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In situ cell surface proteomics reveals differentially expressed membrane proteins in retinal pigment epithelial cells during autoimmune uveitis☆

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ABSTRACT

Retinal pigment epithelium (RPE) builds the outer blood–retinal barrier of the eye and plays an important role in pathogenesis of the sight threatening disease equine recurrent uveitis (ERU). ERU is a spontaneous autoimmune mediated inflammatory disease characterised by the breakdown of the outer blood–retinal barrier and an influx of autoaggressive T-cells into the inner eye. Therefore, identification of molecular mechanisms contributing to changed function of blood–retinal barrier in ERU is important for the understanding of pathophysiology. Cell surface proteins of RPE collected from healthy horses and horses with ERU were captured by *in situ* biotinylation and analysed with high resolution mass spectrometry coupled to liquid chromatography (LC–MS/MS) to identify differentially expressed proteins. With label free differential proteomics, a total of 27 differently expressed cell surface proteins in diseased RPE could be detected. Significant down-regulation of three very interesting proteins, synaptotagmin 1, basigin and collectrin was verified and further characterised.

Biological significance
We applied an innovative and successful method to detect changes in the plasma cell surface proteome of RPE cells in a spontaneous inflammatory eye disease, serving as a valuable model for human autoimmune uveitis. We were able to identify 27 differentially expressed plasma cell membrane proteins, including synaptotagmin 1, basigin and collectrin, which play important roles in cell adhesion, transport and cell communication.

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1. Introduction

Mass spectrometry has become an essential method to investigate quantitative and qualitative changes of the proteome in different biological states [1]. The different types of mass spectrometric analysis are developing rapidly. For example, label-free, non-gel-based tandem mass spectrometry with preceding liquid chromatography (LC–MS/MS) is a highly precise and sensitive high-throughput technique, which enables analysis of complex and widely dynamic protein concentrations [2]. Furthermore, it is a more convenient and efficient method to investigate insoluble proteins such as cell surface proteins in comparison to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [1].

Cell surface proteins, making up 30% of the whole proteome, represent a very important group of proteins [3]. They are, among others, involved in communication and interaction of cells, demarcation of compartments and the transport of ions and dissolved substances [4]. Due to their location on the cell surface, changes in their expression cause dysfunctions of cell interaction and communication. Thereby it happens that immune cells recognize cell surface proteins as foreign substances, which can lead to autoimmune diseases [5].

A very important sight threatening disease of horses is equine recurrent uveitis (ERU). ERU is a spontaneous, organ-specific autoimmune disease characterised by recurrent inflammation of the inner eye [6]. Many matches in clinical as well as immunopathological aspects make ERU the only spontaneous animal model for human autoimmune uveitis [7].

There are two types of blood–retinal barriers in the eye: The inner blood–retinal barrier, formed by capillary endothelia, and the outer blood–retinal barrier, formed by the retinal pigment epithelium (RPE) [8]. Because horses have a widely avascular retina [9], the RPE is the crucial part for immune defence of the eye. Under physiological conditions, it ensures that no leukocytes can move in the inner eye [10]. In case of ERU, leukocytes are allowed to transmigrate the outer blood–retinal barrier with subsequent inflammation and destruction of retinal structures [11]. Until now, molecular pathophysiological mechanisms at RPE, which crucially contribute to ERU [12], are not fully understood.

Therefore, the goal of this study was to compare cell surface proteomes of equine RPE cells of healthy and ERU diseased cases by differential proteomic analysis to identify proteins providing more insights into pathophysiology.

2. Material and methods

2.1. RPE specimen

For this study, RPE of a total of 40 eyes was used (21 healthy controls and 19 spontaneous ERU cases). In detail, four healthy and two diseased eyes were used for mass spectrometric identification of proteins. For flow cytometry, RPE cells of five healthy and four diseased eyes were used. With RPE sections of twelve healthy and thirteen diseased eyes, immunohistochemical stainings were performed. Specimens of healthy equine eyes were obtained at a local abattoir, as well as four ERU diseased eyes. The other uveitic eyes were from horses that had to be enucleated during a therapeutical procedure. Collection and use of equine eyes from animals that were killed due to a research-unrelated cause were approved for purposes of scientific research by the appropriate board of the veterinary inspection office Munich, Germany (Permit number: 8.175.10024.1319.3). No experimental animals were used in this study.

2.2. Sample preparation

Eyes were prepared immediately after collection from the abattoir. First step was to remove the residual periocular tissue carefully with forceps and scissors. Afterwards, the eyes were rinsed in 70% ethanol for 2 min followed by washing in cold phosphate buffered saline (PBS). Then the eyes were stored in sterile Dulbecco's Modified Eagle Medium (DMEM; PAN-Biotech, Aidenbach, Germany) until further use. Subsequent steps were conducted under a laminar flow hood with sterile instruments. Eye globes were cut open circumferentially, and anterior parts of the eye, vitreous and neurosensory retina were carefully removed in order to obtain the posterior eyecups for either in-eye biotinylation for mass spectrometry or RPE cell isolation.

2.3. In-eye biotinylation of cell surface proteins

For discovery of differentially expressed plasma cell membrane proteins, six eyecups from three horses were taken for in-eye biotinylation. Two horses showed one diseased eye each and their contralateral healthy eye was taken as control as well as two more healthy eyes from a third horse. Eyecups were washed once with pre-warmed PBS, then cell surface membrane proteins of the RPE were labelled with biotinylation reagent: 20 mM $NaIO_4$, 100 μM biotin (both Gentaur, Aachen, Germany) and 10 mM aniline (Sigma Aldrich, Deisenhofen, Germany) in PBS pH 6.7. After shaking slowly in the dark for 30 min at 4 °C and two washing steps, the next step was to scrape the RPE cells carefully into ice cold PBS (pH 7.4 with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$), transfer them into a tube and centrifuge them at 130 ×*g* for 5 min. Then the cells were lysed with 1% Nonidet P-40, 1× Roche Complete Protease Inhibitor, EDTA-free (both Roche Diagnostics, Mannheim, Germany), 10 mM Tris–HCl, 5 mM 2-iodoacetamide (SERVA Electrophoresis, Heidelberg, Germany) in 150 mM NaCl pH 7.6. For preparation of integral plasma cell membrane fraction, samples were incubated with high affinity Streptavidin-beads (IBA, Göttingen, Germany) for 2 h at 4 °C on a rotator to capture biotinylated cell surface proteins. Subsequently, extensive washing steps were performed to remove non-specifically bound proteins. Then, proteins were extracted from affinity-purified plasma membrane by digesting beads overnight with trypsin (Promega, Mannheim, Germany) at 37 °C. After centrifugation at 2000 ×*g* for 2 min, supernatant, containing tryptic peptides, was collected. Afterwards, beads were incubated with glycerol-free PNGase F (New England Biolabs, Frankfurt/Main, Germany) for 5 h at 37 °C to elute glycopeptides. Therefore, supernatant was collected after another centrifugation step at 2000 ×*g* for 2 min. Finally, peptides of the two fractions were analysed by LC–MS/MS.