Collection and concentration of tear proteins studied by SDS gel electrophoresis

Presentation of a new method with special reference to dry eye patients

Abstract An obvious obstacle in the analysis of tear film samples from patients with dry eyes is the drastically reduced tear volume. To overcome this problem we have developed a method by which the surface of the eye is flushed with saline, diluting the sparse tear fluid, followed by a concentration of the wash fluid. We compared undiluted tears, diluted tears, tear fluid collected by flushing, reconcentrated diluted tears and reconcentrated flush fluid. Gel electrophoresis of the tear samples obtained showed a representative collection of tear proteins, with bands ranging in molecular weight from about 10 kDa to about 90 kDa. The method may therefore provide a useful alternative to the analysis of undiluted tear fluid in such patients. After storage at -80°C for 1 month, electrophoresis often showed some weakening of band intensities. However, no specific loss of bands was found, and by ultracentrifugation a band profile similar to that of fresh, undiluted tears was obtained. For most purposes, therefore, samples may be kept by this means for later analysis.

Introduction

The tear film is essential for the maintenance of normal corneal function, and disturbances in its composition may lead to corneal dryness, ulcerations or filamentary keratitis, and in serious cases impaired visual function [3]. Tear fluid alterations may be found in disorders localized to the eye region, but may also be seen in systemic diseases, e.g., AIDS, hypervitaminosis A and certain connective tissue diseases such as rheumatoid arthritis, systemic lupus erythematosus and primary Sjögren’s syndrome [2,5]. Knowledge of the composition of tear film in health and in disease is essential when evolving new treatments for tear fluid disturbances, and it is hoped may also be useful in the development of new diagnostic tests.

The study of the composition of tear film in patients with severe dry eye is complicated, however, by the difficulties in collecting sufficient tear fluid. In many cases, no sample at all can be obtained. To overcome this problem, we present a method in which saline is dripped into the eyes and then collected (referred to in the following as “eye flush fluid”), followed by a concentration of protein in the eye flush fluid. For practical purposes, it is often convenient to store tear fluid and eye flush fluid for later analysis. We kept samples at -80°C and analysed by electrophoresis the possible loss after storage of specimens for 1 month.

Materials and methods

Tear samples were collected from 10 healthy volunteers and from five patients with primary Sjögren’s syndrome. All patients were diagnosed according to the Copenhagen criteria [6]. In short, they fulfilled two of three criteria for keratoconjunctivitis sicca (Schirmer-I test < 10 mm/5 min, break-up time ≤ 10 s, Rose Bengal score ≥ 4) as well as two of three criteria for xerostomia (abnormal salivary gland scintigraphy, unstimulated whole sialometry ≤ 1.5 ml/15 min, lower lip biopsy focus score > 1). No transepithelial corneal ulcers or stromal lesions were found in any of the cases. Mean duration of diagnosed disease was 10 years, ranging between 3 and 19 years. Studies were conducted according to the principles established in the Declaration of Helsinki and were approved by an ethics committee.
Tear collection

Samples of pure, undiluted tear fluid were collected in 50-μl glass capillaries placed with one end at the lateral canthus in contact with the tear film. To keep tear stimulation at a low level, contact with the conjunctival epithelium was minimized.

After the subject had rested for approximately 5 min, about two drops of saline were poured into each medial canthus. The subject was asked to blink a few times, and the fluid was collected from the lateral canthus by glass capillaries. The number of drops used was adjusted so that about 150 μl of eye flush fluid, the volume necessary in this study, could be obtained.

Storage

All samples were cooled to +5°C immediately after collection and analysed within a few hours. Some of the samples of undiluted tear fluid, tear fluid diluted with distilled water 1:3 (to minimize deleterious evaporation during storage because of small volumes), as well as eye flush fluid from six volunteers was stored at −80°C for 1 month and then analysed again.

Sample concentration prior to SDS-polyacrylamide gel electrophoresis

Proteins in diluted tear and eye flush fluid were concentrated by ultracentrifugation (2000 g for 30–40 min) of samples in filter units (Ultrafree-MC, Millipore, UFC3 LGC). The Ultrafree-MC unit incorporates a membrane-bottom sample cup in a standard 15-ml microcentrifuge tube and is designed for sample preparation of proteins in diluted tear and eye flush fluid were concentrated by electrophoresis with cut-off values of 5 kDa and 10 kDa.

Electrophoresis

Electrophoresis was carried out by Pharmacia's Phastgel system. The following samples from each person were analysed: undiluted tear fluid, tear fluid diluted with distilled water 1:3, diluted tear fluid (1:3) after concentration, eye flush fluid, eye flush fluid after concentration and molecular weight standards (Pharmacia molecular weight calibration kit). Proteins were denatured before gel electrophoresis by mixing with sample buffer (1 mM Tris/HCl, 0.1 mM EDTA, 2% SDS, 10 mM DTT, 0.02% bromphenol blue, 0.25% glycerol) and heating to 100°C. Immediately after denaturation, samples were applied to a gradient polyacrylamide gel (PhastGel Gradient 8–25) with a 13-mm stacking zone and a 32-mm gradient zone. The gradient zone was continuous 8–25% with 2% crosslinking. The buffer system in the gels is of 0.112 M acetate and 0.112 M Tris, pH 6.4. The buffer strips (PhastGel SDS Buffer Strips) were made of 2% agarose IEF containing a buffer system of 0.20 M tricine, 0.20 M Tris and 0.55% SDS. In the Phast system, operations for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 8–25 separation are preferably carried out at 250 V, 10 mA, 3.0 W, 15°C, 65 Vh. After migration, the gels were stained with Coomassie blue (stain: 0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid in distilled water; destain: 30% methanol and 10% acetic acid in distilled water; preserving solution: 5–8% glycerol and 10% acetic acid in water).

Molecular weight determination

The PhastGel Gradient 8–25 gives a linear relationship between a protein's relative mobility \( R_f \) and the logarithm of its molecular weight (MW) for the MW range 6–300 kDa for SDS-denatured proteins. The relative mobility \( R_f \) is defined as the distance the protein has migrated from origin divided by the distance from origin to reference point (the end of the gradient gel). The approximate MW of each band could therefore be obtained by comparing the relative mobility of each protein with those of standard MW markers (Fig. 1). The migration distance may be estimated as the distance from point of application to the front border or middle of the band. In this study front borders were used as these are well defined, whereas the middle of a band can be difficult to determine, especially when bands are close together and may overlap. However, no significant difference was found between the two methods when MW were calculated, although the values were a little lower using the front border when dealing with broad bands.

Results

A sample size of 0.5–1 μl per well was sufficient to procure well-defined bands in most cases. Increasing the sample volume did not reveal more bands, but in cases displaying very faint bands up to 6 μl per well could increase band intensity.

When denaturing samples, we tested length of boiling period and volume of sample buffer added. In most cases, boiling for 5 min and buffer of volume equal to the samples was adequate. However, this treatment was not always sufficient in samples with high protein concentration, as indicated by the disappearance of bands in such cases after boiling was repeated and more sample buffer added. We therefore recommend it as standard to boil for about 10 min and to add sample buffer in the ratio 2:1 to ensure complete denaturation of proteins.