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The nanomechanical role of melanin granules in the retinal pigment epithelium

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Abstract

Nanomechanical properties of cells and tissues, in particular their elasticity, play an important role in different physiological and pathological processes. Recently, we have demonstrated that melanin granules dramatically modify nanomechanical properties of melanoma cells making them very stiff and, as a result, less aggressive. Although the mechanical effect of melanin granules was demonstrated in pathological cells, it was never studied in the case of normal cells. In this work, we analyzed the impact of melanin granules on nanomechanical properties of primary retinal pigment epithelium tissue fragments isolated from porcine eyes. The obtained results clearly show that melanin granules are responsible for the exceptional nanomechanical properties of the tissue. Our findings suggest that melanin granules in the retinal pigment epithelium may play an important role in sustaining the stiffness of this single cell layer, which functions as a natural mechanical barrier separating the retina from the choroid.
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Key words: Nanomechanical properties; Elasticity; Atomic force microscopy; Retinal pigment epithelium; Melanin granules; Blood-retinal barrier

Retinal pigment epithelium (RPE), a single layer of cells containing the pigment melanin, has several functions, such as light absorption, epithelial transport, spatial ion buffering, visual cycle, phagocytosis, secretion and immune modulation that are of key importance for the survival and proper function of the photoreceptor cells.[1] In pigmented tissues, including the RPE, melanin appears in the form of melanosomes – organelles displaying a range of different shapes and sizes.[2] Interestingly, melanin granules were found to have unusual nanomechanical properties being very stiff and hard to deform.[3] It should be emphasized that nanomechanical properties of cells and tissues play an important role in different physiological processes[4,5] and in certain pathologies.[6,7] Recently, we have shown that the presence of stiff melanin granules in melanoma cells dramatically modifies elastic properties of the cells,[8,9] making them less aggressive.[10] Although the nanomechanical role of melanin granules have been clearly shown in pathological cells, it has never been demonstrated in the case of normal cells.

In normal tissues, melanin presence is most apparent in the skin, where it's content can vary significantly and strongly depends on racial and environmental factors.[11] Moreover, melanin pigmentation in the skin is subjected to continuous metabolisms. This makes it very difficult to examine the mechanical role of melanin granules in the epidermis. On the other hand, melanin content in the retinal pigment epithelium is independent of racial and environmental factors and shows little metabolic turnover after being synthesized during fetal development.[12] Although the main biological function of melanin in pigmented tissues is related to photoprotection,[13] the role of melanin in the RPE is still under extensive scrutiny. It is generally believed that melanin contributes to visual acuity by

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preventing light reflection from the fundus that may otherwise give rise to spurious signals, and protects the retina against light-induced oxidative stress.[14] However, other important functions such as mechanical stabilization of the RPE should also be considered. Importantly, RPE is part of the blood-retinal barrier (BRB) that separates the retina from the choroid.[15] The breakdown of the BRB has severe consequences for proper function of the posterior segments of the eye and occurs in several pathological conditions such as mechanical disruption, hydrostatic factors, metabolic diseases, inflammation and age-related macular degeneration.[16] This points to the unexplored nanomechanical properties of RPE, particularly the role of melanin granules in sustaining the stiffness of the BRB tissue.

We address this issue in an *ex vivo* study of primary retinal pigment epithelium tissue fragments isolated from porcine eyes by analyzing the elastic properties of RPE cells employing atomic force microscopy and spectroscopy (AFM/S) technique. Our findings demonstrate that melanin granules have an important impact on the nanomechanical properties of the RPE tissue.

Methods

Primary Retinal Pigment Epithelium Tissue Fragments

RPE tissue fragments were isolated from porcine eyes based on protocols established for the isolation of human RPE as described elsewhere.[17] In brief, eyecups were prepared by dissecting the anterior segments and removing the vitreous and retina to expose the RPE monolayer. The RPE fragments were scraped from eyecups using a surgical scalpel and placed in multi-well plates containing glass coverslips. Samples were then incubated for 24 hours in MEM culture medium supplemented with 10% fetal bovine serum (FBS) and antibiotics to ensure that the fragments adhere tightly to glass coverslips for AFM analysis.

Melanin Isolation and Determination

For melanosome isolation, RPE cells were homogenized in phosphate buffered solution (PBS) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). Granules were then purified by ultracentrifugation in a discontinuous sucrose density gradient, according to the protocols described elsewhere.[18,19] Purified melanosome fraction, identified as a black pellet at the bottom of the centrifuge tube, was washed and resuspended in a small amount of PBS (pH 7.4). Determination of melanin in the cell samples was made using electron paramagnetic resonance (EPR) spectroscopy.[20] EPR was used because of high specificity of the technique in melanin detection and characterization. Other advantages of EPR, compared to alternative methods, such as photometric analysis, are nondestructive character of the technique and its ability to measure melanin in complex systems with high selectivity and sensitivity. This makes EPR a method of choice for melanin determination. For EPR analysis, 10^9 granules were suspended in PBS, frozen in liquid nitrogen and stored at 77 K. EPR measurements, were carried out in liquid nitrogen, using a standard fingertip quartz dewar and EMX-AA spectrometer (Bruker BioSpin) operating at X-band with 100 kHz magnetic field modulation. Synthetic L-Dopa at a concentration of 0.57 mg/ml was used as standard. Detailed description of EPR analysis can be found elsewhere.[21]

Atomic Force Microscopy

AFM analysis was conducted using a Bioscope Catalyst AFM (Bruker) coupled with an inverted optical microscope AxioObserver Z1 (Zeiss). RPE tissue fragments were analyzed in culture medium at 37 °C. AFM images of the tissue fragments were made using PeakForce Tapping mode. The use of this mode allowed better control of the force exerted on the cells, which is crucial when examining delicate biological samples. The employed mode also enabled high amplitude of cantilever oscillation, which is important when examining samples with high roughness such as tissue fragments. In addition, the PeakForce Capture was turned on, which resulted in acquiring a force-curve in each pixel of an image. For AFM imaging of RPE tissue fragments, a relatively soft cantilever was used with a nominal tip radius of 20 nm and with experimentally determined spring constant of 0.68 N/m (Bruker Probes). AFM analysis of melanosomes was performed on purified, unfixed melanin granules adsorbed onto freshly cleaved mica surface. It should be emphasized that melanin granules are too small for optical microscopy analysis due to limited resolution of the technique. On the other hand, electron microscopy, which is often used, requires the sample to be fixed, dried, covered with metal coating, and for transmission electron microscopy, cut into thin slices. Since AFM analysis can be performed in liquid environment and requires virtually no sample preparation, this method was found ideal for melanosome examination. Images of melanosomes were obtained in Tapping AC mode in PBS buffer at room temperature. Nanomechanical analysis of cells and melanosomes was made in force spectroscopy mode, which consisted of measuring force-displacement curves. In the case of cells, 20–30 force curves were taken from a single cell, which was selected using an optical microscope. 40 pigmented and 40 non-pigmented cells were analyzed. In the case of melanosomes, an AFM image was first acquired to precisely position the AFM tip on top of the granule. Then 5–10 force curves were collected from individual granules. A total number of 20 melanosomes were analyzed. For cells, a soft cantilever was used with a nominal tip radius of 20 nm and with experimentally determined spring constant of 0.01 N/m, whereas for melanosomes, a stiff cantilever with a nominal tip radius of 10 nm and with a spring constant determined to be 45 N/m was chosen (Bruker Probes). Spring constants of the used cantilevers were determined based on the thermal tune procedure as described elsewhere.[22] Data analysis of the obtained force-curves from both PeakForce Tapping and force spectroscopy was performed using AtomicJ software. In brief, force-displacement curves were converted into force-indentation curves and fitted with an appropriate model. In the case of cells, where indentation was large, the Sneddon model was used, whereas in the case of melanosomes in which low indentation was obtained the Hertz model was employed. Detailed information on the analysis of force curves can be found elsewhere.[23]

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