aspirated from the central pupillary area using a 30-gauge needle attached to a tuberculin microsyringe without touching the iris, lens or corneal endothelium with the needle. Samples were immediately frozen and stored at −70 °C until analysed.

For tears collection, the standardized Schirmer strips of filter paper (Alcon, Ft. Worth, TX, USA) were placed in the lateral canthus for 5 min and the patients were advised to look ahead and to blink normally. In order to limit the reflex lacrimal secretion, care was taken to avoid contact between the strip and the cornea. A minimal length 15 mm of moistened strip was necessary. The strips were left in place until the threshold length was reached. In all samples, however, only the first 10 mm of the strip were used for tear extraction. The saturated portion of the filter paper strip was immediately eluted with 100 µl of 0.154 M NaCl at room temperature for one hour in an Eppendorf tube (Eppendorf, Fremont, CA). Tears samples were immediately frozen and stored at −70 °C until analysed. Tears were obtained from 25 patients (mean age 79 ± 4.1; range 64–90 years) with PEX syndrome and 22 normal controls (non-PEX specimens), (mean age 77 ± 3.8; range 56–90 years).

Prior to surgery, all patients were thoroughly examined for the presence of PEX, with maximal pupillary dilation. Diagnosis of PEX was based on the presence of typical grayish pseudoexfoliative deposits on the anterior lens surface and/or on the borders of the typical central disk or the typical peripheral zone after dilating the pupil. Patients included in this prospective study had normal optic disk and visual field and intraocular pressure < 21 mm Hg in both eyes. Patients with pseudoexfoliation syndrome and ocular hypertension were excluded. Exclusion criteria were considered for both groups: (1) topical medication within the past 6 months; (2) a history of laser treatment or other ophthalmic surgical procedures; (3) eye diseases other than PEX; (4) systemic diseases according to the patients case history and clinical evaluation taking into account the intake of any systemic medication.

All human studies were performed following the guidelines of the Helsinki Accords and were approved by our institutional review board. Informed written consent was obtained from all patients before inclusion in the study.

2.3. Analytical methods

Total protein was determined by the Bradford assay [21] by using 3 µl of sample volume. Bovine serum albumin was used as the reference compound.

Lysozyme activity was measured kinetically. Five µl of tears were mixed with 2.6 ml of a suspension of micrococcus leisodeikticus with initial turbidity at 450 nm of 1.0 absorbance unit. The final suspension was incubated for 30 min at room temperature with concomitant monitoring of its absorbance. From the decrease of the absorbance, the units of lysozyme were calculated.

2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Tear protein composition was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, T: 10%, C: 2.7%) [22]. Samples from tears (1, 2, or 3 µl of initial volume of tears’ eluate) were diluted with water to a final volume of 5 µl, and then mixed with 5 µl of 0.125 M Tris/HCl pH 6.8 containing 2% (w/v) SDS and 20% (v/v) glycerol. The samples were heated to 95 °C for 3 minutes. Electrophoresis was performed in a mini-Protein apparatus (BioRad) at a constant voltage of 200V. The gels were stained with 0.25% Coomassie Blue R 250 in methanol/acetic acid/water (50:10:40) for 30 min at room temperature. Excess dye was removed after three washes with methanol/acetic acid/water (50:10:40).

2.5. Two-dimensional electrophoresis

Isoelectric focusing followed by SDS-PAGE was performed as described by Robertson et al. [23], using the mini protein apparatus. Samples from the aqueous humour (1, 2, or 5µl of initial volume) were diluted with water to a final volume of 5 µl, and then mixed with 5 µl of 60% (v/v) glycerol and 4% ampholyte of pH range 3.5 to 10. The gels were either silver stained [24] or used for Western blotting.

2.6. Western and dot blotting

Electrophoretic transfer of proteins separated by SDS-PAGE or by two-dimensional electrophoresis onto the surface of an immobilizing membrane (Immobilon P, Millipore) was performed according to standard protocols [25]. In
order to monitor visually the success of the transfer and to determine simultaneously the molecular mass of the transferred proteins, prestained protein markers (Pierce) were included in SDS-PAGE along with the tear samples.

After the transfer, the membranes were washed with 0.14 M NaCl in 0.01M phosphate buffer pH 7.2 containing 0.1% Tween 20 (PBS-T) and blocked with 5% dry defatted milk in PBS-T for 1 h at room temperature. Then they were immersed in the appropriate antibody solution diluted (1:1000) in PBS-T and incubated for 1 h at room temperature. The immunoreaction was visualized by using the enhanced chemiluminescence reagents (Amersham, UK) according to the manufacturer’s instructions and by exposure to Agfa Curix X-ray film. The results of the western blots were semiquantified using the Scion Image PC program.

2.7. Statistical analysis

Normality of distribution of values was tested with Kolmogorov–Smirnov test. Results were statistically analysed using the unpaired t-test to detect differences between tears collected from the two patient groups. \( P < 0.05 \) was regarded as statistically significant. For the statistical analysis, the Prism statistical software was used (Prism 3.02 for Windows, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Total protein and lysozyme activity in tears

Since there is no information concerning differences in tears protein composition and alteration in PEX patients, a protein pattern of tears was firstly obtained for normal and PEX samples. As it is shown in Fig. 1, a variation in the protein content of samples could be observed, which would be due to different dilution of protein components into the tears. Therefore, any variation subsequently measured in other macromolecular component(s) might be similarly explained. To overcome this and to obtain results in quantitative terms, tears samples were further analysed for their protein content.

The amount of total protein measured in normal and PEX tear samples ranged from 0.400 mg/ml to 1.442 mg/ml (mean value: 0.921 ± 0.52 mg/ml) and from 0.526 mg/ml to 1.792 mg/ml (mean value: 1.159 ± 0.63 mg/ml), respectively. This corresponded to an increase of protein content by 25% in PEX tears, which was not statistically significant compared to normal samples (\( P = 0.35 \)) (Figs. 1A).

Since no statistical difference was observed in protein, we proceeded to the quantitative analysis of a specific component of tears, lysozyme, which in addition is produced by the lacrimal glands; therefore it might be regarded as more characteristic macromolecule of tears. Similarly as above, the units of lysozyme measured in the various samples were not statistically different between normal and PEX samples (\( P = 0.43 \)), although high variations appeared to be present in both groups, i.e., from 0.936 units/\( \mu l \) to 3.043 units/\( \mu l \) in normal and from 1.154 units/\( \mu l \) to 3.509 units/\( \mu l \) in PEX samples (Fig. 1B).

3.2. Extracellular matrix components in tears

The work was focused in those components that had previously been identified in the aqueous humour [18], collagen type IX and HNK-1 being the most characteristic. Collagen type IX was immunodetected in four bands corresponding to molecular mass of 120, 113, 56 and 43 kDa (Fig. 2, A, inset), identical to which was observed when examined in aqueous humour samples. A slightly different migration pattern was observed in the case of HNK-1 epitope, which was immunodetected in three bands corresponding to molecular masse of 120, 113 and 56 kDa (Fig. 2B, inset). Semiquantitative analysis of the results (Fig. 2) suggested a surprisingly high increase of collagen type IX in PEX samples as compared with that of HNK-1 epitope. Collagen type IX increase was about 270% to normal (\( P < 0.05 \)), whereas HNK-1 epitope was increase by only 25%, a figure similar to that of total protein.

The observation of the different immunostaining patterns between collagen type IX and HNK-1 epitope might be explained by the presence in tears of a collagen type IX band lacking HNK-1 epitope. The substantially higher
increase of collagen type IX in tears as compared with that of HNK-1 epitope, however, suggested that these components would be located at different macromolecules, at least in tears. This observation was further examined in aqueous humour samples by the application of two-dimensional electrophoretic analysis (Fig. 3). Indeed, the results obtained suggested that immunostaining for collagen type IX occurred in spots of pI ranging from 6.5 to 7 with molecular mass of 120 kDa, whereas that for HNK-1 epitope occurred in spots of pI 5.4 and molecular mass of 120 kDa, 113 kDa and 93 kDa and in spots of pI from 5.1 to 4.6 with a molecular mass of 93 kDa.

4. Discussion

PEX syndrome is an age-related disease with many ocular manifestations and it has been proposed that extra-ocular manifestations are also observed. Among the ocular manifestations, the most common is the precipitation of the pseudoexfoliation material (XFM) in ocular tissues. XFM is constituted by a variety of basement membrane components and most of these have also been identified in aqueous humour [7,10,18]. In addition, MMPs [12] and growth factors [26] have also been identified in aqueous humour, in increased concentrations in PEX compared with normal samples. Growth factors might alter the surrounding cell behaviour leading to their abnormal metabolic properties [11], one of these being the increased MMPs concentration. The possibility that the disease occurred before the precipitation of XFM in the anterior segment of the eye has also been proposed, since the presence of this material has been observed in the conjunctiva of the apparently unaffected eye in unilateral cases of this syndrome [16]. Thus, the initial PEX manifestations could be observed extra-ocularly and consequently, tear film constituents might be totally affected.

Based on this information, the present study was undertaken to investigate the presence of specific macromolecular components in tears and to examine their possible relation with PEX. Tear samples were collected by absorption with a Schirmer filter paper strip that directly irritates the non-anesthetized conjunctiva without any other stimulation of tearing, e.g., by irritation of the nasal mucosa. Although minor reflex tearing induced by this collection procedure is difficult to control, several techniques have been used to avoid overt reflex tear stimulation. The capillary tube collection method has been proposed as an alternative method of choice for reflex tear collection for biochemical studies. Previous studies have been demonstrated no significant differences between levels of the lacrimal proteins, lactoferrin and lysozyme, in tear samples obtained either with Schirmer strips or capillary tubes [27]. Therefore, bias from collection method probably has exerted only a minimal effect on the results.

The total protein and lysozyme contents were initially analysed to investigate any effect of PEX syndrome in the most characteristic constituent of tears. A small but not significant increase of total protein in PEX tears was observed, which correlates with lysozyme catalytic units. This increase might be
related to xerophthalmia, which was also observed by others [28] in PEX patients, but it was not significant in the samples examined. By examining the other constituents, also identified in the aqueous humour of PEX eyes [18], a substantial increase of collagen type IX was observed. This specific type of collagen is present in vitreous humour and cornea and its identification in other ophthalmic sites might be due to degenerating conditions. Other macromolecular constituents, such as MMPs and dermatan sulphate, identified in the aqueous humour of PEX patients in increased amounts as compared with normal samples, did not show any variations when examined in tear film. This observation was unexpected, since HNK-1 epitope was found to co-migrate with collagen type IX and it was postulated that HNK-1 epitope might be a component of this type of collagen [18]. This was further examined by analysing collagen type IX and HNK-1 epitope with two-dimensional electrophoresis, from which it was found that these components were well separated upon applying the isoelectric focusing step. This observation clearly explained the differential increase of collagen type IX and HNK-1 epitope and the different protein bands migration observed in PEX tears. Whether this is due to the altered basic features of goblet cells [29], it is not investigated.

Since collagen type IX might be identified in normal tears and its amounts were increased during PEX syndrome, the results of the present study suggested that this constituent might be applied for diagnostic purposes.

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References