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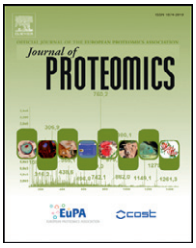

Altered expression of talin 1 in peripheral immune cells points to a significant role of the innate immune system in spontaneous autoimmune uveitis☆

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ABSTRACT

The molecular mechanism which enables activated immune cells to cross the blood–retinal barrier in spontaneous autoimmune uveitis is yet to be unraveled. Equine recurrent uveitis is the only spontaneous animal model allowing us to investigate the autoimmune mediated transformation of leukocytes in the course of this sight threatening disease. Hypothesizing that peripheral blood immune cells change their protein expression pattern in spontaneous autoimmune uveitis, we used DIGE to detect proteins with altered abundance comparing peripheral immune cells of healthy and ERU diseased horses. Among others, we found a significant downregulation of talin 1 in peripheral blood granulocytes of ERU specimen, pointing to changes in β integrin activation and indicating a significant role of the innate immune system in spontaneous autoimmune diseases.
This article is part of a Special Issue entitled: SI: Farm animal proteomics.

1. Introduction

Recurrent autoimmune uveitis is a sight threatening disease characterized by recurrent inflammation of the inner eye [1]. Leukocytes are activated in periphery and enter the eye by crossing the blood–retinal barrier immediately before an uveitic attack [2]. However, the molecular mechanisms involved in autoimmune mediated transformation of leukocytes are not fully understood [3]. In ERU, as well as in other autoimmune diseases the target antigens of the adaptive immune system become more and more diverse during the onset of disease [4,5]. Responses to different proteins probably contribute significantly to the heterogeneity of clinical manifestations of such diseases. In addition, epitope spreading is thought to be essential for disease progression as well as inflammatory relapses in a variety of diseases, including autoimmune uveitis [6]. Thus, epitope spreading in autoimmune diseases results in the detection of an increasing diversity of target antigens in the immune response pattern against various target antigens over time [4,6].

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Many target antigens may still not be discovered, but although target epitopes differ between individuals, immune cell subsets responsible for inflammation seem to be essentially the same in every autoimmune disease. Therefore, characterization of the immunoproteome in spontaneously diseased patients versus healthy controls could provide a systemic understanding of underlying changes in cell subsets contributing to pathogenesis. This approach aims at understanding the molecular processes of the disease, irrespective of individual autoantigen specificity. We investigated fundamentals of immune response in a spontaneous autoimmune disease model in the horse, equine recurrent uveitis (ERU) [7,8]. This is the only spontaneous animal model for autoimmune uveitis, and ERU closely resembles the human disease in many clinical as well as immunopathological aspects [9,10]. Differences in protein abundance, modifications and functions can act as useful indicators of pathological abnormalities. Comparison of protein expression patterns between healthy and diseased state creates new insights in pathogenesis. Systematic evaluation of target organ proteome in ERU in contrast to physiological protein inventory made it possible to detect previously unknown molecules that change their expression and function in ERU affected tissues [7,8,11,12]. This enabled us to understand cellular behavior on the protein level in target tissue proteome [12]. Since changes in target tissue are only one side of the story, we analyzed the immunoproteome of peripheral blood derived leukocytes (PBL) in this study. Breakdown of the blood–retinal barrier and the immigration of activated leukocytes into the eye indicate that differentially expressed proteins are crucial to the disease process. Therefore, the goal of this study was the identification of abundance changes of immune cell proteins to find candidates that alter leukocyte function in this spontaneous autoimmune disease.

2. Material and methods

2.1. Preparation of equine PBL

Blood samples of 46 controls and 39 ERU diseased horses were used in this study. In detail, PBL of 6 ERU cases and 6 healthy control horses were used for DIGE experiment identifying differentially expressed proteins. For validation of talin 1 regulation with Western blots, 36 additional controls and 29 ERU samples were used. Identification of cellular subset showing talin 1 downregulation was performed with 4 additional ERU specimen and 4 controls. All ERU diseased horses were those brought to the Equine Clinic in Munich without further selection for a respective experimental condition (DIGE profiling, Western blot verification, flow cytometry). Venous whole blood was collected in lithium-heparin coated tubes. After sedimentation of erythrocytes, PBL were isolated from plasma by density gradient centrifugation (RT, 290 rcf, 25 min, brake off) using Biocoll Separating Solution (Biochrom, Berlin, Germany). Cells were extracted from intermediate phase and washed twice in PBS (4 °C, 453 rcf, 10 min). Pellets were then resuspended in PBS and either used immediately or stored at −20 °C until further usage. Samples with a granulocyte/lymphocyte ratio of 1 as assessed by analysis of cytospin preparations were used in this study.

2.2. 2D DIGE (minimal labeling technique)

PBL of ERU diseased horses and controls (6 different individuals per group) were dissolved in DIGE lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris; pH 8.5) and processed using QIAshredder homogenizers (Qiagen, Hilden, Germany). Samples were portioned into aliquots of 50 μg protein and then labeled separately with 400 pmol Cy3 or Cy5 fluorescent CyDyes (GE Healthcare, Freiburg, Germany) according to minimal labeling technique. Reverse labeling was applied; therefore we labeled 3 controls with Cy5 and 3 with Cy3 and vice versa in ERU cases in order to exclude bias resulting from different labeling intensities. Additionally, a pooled internal standard containing all ERU and control samples used in the experiment was labeled with Cy2 (GE Healthcare). Labeling of proteins at 4 °C was terminated after 30 min by addition of lysine. After 10 min of incubation, samples were pooled into sets, each comprising three 50 μg aliquots (diseased/control/internal standard). The 150 μg protein compounds were adjusted to a volume of 460 μl with lysis buffer and processed. Each of the sample sets was then loaded on 24 cm pH 3–11 NL IPG strips (GE Healthcare) and subjected to IEF followed by equilibration and SDS-PAGE as described before. Resulting gels were first scanned at different wavelengths (488 nm, 532 nm, 633 nm) with Typhoon Trio69 Scanner (GE Healthcare) and then silver stained for visualization of protein spots. After image analysis, differentially expressed spots were cut and processed for mass spectrometry.

2.3. Image analysis and detection of differentially expressed proteins

Data of scanned DIGE gels were imported into DeCyder 6.5 software (GE Healthcare) and processed in DIA module for intra gel analysis of each gel separately, comprising the assignment of dye tag to images (internal standard: Cy2, control: Cy3 or Cy5, diseased: Cy5 or Cy3 due to reverse labeling), spot detection and normalization of Cy3 and Cy5 labeled spot abundances to the internal standard as well as comparison of spot abundances between control and ERU data sets. To avoid false positive spots, inclusion and exclusion criteria for spot detection were set as follows: spot slope>2, spot volume<30,000 and threshold 2.5. Detection and matching of spots were performed automatically by software but were manually verified and corrected if necessary.

Inter gel analysis was performed in BVA module, comparing standardized protein spot abundances from all gel data sets in the experiment in order to detect relevant differences between controls and ERU. We used Student's *t*-test for statistical analysis. Differences in spot volume between ERU and controls were considered significant at $p \leq 0.05$. Standardized log abundances as calculated for each spot with DeCyder 6.5 were hierarchically clustered using R-statistics. The heatmap.2 function of the gplots package in the R software environment for statistical computing and graphics was used with the default parameters to create a heatmap with hierarchical clustering (Ward's method).

Differentially abundant protein spots on digital DeCyder map were then located on matching silver stained gels and excised for subsequent identification by mass spectrometry