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Extracellular vesicle-mediated long-range communication in stressed retinal pigment epithelial cell monolayers

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ABSTRACT

Retinal pigment epithelium (RPE) alterations in age-related macular degeneration occur in patches, potentially involving long-distance communication between damaged and healthy areas. Communication along the epithelium might be mediated by extracellular vesicles (EVs). To test this hypothesis, EVs were collected from supernatants of polarized ARPE-19 and primary porcine RPE monolayers for functional and biochemical assays. EVs from oxidatively stressed donor cells reduced barrier function in recipient RPE monolayers when compared to control EVs. The effect on barrier function was dependent on EV uptake, which occurred rapidly with EVs from oxidatively stressed donor cells. Mass spectrometry-based proteomic analysis of EVs identified HDAC6, which is known to reduce tight junction stability. Activity assays confirmed the presence of HDAC6 in EVs, and EV transfer assays using HDAC6 inhibitors confirmed its effect in monolayers. These findings demonstrate that EVs can communicate stress messages to healthy RPE cells, potentially contributing to RPE dysfunction.

1. Introduction

The bystander effect has garnered a lot of attention in biology in particular in the context of the spreading of pathology. Specifically, it refers to the phenomenon of the induction of biological effects in cells that are not directly targeted [1]. The impacts in neighboring cells can be mediated via direct cell-cell communication via gap junctions (GJs), or long-distance via secreted material. The first mechanism typically occurs in cells that are part of a network and information can spread from the treated to the untreated cell via GJs or across membranes. GJs allow small signaling molecules to pass from one cell to another [e.g., [2,3]], whereas membrane current or reactive oxygen species (ROS) can be transmitted directly across membranes [e.g., [4]]. Long-distance communication on the other hand involves the secretion of biologically active material, with recipient cells being either part of the same network or cell type, or of different origin. The biologically active material might be secreted as individual molecules, or packaged in extracellular vesicles such as exosomes [5–7].

The retinal pigment epithelium (RPE) is a monolayer of cells localized between the light-sensitive layer of the retina and the choroidal blood supply, forming the outer blood-retina barrier. In this network, intercellular communication is mediated by GJs made up of connexin43 and connexin46 [8], whereas it's barrier function is dependent on tight- and adherence junctions.

In age-related macular degeneration (AMD), the leading cause of visual impairment and blindness in the elderly worldwide, the RPE appears to be a primary site of damage. Persistent oxidative stress is recognized as one of the important underlying risk factors that lead to AMD pathology. Oxidative stress in the RPE is thought to originate from multiple sources and relates to the biological functions of the tissue. Specifically, the RPE's biological role is to digest photoreceptor outer segments in a diurnal fashion. These outer segments contain high levels of polyunsaturated fatty acids and photosensitizers, which together increase the production of ROS in the presence of oxygen as well as light [9,10]. Additional risk factors such as smoking, hypercholesterolemia, excessive light exposure and dysregulated para-inflammation, augment the generation of ROS [11,12].

However, RPE pathology does not get triggered in a single location to spread uniformly from there; but rather RPE damage occurs in multiple locations within the central part of the eye, that finally

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coalesce to form a region of atrophy (geographic atrophy, GA) [13]. This observation suggests that pathology occurs in susceptible regions, while healthy regions are protected; and that damage may occur randomly, in a stochastic fashion, or once triggered in a susceptible area, might spread via yet to be discovered means to other receptive areas.

This bystander effect in the RPE might be mediated via extracellular vesicles (EVs). These EVs might represent microvesicles, exosomes or apoptotic bodies, which can be distinguished based on size, the direction of blebbing (outward or inward), their content [5] as well as their sedimentation. Apoptotic bodies, due to their large size (1–5 μm in diameter) sediment at 1200 ×*g* (summarized in [14]), whereas microvesicles and exosomes are smaller (0.1–1 vs 0.04–0.15 μm, respectively [5]) and sediment by high-speed centrifugation (10,000–20,000 ×*g*) or ultracentrifugation (100,000 ×*g* for 70 min), respectively. EVs are secreted by almost every cell type in the body, and are involved in several biological and pathological processes [15,16]. EVs are key facilitators of intercellular communication with broad biological and medical implications [17,18], including cell growth, migration, differentiation, neuronal signaling, and immune cell modulation. EVs transport functional molecules, such as messenger RNA (mRNA), microRNA (miRNA) and proteins into target tissues or cells [19–21]. These bioactive molecules are considerably stable and can modulate cell behaviors in recipient cells [22]. The biogenesis of exosomes within a donor cell has four major steps, membrane formation (initiation), endocytosis and multivesicular body (MVB) formation, MVB fusion with the plasma membrane, followed by the release of the internal vesicles (i.e., exosomes) into the extracellular milieu [15]. The biogenesis of microvesicles on the other hand involves trafficking of the cargo to be packaged to the plasma membrane, a redistribution of membrane lipids, followed by the pinching off of vesicles. Surface markers of microvesicles hence tend to be reflective of the composition of the membrane of origin [23]. Due to the complications to unequivocally identify the EVs as exosomes or microvesicles, many reports will not aim to distinguish between the two types, but rather refer to them combined as EVs. The same strategy is followed here. In recipient cells, EVs can mediate their effects in multiple ways. Proteins integral to the EV membrane or bound on the extravesicular side might serve directly as ligands for receptor-mediated effects via EV binding to the recipient cell, or these proteins might get cleaved off and serve as soluble ligands. Alternatively, EVs might fuse directly with the recipient cell membrane to release their content into the cytosol, or are taken up by the recipient cells via one of multiple mechanisms, including endocytosis or phagocytosis, with the EV content gaining access to the endosomal/lysosomal compartment of the cell.

Here, we investigated whether oxidatively stressed highly polarized RPE cells can communicate stress messages to healthy neighboring cells (recipient cells) via EVs, and the potential cargo it might involve.

2. Material and methods

2.1. Cell culture

Human ARPE-19 cells and primary porcine RPE cells were used for this study. All cell culture products were obtained from Gibco/ThermoFisher Scientific. ARPE-19 cells were grown as monolayers on 6-well transwell filters as described previously [24]. Primary porcine RPE (pRPE) were cultured according to a published protocol [25], using eyes obtained from a local abattoir. Pooled cells from 10 eyes were suspended in 10% FBS Growth Medium (high glucose DMEM with L-glutamine, sodium pyruvate, 1% nonessential amino acids, 10% FBS and Penicillin and Streptomycin), expanded in T25 cell culture flasks and transferred to 12-well plates (3×10^5 cells/cm^2). For both cell types, upon reaching confluency, tight junction formation was enabled by step-wise FBS reduction to 1%. Monolayer integrity was assessed by transepithelial resistance (TER) measurements using an EVOM volt-ohmmeter (World Precision Instruments), with monolayers being considered stable when TER was repeatedly measured as ~40–45 Ω cm^2 (ARPE-19 cells) or 150 Ω cm^2 (pRPE cells).

Prior to each experiment, monolayers were washed with FBS-free medium and maintained with FBS-free medium for 24–48 h to avoid the contamination with FBS-derived EVs.

2.2. Isolation of extracellular vesicles

To trigger the release of EVs, monolayers on transwells were stimulated apically with 0.5 mM H_2O_2 (Sigma Aldrich, 216763) once a day for 3 days without changing medium. Pulsed exposure of H_2O_2 leads to rapid depletion of the oxidant rather than build-up over time, while eliciting the desired effect [26]. Supernatants were collected from both apical and basal sides, spun at 3000*g* and used for EV isolation using Exoquick-TC (Systems Biosciences) according to the manufacturer's instructions. In short, 2 mL of Exoquick-TC was added to 10 mL of culture media and incubated overnight at 4 °C, centrifuged at 1600*g* for 35 min to collect the EV pellet and resuspended in 50 μL of sterile PBS.

2.3. Zetaview nanoparticle tracking analysis (NTA)

NTA was performed using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and its corresponding software (ZetaView 8.02.28), using instrument settings previously described by us [27]. For each sample, 5–10 μL of the resuspended pellet were diluted into 2 mL of 1× PBS and loaded into the NTA cell for analysis to obtain the diameter size (modal) and EV particle concentration of each sample.

2.4. Transmission Electron microscopy (TEM)

For negative staining of EVs, freshly isolated EV suspensions were applied to copper mesh Formvar coated carbon stabilized grids, fixed in 4% paraformaldehyde for 1 h, and stained with 1% aqueous uranyl acetate. For labelling with anti-CD81 (BioRad, 1D6) [28], EV samples were fixed in 4% paraformaldehyde diluted in 0.1 M cacodylate buffer (pH 7.4). Grids were floated sequentially onto drops of 1 M ammonium chloride, blocking buffer (0.4% BSA in PBS), blocking buffer (negative control) or primary antibody (CD81; 1:100), 1.4 nm anti-rabbit nanogold (Nanoprobes, Inc.; 1:1000), HQ Silver (gold enhancement reagent, Nanoprobes, Inc.) and 2% aqueous uranyl acetate. After air drying, TEM examination was performed using a JEM 1230 transmission electron microscope (JEOL USA Inc., Peabody, MA) at 110 kV and imaged with an UltraScan 4000 CCD camera & First Light Digital Camera Controller (Gatan Inc., Pleasanton, CA) [27].

2.5. Transfer assays

Transfer assays were performed to study cell-cell communication using either EV-containing media, or purified EVs. EV-containing media were prepared by sequential centrifugation at 2000*g* followed by 20,000*g* [29]; alternatively, EVs were isolated by Exoquick-TC as described above and resuspended in fresh media equal in amount to the starting material. Material (2 mL or 0.5 mL of EV-containing media or resuspended EVs for 6- and 12-well plates, respectively) collected from donor monolayers were transferred to recipient monolayers of the same age and TER as donor cells. TER measurements were performed prior to the transfer (0 h) and after incubation of 4 h.

2.6. Endocytosis/exosome uptake assays

EVs were labelled with ExoGlow according to the manufacturer's instructions (Systems Biosciences, EXOC300A-1) and tracked with live-cell imaging of recipient cells. Annexin A2 is localized on the surface of EVs and is required for EV uptake by recipient cells [30]. Annexin A2 was knocked down in ARPE-19 cells using lentiviral vector-mediated