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The Influence of Substrate Elastic Modulus on Retinal Pigment Epithelial Cell Phagocytosis

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Abstract

To better understand if a complex process such as phagocytosis is influenced by substrate stiffness, we investigated the influence of substrate elastic modulus on phagocytosis in the retinal pigment epithelial (RPE) cell line ARPE-19. RPE cells lie on Bruch's membrane, directly under the retina, and phagocytose the shed photoreceptor outer segments. Bruch's membrane is known to increase in stiffness by an order of magnitude with age and thus, this study has potential relevance in explaining retinal changes in age-related macular degeneration.

ARPE-19 cells were plated on laminin-coated polyacrylamide substrates of varying elastic modulus. After 14 days in culture, a solution of latex fluorescent beads suspended in PBS was placed in each well. After an incubation time of 4 hours, flow cytometry was performed to determine the number of cells that phagocytosed a bead. The number of ARPE-19 cells that phagocytosed a bead decreased continuously as a function of increasing substrate elastic modulus ($p=0.0135$), and this was found to be a linear relationship (slope$=-0.03305 \pm 0.01104$, $R2 =0.4726$ per 10,000 cells).

Our results suggest that RPE cells display decreased phagocytosis when grown on firmer substrates, and thus, RPE cells in older eyes, in which Bruch's membrane is stiffer, may demonstrate decreased phagocytosis. Impaired phagocytosis by RPE cells may contribute to impaired metabolism of photoreceptor outer segments and to development of macular

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degeneration. Material stiffness may be a critical parameter in the development of neural therapies, including retinal prosthetics and stem cell therapies.

Keywords

Mechanotransduction; Retinal Pigment Epithelium; Phagocytosis; Flow Cytometry

Introduction

The elastic modulus of the environment in which a cell exists has recently been shown to influence cell processes such as proliferation (Janmey *et al*, 2009), motility (Pelham and Wang, 1998), and gene expression (Pelham and Wang, 1998) in many cell types. We sought to study the influence of substrate stiffness upon a complex cellular process and, as a model, selected phagocytosis by retinal pigment epithelial (RPE) cells.

The biomedical relevance of this research is based upon the fact that Bruch's membrane, the basement membrane of the RPE cells, is directly under the retina and is known to increase in stiffness by an order of magnitude (from 1,000 Pa to 10,000 Pa) with age (Fischer, 1987). To better understand whether phagocytosis by RPE cells is influenced by substrate stiffness, we investigated the influence of substrate elastic modulus on phagocytosis in the RPE cell line ARPE-19.

The most common form of age-related macular degeneration is the non-exudative, or dry form. RPE cells are thought to play an important role in the development of dry macular degeneration because they are located on Bruch's membrane, directly under the retina and provide metabolic support for the photoreceptor cells (rods and cones) (Kevany and Palczewski, 2010). As patients age, RPE cells have been found to undergo structural changes including accumulation of the pigment lipofuscin, loss of melanin, and accumulation of drusen on Bruch's membrane (Dunn, 1996).

The process of non-exudative macular degeneration may be initiated by the failure of RPE cells to phagocytose the shed outer segments of photoreceptors (Green, 1999). This may result in an accumulation of drusen (deposits of lipids and calcium (Dunn, 1996)) adjacent to the basement membrane of RPE cells (Boulton and Wassell, 1998), atrophy of the retinal pigment layer below the retina, and loss of photoreceptors in the macula (Green, 1999), leading to vision loss.

The aim of the present study was to examine the influence of the mechanical properties of the cell culture substrate upon phagocytosis in RPE cells.

Methods and Materials

Preparation of glass coverslips

Cover slips were chemically activated to allow stable, covalent formation of polyacrylamide sheets according to the protocol of Pelham and Wang (Pelham and Wang, 1998), with modifications (Davis *et al*, 2012). Briefly, a glass cover slip (No. 1, 15 mm diameter; Fisher

Scientific, Pittsburgh, PA) was coated with a small drop of 3-aminopropyltrimethoxysilane (TESPA, Sigma, St. Louis, MO), which was spread evenly on the surface. After 5 minutes, the coverslips were washed extensively with distilled water and then were autoclaved (121°C at 1.5 atm) for 1 hour. The coverslips were transferred, treated side up, into plastic petri dishes and covered with 0.5% glutaraldehyde in phosphate buffered saline (PBS) (prepared by diluting 1 part of 70% stock solution, Polysciences, Inc., Warrington, PA, with 140 parts of PBS). After incubation at room temperature for 30 minutes, the coverslips were washed 5 times in distilled water on a shaker, for 10 minutes per wash, and allowed to dry in air. The treated coverslips were stored in a petri dish for up to 48 hours after preparation.

Creation of Polyacrylamide Gels

Thin sheets of polyacrylamide gel were prepared and bonded to the activated glass surface of the cover slips according to Pelham and Wang (Pelham and Wang, 1998) with modifications (Davis *et al*, 2012). Acrylamide (Bio-Rad, Hercules, CA, 30% w/v) was mixed with N, N-methylene-bis-acrylamide (BIS, Bio-Rad, 2.5% w/v) and distilled water to obtain a final concentration of 10% acrylamide and 0.03% BIS. For more rigid or more flexible substrata the percentage of BIS was increased or decreased according to a previously established nonogram (Pelham and Wang, 1998). After the acrylamide/BIS solution was combined, polymerization was initiated by addition of ammonium persulfate (10% w/v solution, Bio-Rad, 1:200 volume) and N,N,N,N-tetramethylethylenediamine (TEMED, Bio-Rad, 1:2000 volume). Twenty-five microliters of the acrylamide solution was immediately placed onto the surface of an activated coverslip and the droplet was flattened using a large circular cover slip (No. 1, 22 mm diam., Fisher Scientific) covered in Sigma-Cote (SL-2 100ml, Sigma-Aldrich). After polymerization for 30 minutes, the circular cover slip was removed, and the gels were agitated on a shaker in 500 μl HEPES (50 mM, pH 8.5) and then transferred to a 24 well plate. The HEPES was removed from the wells and a solution of 40 μl of Sulfo-SANPAH (0.5mg in 1ml of distilled water, Pierce Chemicals, Rockford, IL) was applied to each gel. The surface of each gel was then exposed to UV light from a 30 W germicidal lamp for 10 minutes. The glass-supported polyacrylamide sheets then underwent three washes of 500 μl HEPES (50mM, pH 8.5). The polyacrylamide sheets were then covered with a 200 μl solution of laminin (1mg natural mouse laminin in 5.95ml HEPES buffer, Invitrogen™) and were incubated at 37°C for 4 hours in 5% CO_2. The gels were then washed three times with 500 μl of PBS to prepare the polyacrylamide coated cover slips for cell culture. The gels were immersed in 500 μl of RPE media (225ml Dulbecco's modified eagle medium, 25ml of fetal bovine serum, and 1% penicillin streptomycin) 15 minutes prior to applying ARPE-19 cells at 20,000 (cells/ ml) per gel. Substrates of 500 Pa, 1000 Pa, and 5000 Pa were created for this study.

Cell Culture

ARPE-19 cells (American Type Culture Collection (ATCC), Manassas, VA) were grown on the gels at 37°C for 14 days in 5% CO_2, in 500 μl of RPE media and the media was changed every other day. After 14 days in culture, 35 μl of bead solution [20μl of FITC-labeled latex fluorescent beads (Polysciences Inc., diameter 1.967μm, initially packaged as a 2.5% aqueous suspension) suspended in 4ml of PBS] were added to each well, and incubated at 37°C for 4 hours in 5% CO_2.

The ARPE-19 cell line is well characterized and we have previously found that the doubling time for these cells to be 60 hours. Given the plated density of cells, we thus predicted a plated cell density of 70,000 cells/well at the time of the experiments. The added solution thus had a concentration of 2.84×10^7 beads/ml and 9.94×10^6 beads total or 142 beads/cell were added to each well.

Flow Cytometry

After a 4-hour incubation period with the beads, all media were removed and the gels were washed three times with 500 μl of PBS. Trypsin-EDTA (200μl of 0.05%) was added to each well and the gels were incubated at 37°C for 15 minutes in 5% CO_2. The cells were then collected into separate, sterile, 15ml conical tubes. Each tube was centrifuged for 8 minutes at 800 RPM, at room temperature. Excess media was removed from the conical tubes and the cells were resuspended in 3ml of PBS and 350μl of the resulting solution were transferred to flow cytometry tubes for processing. RPE suspensions were analyzed in a flow cytometer (BD FACSCanto II, BD Biosciences) to obtain the number of cells with phagocytosed beads. The signal intensity obtained from the beads allowed quantification of the number of cells that phagocytosed a fluorescent bead.

Three biological repeats were performed per substrate stiffness. Each gel was plated with a 30μl solution of 20,000 (cells/ml). ARPE-19 cells were studied at 14 days because the results from Guidry (2005) and Davis (Davis *et al*, 2012) suggested that that time scale was required for cells to adapt to a given substrate stiffness. We studied the cells after 4 hours of incubation with beads because, cultured RPE cells have been found to phagocytose rod outer segments at a steady rate for at least 3 hours, before decreasing their rate of phagocytosis (Kennedy *et al*, 1996).

Recent data (Provenzano *et al*, 2009) suggest that, for any given cell type, there are two cell culture regimes: ‘hard’ and ‘soft’. Therefore, cells cultured on glass substrates were characterized by markedly distorted cellular morphology and were not used, because their results would be less physiologically relevant.

Microscopy

Cells were imaged by epifluorescence and Hoffman illumination using a 20x objective on an inverted microscope (IX71; Olympus, Tokyo) and captured using a monochrome, cooled CCD digital camera (Rolera-XR; Q-Imaging, Surrey, BC, Canada). Epifluorescence images were captured at the optimal focal plane for the majority of beads (adherent cells) or at the surface of the cells (trypsinized, non-adherent cells) and multiple fields were sequentially captured. Digital images for the fluorescence (beads) and Hoffman illumination (cells) were over-laid to identify the beads associated with individual cells. Beads that were located on the cell surface or not associated with a cell demonstrated the brightest fluorescence and crisp boundaries. Beads that were inside a cell were not in the same focal plane as the cell surface and demonstrated dimmer fluorescence with blurred outlines.

Using these criteria we found that prior to trypsinization 2.1294% ($p=0.0012$) cells contained an internalized bead. Results were analyzed using an unpaired Student’s t-test and

are representative of 3 independent experiments. This is consistent with the results from flow cytometry where 5.83% (583 out of 10,000) cells cultured on substrates of the same elastic modulus internalized a bead.

Data Analysis

The number of cells that phagocytosed a bead was corrected for the total number of events analyzed. Only cells with one phagocytosed bead were analyzed.

Out of 10,000 events counted, approximately 70% of the events had a form factor consistent with a single cell and only approximately 6% of all the events were consistent with a cell with one or more beads. Of those, consistently, less than 10% of the cells with one or more beads had more than one bead. This means that less than 0.6% of events were consistent with a cell containing more than one bead. This is on the order of magnitude of our measurement error and thus analysis of cells that have phagocytosed only one bead is appropriate. In addition, a cell containing more than one strongly-scattering bead will have an abnormal form factor and excluding counts with an abnormal form factor in our analysis also allows us to exclude cellular debris that is adherent to one or more beads.

Linear regression was performed to evaluate the dependence of RPE cell phagocytosis on varying substrate stiffness. The forward scatter channel (FSC) and side scatter channel (SSC) were gated to select viable cells of interest (Rahman, 2006). ARPE-19 cells not exposed to latex beads served as the negative control, and facilitated determination of the negative threshold in the fluorescence channel. Events exceeding the threshold and consistent with the fluorescence of a single bead were counted as phagocytosing cells.

Results

ARPE-19 cells demonstrated decreased phagocytosis on higher elastic modulus gels and a linear relationship between substrate elastic modulus and phagocytosis (Figure 1). ARPE-19 cells cultured on the highest elastic modulus exhibited the least amount of phagocytosis.

Fluorescence microscopy images indicate that the latex beads are phagocytosed. Given that we only analyzed cells that are associated with one bead (ie, are characterized by the form factor of a cell and the fluorescence of a single bead), we avoided problems associated with multiple beads being phagocytosed or beads adherent to the cell surface.

Discussion

The results of this study, together with prior results (Janmey *et al*, 2009; Pelham and Wang, 1998; Pelham and Wang, 1998; Davis *et al*, 2012; Raghunathan *et al*, 2013; Georges *et al*, 2007), suggest that substrate elastic modulus influences cell behavior, including complex cell behaviors such as phagocytosis, in a variety of cell types.

Currently, there is substantial interest in developing synthetic substitutes for Bruch's membrane for the culture of stem-cell derived RPE cells, for eventual transplantation into patients suffering from macular degeneration (Bharti *et al*, 2010). Our study suggests that the elastic modulus of these substrates may be a critical design parameter. In addition, sub-

retinal implantation of prosthetic devices has recently come into clinical use and, in designing the device-tissue interface, the stiffness of the device surface should be considered.

A weakness of this study is the use of a cell line, rather than primary RPE cells. However, it is known that a variety of cell types including retinal Müller cells (Davis, 2012), primary human trabecular meshwork cells (Raghunathan *et al*, 2013), kidney epithelial cells (Pelham and Wang, 1998), and liver cells (Georges *et al*, 2007) and numerous cellular processes, including cell spreading and motility (Georges *et al*, 2005) are influenced by substrate elastic modulus.

Decreased phagocytosis by ARPE-19 cells cultured on substrates of higher elastic moduli suggests that changes in the stiffness of Bruch's membrane may affect RPE cells *in vivo*. As Bruch's membrane ages, RPE cells may be less able to phagocytose the outer segments of photoreceptor cells, and this may eventually lead to RPE dysfunction. In turn, RPE dysfunction may affect photoreceptor function, causing the loss of central vision in the macula.

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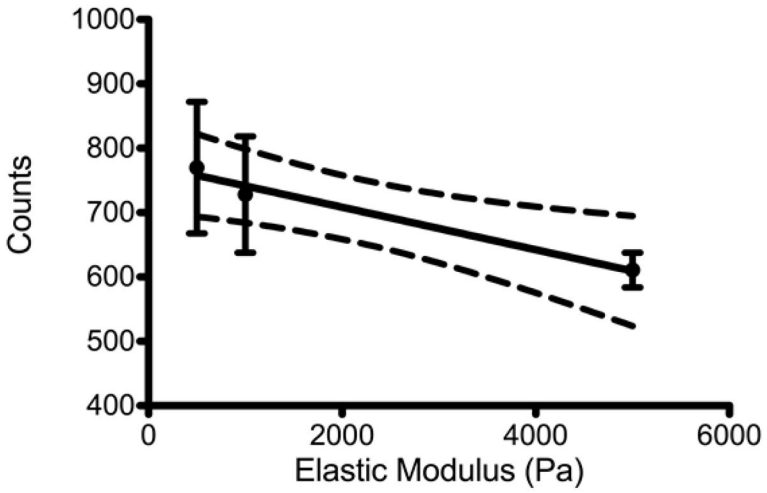

Figure 1.

The number of ARPE-19 cells that phagocytosed a bead, per 10,000 cells (Counts) plotted versus the substrate stiffness (Elastic Modulus). The mean and standard error of the mean plotted with the results of a linear regression analysis. The dashed lines indicate 95% confidence intervals; $p=0.0135$; $R^2=0.4726$.

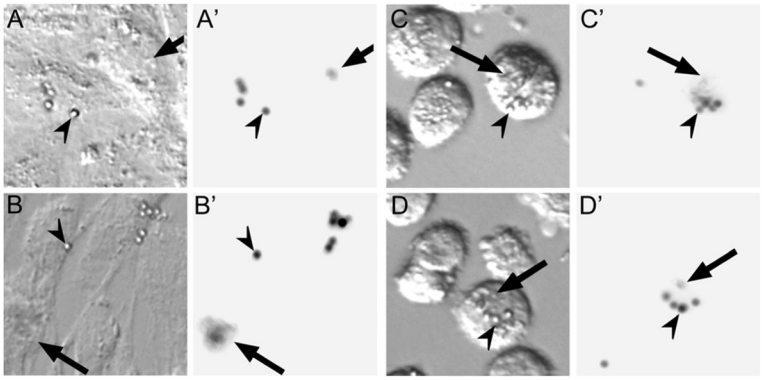

Figure 2.

Imaging of ARPE-19 cells, grown on a 1000 Pa gel, after incubation with beads. A&B: phase contrast image before trypsinization, A′&B′: fluorescence image before trypsinization, C&D: phase contrast images after trypsinization, C′&D′: fluorescence images after trypsinization. Arrowheads indicate beads that were not phagocytosed while arrows indicate beads that have been phagocytosed by the RPE cells.