Immunobiology 215 (2010) 949–955

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio

Short Communication

Complement factor B expression profile in a spontaneous uveitis model

Johanna K. Zipplies [a], Michael Kirschfink [b], Barbara Amann [a], Stefanie M. Hauck [c], Manfred Stangassinger [a], Cornelia A. Deeg [a,*]

[a] Institute of Animal Physiology, Department for Veterinary Sciences, Ludwig-Maximilians University, Veterinärstr. 13, D-80539 Munich, Germany
[b] Institute of Immunology, University of Heidelberg, Im Neuenheimer Feld 305, D-69120 Heidelberg, Germany
[c] Department of Protein Sciences, Helmholtz Center Munich, Ingolstaedter Landstr. 1, D-85764 Neuherberg, Germany

ARTICLE INFO

Article history:
Received 11 November 2009
Received in revised form
10 February 2010
Accepted 10 February 2010

Keywords:
Autoimmune
Complement system
Serum
Uveitis
Vitreous

ABSTRACT

Equine recurrent uveitis serves as a spontaneous model for human autoimmune uveitis. Unpredictable relapses and ongoing inflammation in the eyes of diseased horses as well as in humans lead to destruction of the retina and finally result in blindness. However, the molecular mechanisms leading to inflammation and retinal degeneration are not well understood.

An initial screening for differentially regulated proteins in sera of uveitic cases compared to healthy controls revealed an increase of the alternative pathway complement component factor B in ERU cases. To determine the activation status of the complement system, sera were subsequently examined for complement split products. We could demonstrate a significant higher concentration of the activation products B/Ba, B/Bb, Bb neoantigen, iC3b and C3d in uveitic condition compared to healthy controls, whereas for C5b-9 no differences were detected. Additionally, we investigated complement activation directly in the retina by immunohistochemistry, since it is the main target organ of this autoimmune disease. Interestingly, infiltrating cells co-expressed activated factor Bb neoantigen, complement split product C3d as well as CD68, a macrophage marker.

In this study, we could demonstrate activation of the complement system both systemically as well as in the eye, the target organ of spontaneous recurrent uveitis. Based on these novel findings, we postulate a novel role for macrophages in connection with complement synthesis at the site of inflammation.

Introduction

Equine recurrent uveitis is an autoimmune disease affecting the eye (Deeg et al., 2008). It currently represents the only spontaneous model for human autoimmune uveitis (Deeg et al., 2006b). Autoimmune reactions are directed towards retina-expressed proteins in both humans and horses (Deeg et al., 2006a; Deeg et al., 2006b; Deeg et al., 2004; Deeg et al., 2002b; Nussenblatt, 1990). The remitting-relapsing disease of the inner eye is characterized by the breakdown of the blood–retinal barrier, infiltration by inflammatory cells and tissue destruction finally leading to blindness (Deeg et al., 2007; Deeg et al., 2001; Hauck et al., 2007). Molecular processes and pathogenesis of the disease are largely unknown. Under physiological conditions, the eye is an immune privileged organ with a special relationship to the immune system to be protected from inflammatory responses, which could threaten vision (Caspi, 2006).

Differential analysis of proteomes of vitreous, retina and sera revealed several differentially expressed proteins, which point to the breakdown of the blood–retinal barrier and are related to immune response (Deeg et al., 2007; Hauck et al., 2007; Zipplies et al., 2009a; Zipplies et al., 2009b). In all experiments, a differential expression of complement factors was noted, which prompted us to further investigate the expression pattern of complement factors in uveitic condition.

The complement system represents a major component of the innate immune system (Sjoberg et al., 2009). It comprises more than 30 serum and surface proteins that play a central role in host defence against infection, modulation of antigen-specific immune and inflammatory responses (Frank and Fries, 1991; Kinoshita, 1991; Walport, 2001). Complement activation occurs via three distinct pathways, referred to as the classical, alternative and lectin pathway. Under normal conditions activation of the complement system is kept under tight control by multiple complement regulatory proteins preventing serious damage of the host tissues. It is well known that the complement system and its regulation contributes to the pathology of many diseases

Abbreviations: AMD, age related macular degeneration; FB, complement factor B; ERU, equine recurrent uveitis; 2D-DIGE, two dimensional-difference gel electrophoresis

* Corresponding author. Tel.: +49 89 21 801630; fax: +49 89 21 802554.
E-mail address: deeg@tiph.vetmed.uni-muenchen.de (C.A. Deeg).

0171-2985/$ - see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.imbio.2010.02.003

(Zipfel, 2009), including ocular disorders (Bora and Bora, 2008). Complement factors are mainly synthesized by the liver but an extrahepatic, local synthesis of complement components is also known, which includes a wide range of cell types like endothelial cells, epithelial cells, blood cells, fibroblasts, brain cells and monocytes/macrophages (Laufer et al., 2001; Morgan and Gasque, 1997). The purpose of our study was to determine the expression pattern of factor B and complement split products in sera and the ERU target tissue, the retina, to evaluate the possible involvement of complement in the disease process.

Material and methods

Samples

A total of 28 ERU cases and 22 healthy controls were used for this study. The sera of horses diagnosed with ERU were chosen in the order presented to the Equine Clinic of the Ludwig-Maximillians-University of Munich. ERU was diagnosed according to clinical criteria as described (Deeg et al., 2001). Healthy controls were matched in sex and age. All sera samples were taken from the jugular vein directly into 10 ml tubes. Sera was allowed to clot for 1 h at room temperature and then centrifuged for 10 min. Sera was separated and the samples were stabilized with protease inhibitors (Roche, Mannheim, Germany) and stored immediately at −20 °C. Protein content was quantified with the Bradford assay (Sigma, Deisenhofen, Germany). For the initial 2D-DIGE screening experiment we processed sera of five healthy horses and five ERU cases. For determination of complement split products, sera of 12 ERU cases and 12 healthy controls were used. For candidate validation with immunohistochemistry, we used eyes of 11 additional ERU cases and five healthy controls were used, which were fixed in Bouin's solution (Sigma, Deisenhofen, Germany) embedded in paraffin (Micron, Walldorf, Germany) and sectioned.

Two dimensional-difference gel electrophoresis (2D-DIGE)

Sera of ERU cases and healthy controls were labelled with fluorescence dye and separated by two dimensional gel electrophoresis. The initial 2D-DIGE screening experiment for the identification of differentially regulated candidates in sera of ERU cases, revealed a total of 15 differentially expressed spots, detected by DeCyder 6.5 analysis (GE Healthcare, Freiburg, Germany) and subsequently identified by mass spectrometry (MALDI–TOF/TOF) (Zipplies et al., 2009b).

Dot blots

Serum samples ($n=12$ healthy controls, $n=12$ ERU diseased) were diluted in PBS (1:100) and 3 μl from the diluted sera was applied on nitrocellulose membranes (GE Healthcare, Freiburg, Germany). Membranes were blocked with 4% BSA in PBS-T for 30 min. Blots were then incubated with primary antibodies for 1 h. In detail, monoclonal mouse antibodies specific for human complement factor B/Ba (clone 014III-33.2.4.3), human complement factor B/Bb (clone 10-09) and human complement factor Bb neoantigen (clone 032B-22.1X) (all from Serotec AbD, Düsseldorf, Germany, 1:1000), human complement C5b-9 (clone aE11) (Dianova, Hamburg, Germany; 1:1000), human complement fragment iC3b neoantigen (clone 013.III-1.1.6) (BioMérieux, Nürtingen, Germany; 1:1000), and polyclonal rabbit antibody to C3d (DAKO Cytomation, Hamburg; Germany; 1:1000) were used. After incubation, membranes were washed three times with PBS-T and then incubated with the HRP-coupled secondary antibodies (anti-mouse IgG-HRP 1:5000 and anti-rabbit IgG-HRP 1:3000, Sigma, Deisenhofen, Germany) for 1 h. Signal was developed with ECL kit (GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions and detected on Hyperfilm ECL (GE Healthcare, Freiburg, Germany). To evaluate the cross reactivity of complement antibodies with equine complement proteins a dilution series of human and equine sera was pretested. The signal intensities of the horse sera were similar to the human sera (data not shown).

Image analysis and signal quantification

Exposed films with dot blots signals were scanned on a transmission scanner (GE Healthcare, Freiburg, Germany). Quantification of dot blot signals was performed with ImageQuant TL software (GE Healthcare, Freiburg, Germany). Images of dot blot signals were imported to image analysis software and dot volume intensities were subsequently calculated.

Candidate validation with immunohistochemistry

Paraffin-embedded ERU and healthy retinal tissue samples were sectioned at 8 μm. Heat antigen retrieval was performed at 99 °C for 15 min in 0.1 M EDTA–NaOH buffer pH 8.8. We used monoclonal mouse antibody specific for Bb neoantigen (1:50), polyclonal rabbit anti-C3d antibody (1:200) and monoclonal mouse anti-CD68 antibody (clone EBM11) (DAKO, Cytomation, Hamburg; Germany 1:50) for candidate detection in tissues. For fluorescence labelling, Bb neoantigen was stained with an anti-mouse IgG antibody coupled to Alexa 568 (Invitrogen, Karlsruhe, Germany; 1:500), C3d with anti-rabbit IgG coupled to Cy5 (Linaris, Wertheim-Bettingen, Germany; 1:500) and CD68 with anti-mouse IgG antibody coupled to Alexa 488 (Invitrogen, Karlsruhe, Germany). Cell nuclei were counter-stained with DAPI (Invitrogen, Karlsruhe, Germany; 1:1000). Fluorescence stainings were photographed with an Axio Imager M1 (Zeiss, Göttingen, Germany) and visualized with the Axio Vision 4.6.3 software (Zeiss, Göttingen, Germany).

Statistical analysis

Dot blot signals of uveitic cases and healthy controls were compared using the Mann–Whitney test (free software package Past; http://flok.uio.no/ohammer/past/). Significance levels were defined as follows: $^{***}p < 0.001$.

Results

Mass spectrometric identification of differentially detected proteins reveals higher levels of complement factor B in sera of spontaneous uveitis cases

In a 2D-DIGE screening experiment with subsequent DeCyder analysis and mass spectrometry (MALDI–TOF/TOF) (Fig. 1), FB was identified as differentially expressed protein between uveitic cases and healthy controls. Spots representing FB were matched with the help of DeCyder 6.5 software in 4 out of 5 analyzed sample sets (one individual ERU case compared to one healthy control in each set). Factor B was significantly upregulated in uveitic sera tested compared to healthy controls (Fig. 1).