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Research article

Protein profiles in cortical and nuclear regions of aged human donor lenses: A confocal Raman microspectroscopic and imaging study

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ABSTRACT

A combination of Raman spectroscopy, imaging, hierarchical cluster analysis (HCA) and peak ratio analysis was used to analyze protein profiles in the superficial cortex (SC), deep cortex (DC) and nucleus of old human lenses with cortical, nuclear and mixed cataracts.

No consistent differences were observed in protein spectra and after cluster analysis between the three locations irrespective of the presence or absence of cortical opacities and/or coloration. A sharp increase (~15%–~33%) in protein content from SC to DC, normal for human lenses, was found in 7 lenses. In 4 lenses, characterized by the absence of cortical opacities, the SC has a protein content of ~35%. A significant increase in the disulfide-to-protein ratio is found only in the SC of the 7 cortical cataracts. No changes were found in sulfhydryl-to-protein ratio. The relative contents of α-helices and β-sheets increase from SC to nucleus. β-Sheets are more common in the SC of lenses with cortical cataract.

The absence of significant and consistent changes in protein profiles between nucleus and cortex even in cases of severe coloration is not favoring the prevailing concept that ubiquitous protein oxidation is a key factor for age related nuclear (ARN) cataracts. The observations favor the idea that multilamellar bodies or protein aggregates at very low volume densities are responsible for the rise in Mie light scatter as a main cause of ARN cataracts leaving the short-range-order of the fiber cytoplasm largely intact. The absence of significant changes in the protein spectra of the deep cortical opacities, milky white as a result of the presence of vesicle-like features, indicate they are packed with relatively undisturbed crystallins.

1. Introduction

The purpose of the eye lens is to project a sharply focused, undistorted image of the visual surround on the neural retina. Requirements securing this purpose are transparency, a high refractive index, accommodative power and low scattering properties which are realized by specific structural and functional properties of the lens.

The inherent transparency of the eye lens is due to the absence of blood cells and blood vessels and the large organelle free zone encompassing the deep cortex and core attributable to the pre-programmed elimination of nuclei and cell organelles of elongating lens fibers (Bassnett, 2009; Dahm et al., 2011; Vrensen et al., 2004).

The human eye lens is characterized by a sharp gradient in refractive index (RI) and protein content. Within about 0.5 mm from the capsular surface RI is rising from ~1.37 in the epithelium and elongating fibers plateauing to a constant level of ~1.42 in the cortical and nuclear regions (Augusteyn, 2008). Accommodation amplitude (AA) decreases with age from 19 to 2 D due to changes in the nuclear stiffness and decline in the functioning of the ciliary body (Dubbelman et al., 2003). This decline in AA is a main factor for presbyopia.

The low scattering properties of lens fibers are due to the short-range-order of the crystallins (Delaye and Tardieu, 1983), the absence of refractive index differences between cytoplasm and membranes of the fibers (Michael et al., 2003) and the refractive

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index matching between the cytoplasm of adjacent cells achieved through the formation of cellular fusions that allows the intermingling of proteins (Bassnett et al., 2011).

The eye lens grows throughout life. Post-mitotic epithelial cells in the equatorial zone continuously form new fibers all be it at a low rate. Cohorts of fibers are stacked one upon another in rows and are held together by complex interdigitating protrusions (Bassnett et al., 2011; Dickson and Crock, 1972; Kuszak et al., 2004; Vrensen and Willekens, 1990). The lens is a closed system and, in contrast with most other tissues, there is no shedding of fibers during its lifetime. Furthermore in the absence of metabolic activity in the organelle free zone there is no turnover of the crystallins. Hence lens fiber proteins are as old as their time of origin i.e. central fibers are as old as the individual. They are therefore subject to aging factors for long periods which eventually lead to cortical and nuclear cataracts (Lou, 2003; Michael and Bron, 2011; Vrensen, 2009). It is advocated that these changes are accompanied or induced by conformational changes and aggregation of crystallins and disturbance of the short range order (Truscott, 2005).

Although many factors have been proposed to contribute to age related cataract in humans, recent data indicate that aging as such is by far the most prominent causative factor (Michael and Bron, 2011; Truscott, 2000, 2005). Based on slitlamp, Scheimpflug and retro-illumination images three primary types of age-related cataracts can be distinguished; nuclear (NC), cortical (CC) and posterior sub-capsular (PSC) cataracts. In addition many lenses show mixed types of cataract. The sample of donor lenses used in this study, indeed represent a broad spectrum of age related nuclear and cortical cataracts (Fig. 1). PSC's are far less common in the population (<1%) (Sasaki et al., 2004) and are absent in the sample.

Nuclear cataracts are clinically characterized by an age-related increase in straylight, coloration and fluorescence of the lens core, starting to have significant vision effects around the age of 40–50 years (Burd et al., 2012; Truscott, 2000, 2005; van den Berg, 1996; van den Berg and IJspeert, 1995). Coloration and fluorescence of the lens nucleus are caused by accumulation of chromophores induced by UV-irradiation and oxidative stress (Davies and Truscott, 2001). Oxidative stress also leads to changes in crystallins as racemization, deamidation and truncation of crystallins, loss of sulfhydryl groups of cysteine and methionine and to aggregation of proteins. A decline in the availability of glutathione (GSH) around the age of 40–50 years is proposed as being a main cause of this effect of oxidation in old age (Michael and Bron, 2011; Truscott, 2005).

In a recent paper Michael et al. (2014) showed that Raman microspectroscopy is a powerful analytical tool to approach local changes in protein and lipid profiles with a resolving power at the sub-μm level. In the present study we used this technique to analyze the protein profiles of superficial cortical, deep cortical and nuclear regions of old human autopsy lenses with nuclear, cortical and mixed cataracts.

2. Material and methods

Lenses from 11 donors (Fig. 1) were provided by the Banco de Ojos para Tratamientos de la Ceguera, Barcelona in collaboration with the Neurological Tissue Bank of the Biobank-Hospital Clinic-IDIBAPS (NTB-IDIBAPS), Barcelona. Written informed consent for removal of the eyes for diagnostic and research purposes was obtained from the patients and/or their relatives. The research adhered to the tenets of the Declaration of Helsinki on research involving human subjects.

Cortical cataracts were graded for percentage of lens circumference affected by cortical opacities and their extension from the lens periphery towards the optical axis. Nuclear cataracts were graded in a combined scale for opacity and coloration on a scale between 0 and 10. Grading was performed by three independent ophthalmologists, and the mean grading values are given in Table 1. Lens tissue was fixed in 3.6% PBS buffered formaldehyde for 4 weeks. Half lenses were cut with parallel placed razorblades in slices of about 0.5 mm thickness and stored in PBS for Raman analysis. The cortex of the lenses was analyzed at about 0.15 mm (superficial cortex) and 0.7 mm (deep cortex) below the equatorial lens capsule. Cortical opacities are usually located at 0.7 mm. The nucleus was analyzed at about 4 mm below the equatorial lens capsule (Fig. 2).

Non-resonant Raman spectroscopy and imaging experiments were performed on a previously described laser-scanning confocal Raman microspectrometer (Pully et al., 2010). The system consist of a Krypton laser (Innova 90-K; Coherent, SantaClara, CA, λ_{exc} 647.1 nm), an upright microscope BX41 from Olympus with an objective Olympus, Plan Apochomat, 40×, 0.95NA, coverslip corrected for illumination of the sample as well as for collection of Raman scattered photons. The home-built spectrograph was optimized for broadband (20–3670 cm^{-1}) high-wave number-resolution (1.85–2.85 cm^{-1}/pixel) Raman microspectroscopy. Imaging experiments were performed by raster-scanning the laser beam over a region of interest with a step size of approx. 0.5 μm (30 × 30 μm^2 containing 64 × 64 spectra) with a dwell time per pixel of 50 ms. A full image of each area was thus acquired in ~205 s with a laser power of 35 mW. Noise in the resulting 3D (spatial × spatial × spectral dimension) data matrix of 4096 spectra times 1600 frequencies was reduced by singular value decomposition (Uzunbajakava et al., 2003). In the three areas (Fig. 2) of each lens three Raman datasets per area were acquired to a total of 99 Raman datasets, each containing 4096 Raman spectra. All together the analysis concerned 405,504 Raman spectra.

The main objective of this study is the analysis of the difference in protein profiles of the three lens regions indicated. Because the lens mainly consists of lens fibers with a high protein content and have no cell organelles, except for a small equatorial region, integrated spectra over the 30 × 30 μm^2 areas can be considered to reflect the protein profile of the region measured. Three measurements per region are averaged and the background, mainly due to fluorescence, is removed by subtracting a linear function (Fig. 2). For the so-called fingerprint region (700–1800 cm^{-1}), this function was obtained by a linear fit through the intensity at 800 cm^{-1} and at 1830 cm^{-1}. For comparison of the profiles of the three areas, the spectra are normalized for protein using the 1450 cm^{-1} protein band as described by Siebinga et al. (1992) (Fig. 2). For the high frequency region (2700–3700 cm^{-1}), this function was obtained by a linear fit through the intensity at 2440 cm^{-1} and at 2770 cm^{-1} (Fig. 2).

Protein mass percentage (PMP) was calculated according to Huizinga et al. (1989) from the background corrected ratios of the Raman peaks at 3390 cm^{-1} (water peak) and 2935 cm^{-1} (CH-stretch of proteins). For the relative contributions of β-sheets (1668 cm^{-1}), α-helices (1265 cm^{-1}), phenylalanine (1000 cm^{-1}), tyrosine (857 cm^{-1}), disulfide (S–S)(508 cm^{-1}) and sulfhydryl (SH)(2581 cm^{-1}) the ratios of the corresponding Raman signals and the protein Raman signal at 1450 cm^{-1} were calculated for all the lenses and sites. Mean values of the ratios were calculated as well as the coefficient of error. We also calculated the coefficient of error for the full spectra of the 11 lenses. Differences were tested using Student's t-test.

Another point of interest is the problem of the occurrence of specific objects within the measured areas as for instance protein aggregates. To this end hierarchical cluster analysis (HCA) was performed on Raman imaging data matrices to visualize regions with high Raman spectral similarities. No baseline subtraction was performed prior to principal component analysis (PCA). In the cluster analysis routine scores derived from PCA were taken as input variables, squared Euclidean distances were used as distance