Kininogen in Autoimmune Uveitis: Decrease in Peripheral Blood Stream versus Increase in Target Tissue

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PURPOSE. Equine recurrent uveitis (ERU) is an incurable disease affecting the inner eye that leads to blindness, through activated T cells that pass the blood-retinal barrier and destroy the retina. Serum markers are a desirable choice for monitoring development of disease, as serum is easy accessible and the markers could serve to predict the beginning of disease or an imminent relapse.

METHODS. In this study, serum proteomes (depleted of high-abundance serum proteins) of horses with ERU and healthy controls were compared with the 2-D DIGE (two-dimensional gel electrophoresis) technique to identify differentially expressed proteins. The expression pattern of a candidate protein in retina and vitreous was validated by Western blots and immunohistochemistry.

RESULTS. Ten differentially expressed proteins could be identified by mass spectrometry (MALDI-TOF/TOF). Five proteins—IgM, IgG4 hc, serotransferrin, α2HS-glycoprotein, and complement factor B—were upregulated in the uveitic state, whereas the five proteins albumin, apolipoprotein A-IV and H, IgG5 hc, and high-molecular-weight kininogen (HK) showed a significantly lower expression in sera of uveitis cases. Of interest, kininogen was significantly upregulated in the target tissues vitreous and retina. HK is a plasma protein with multiple physiologic functions, with an important role in inflammation and promoting neovascularization. Most interesting is the as of yet unaddressed association of HK with uveitis. Immunohistochemistry showed coexpression of kininogen and VEGF in inflamed eyes.

CONCLUSIONS. Since neovascularization plays a major role in the pathogenesis of uveitis, the identification of a proangiogenic factor in the retina presents an important finding and may contribute to elucidating the pathogenesis of uveitis. (Invest Ophthalmol Vis Sci. 2010;51:375–382) DOI:10.1167/iovs.09-4094

Equine recurrent uveitis (ERU) occurs worldwide and affects up to 10% of the equine population.1 The disease is characterized by recurrent inflammatory episodes in the inner eye that are interrupted by variable periods of quiescence.2 ERU serves as a valuable model for human uveitis, because it closely resembles human autoimmune uveitis in clinical and immunopathologic features.3 Moreover, ERU is the only spontaneous animal model for uveitis.4 The recurrent inflammatory episodes lead to the destruction of the retina and result in blindness. Only activated T cells are able to cross the blood-retinal barrier; however, the mechanism that enables the T cells to pass is as yet unknown. The autoaggressive T cells are directed against various retinal autoantigens such as S-antigen, interphotoreceptor binding protein (IRBP), or cellular retinaldehyde-binding protein (CRALBP).6–8

Alterations of protein abundance, structure, and function act as useful indicators of pathologic abnormalities. Differences between protein expression levels of healthy and diseased individuals can form the basis for elucidating causative factors of disease and identifying biomarkers for diseased states.9 Former proteomic analysis of retina and vitreous from ERU-diseased horses and healthy control animals revealed several differentially expressed proteins associated with disease.10,11 The breakdown of the blood-retinal barrier and the immigration of activated T cells into the eye allowed the assumption that differentially expressed proteins that are related to the disease process are present in the peripheral blood. Serum is a valuable specimen, because it stays in touch with many cells of the body and therefore possesses a wealth of information concerning the pathophysiology of a disease,12 and moreover it is easily accessible, which allows tight monitoring. The comparison of serum proteomes of ERU cases and healthy controls presented a successful approach identifying a potential uveitis marker in serum.13 In the previous study, PEDF, a neurotrophic factor and potent inhibitor of angiogenesis, was significantly downregulated, not only in the target tissues of disease, vitreous, and retina,10,11 but also in uveitic serum.13

Focus in biomarker research is shifting from methods that can analyze one marker at a time to so called profiling methods. Representative marker patterns will allow a statistically more stringent differentiation and a better classification of patient groups.14

Although serum comprises a wealth of information, only a few proteins make up approximately 99% of the protein content.15 The reduction of high-abundance proteins avoids overloading of the analytical system and enables the enrichment of proteins with a low concentration range. The purpose of this
study was to compare sera of ERU cases and healthy controls depleted of high-abundance serum proteins with a comprehensive approach for identifying proteins, which are involved in the pathology of autoimmune uveitis or may serve as marker for disease monitoring.

Methods

Samples

A total of 20 ERU cases and 20 healthy control subjects were used. ERU was diagnosed according to clinical criteria as described. For the 2-D DIGE screening experiment we processed sera of five healthy horses and five ERU cases. All serum samples were stabilized with protease inhibitors (Roche, Mannheim, Germany) and stored immediately at −20°C. Nine vitreous and nine retinal specimens for 1-D gel electrophoresis were homogenized, stabilized with protease inhibitors, lyophilized, and stored at −80°C. For immunohistochemistry, Bouin-fixed (Sigma-Aldrich, Deisenhofen, Germany) eyes (ERU, n = 10; controls, n = 10) were embedded in paraffin (Micron, Walldorf, Germany) and sectioned. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All horses had been presented as patients to the Equine Clinic of LMU Munich; no experimental animals were used.

Serum Depletion of High-Abundance Proteins

Serum was depleted of high-abundance proteins (ProteomeLab IgY-12 kit; Beckman Coulter, Krefeld, Germany). The column is charged with resin-coupled polyclonal avian antibodies directed against 12 high-abundance serum proteins. Serum depletions were performed according to instructions on column usage and loading capacity. The depletion scheme consisted of sample loading, washing, eluting and neutralization. Three buffers were used: dilution/washing buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4), stripping buffer (0.1 M glycine HCl), and neutralization buffer (1M Tris-HCl). The column was alternated between specimens from ERU cases and healthy controls to avoid differences in depletion quality between both groups of samples. The protein content of sample sets was quantified in the same Bradford assay (Sigma-Aldrich) using the same standard curve to minimize technical variation.

Sample Preparation and Fluorescence Labeling

For the 2-D DIGE experiment, a minimal labeling technique was performed. Aliquots of the depleted serum samples containing a total of 50 μg protein each were prepared. Equal amounts of DIGE buffer (pH 8.5; 30 mM Tris-HCl, 7 M urea, 2 M thiourea, and 4% CHAPS) were added to each sample. The samples of the ERU diseased horses and the healthy controls were labeled with the fluorescent dyes Cy3 or Cy5 (CyDye; GE Healthcare). Cy2 was used to label the internal standard (comprised a mixture of all samples used in the experiment). The dye/protein ratio was 400 picomoles/50 μg protein. The labeling reaction was stopped after 30 minutes by addition of 1% lysine. After an additional 10 minutes of incubation, samples were combined into five sample sets, each containing one ERU, one control, and one internal standard. Each sample set was filled to a final volume of 460 μL with DIGE buffer and 1% carrier ampholytes (pharmalyte pH 3–10; GE Healthcare, Freiburg, Germany) and 0.5% bromophenol blue were added. Dry strips (pH 3–11, 24 cm, Immobiline; GE Healthcare) were rehydrated with the samples overnight. Isoelectric focusing was performed on an electrophoresis system (Multiphor; GE Healthcare) for 45 kVh at 20°C, followed by separation on SDS gels (12%) at constant 45 V per gel. After the SDS-PAGE, the gels were scanned (Typhoon Trio scanner; GE Healthcare) at the emission wavelengths of the fluorescent dyes (Cy2–488 nm; Cy3–532 nm; Cy5–653 nm). After scanning, all gels were silver-stained and stored for subsequent mass spectrometric identifications.

Image Acquisition and Biological Variation Analysis

Scanned fluorescent images were imported into image-analysis software (DeCyder 6.5 software; GE Healthcare). The volume of a spot for a given dye is defined as the fluorescence intensity of the corresponding dye integrated over the area of a spot. First, the differential gel analysis (DIA) program for intergel analysis was used. Spot detection (maximum, 2,500 spots; exclusion filter slope, >2; maximum volume, 30,000) and normalization (Cy3/Cy2 and Cy5/Cy2) was performed and for each gel the number and distribution of spots according to their volume/ratio were displayed in a histogram. The histogram indicated the type of the detected spot: increased, unchanged, or decreased. The gels were imported in the biological variation analysis (BVA) program for an intergel analysis. The gel with the most spots was set as master gel. The software provides a ratio of normalized spot volumes, also called the standardized abundances. The standardized abundances of all matched protein spots were compared across the five analytical gels per experiment. Spot matching was automatically performed by the software, but the matching was checked manually and corrected if necessary. For statistical analysis, Student’s t test (P < 0.05) was used to detect significant differences between spot volumes from uveitic and healthy control serum. The detected spots were highlighted on the map created by the software. This map was transferred onto a transparent film and used as the reference to localize the spots of interest on the respective silver-stained gels to cut and identify by mass spectrometry.

Mass Spectrometry

Selected spots from 2-DE were excised, destained, and processed by proteolysis with trypsin, as described before and analyzed by MALDI-TOF peptide mass fingerprinting and MS/MS on a MALDI-TOF/TOF tandem mass spectrometer (ABI 4700 Proteomics Analyzer; Applied Biosystems, Inc. [ABI], Foster City, CA). For positive-ion reflector mode spectra 2500 laser shots were averaged and processed with external calibration. PMF spectra were not smoothed, and background was not subtracted. Monoisotopic peak masses were automatically determined within the mass range 800 to 4000 kDa with a signal-to-noise ratio minimum set to 5 and the local noise window width m/z 200. Up to seven of the most intense ion signals with signal-to-noise ratio above 30 were selected as precursors for MS/MS acquisition excluding common trypsin autolysis peaks and matrix ion signals. In MS/MS positive ion mode 4000 spectra were averaged with 1 kV collision energy, collision gas air at a pressure of 1.6 × 10−6 torr and default calibration. Monoisotopic peak masses were automatically determined with a signal-to-noise ratio minimum set to 10 and the local noise window width m/z 200. Combined PMF and MS/MS queries were performed (Mascot Database search engine v1.9; Matrix Science Ltd., UK; http://www.matrixscience.com, embedded in GPS-Explorer Software; ABI) on the SWISS-PROT database (version 20070531; 270778 sequences; 99412397 residues; http://expasy.hcuge.ch/tools/scnpsite.html) provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland), the MSDB metadata database (version 20061115; 3239079 sequences; 1079594700 residues; http://proteomics.leeds.ac.uk/bioinf/msdb_simple.html), or the horse genome database (retrieved from UCSC, version 20070101: 39612 sequences; 107365 residues; provided in the public domain by the University of Santa Cruz, CA; http://genome.ucsc.edu/cgi-bin/hgGateway) with the following parameter settings (if applicable): entries restricted to mammalian, 65 ppm mass accuracy, 0.3-Da fragment mass tolerance, trypsin cleavage, one missed cleavage allowed, carbamidomethylation was set as fixed modification and oxidation of methionines was allowed as variable modification. A protein was regarded as identified, if the probability-based MOWSE score was significant for the respective database (protein scores >58 were significant for SwissProt, scores >67 for MSDB, and scores >54 for the horse genome database; P < 0.05), if the matched peptide masses were abundant in the spectrum and if the
theoretical masses of the significant hit fit the experimentally observed values.

1-D Gel Electrophoresis and Western Blot Analysis

For the Western blot analysis, we normalized the sample input to equal total protein loading onto the gels. Vitreous and retina samples were resolved by 10% sodium SDS-PAGE and blotted semidry on PVDF membranes (GE Healthcare). Unspecific binding was blocked for 1 hour with 5% BSA in PBS-T. Blots were incubated with the primary antibody overnight at 4°C (anti-kininogen 1:500; Santa Cruz, Heidelberg, Germany), washed and incubated with HRP-coupled secondary antibodies (1:5000, Sigma-Aldrich). Signal was detected with enhanced chemiluminescence (Hyperfilm ECL; GE Healthcare).

Image Analysis and Signal Quantification

Films were scanned on a transmission scanner (GE Healthcare). Quantification of Western blot signals was performed (ImageQuantTL software; GE Healthcare). Images of the Western blot signals (8 bit/600 dpi) were imported into image-analysis software and band volume intensities were subsequently calculated. Standardization of the band volume intensity was performed with the calibration tool of the scanning software (LabScan ver. 5.0 software; GE Healthcare; densitometric calibration by Kodak No. 2 Step tablet film; Eastman Kodak, Rochester, NY).

Statistical Analysis

The image-analysis software (DeCyder; GE Healthcare) and Student’s t-test (P ≤ 0.05) were used to detect significant differences between spot volumes from uveitic and healthy control serum.

I-D Western blot signals of uveitic cases and healthy controls were compared by using the Mann-Whitney test (Past; by Øyvind Hammer, David A. T. Harper, and P. D. Ryan; offered in the public domain from the University of Oslo, Norway, at http://folk.uio.no/ohammer/past/).

Candidate Validation with Immunohistochemistry

Heat antigen retrieval was performed at 99°C for 15 minutes in 0.1 M EDTA-NaOH buffer (pH 8.8). We used monoclonal mouse antibody specific for kininogen (5 µg/mL; Santa Cruz) and monoclonal mouse antibody for VEGF (10 µg/mL; BD Pharmingen, Heidelberg, Germany) for candidate detection in tissues. For fluorescence labeling, kininogen

was stained with an anti-mouse IgG antibody coupled to AlexaFluor 568 (1:500; Invitrogen, Karlsruhe, Germany), VEGF with anti-mouse IgG coupled to AlexaFluor 488 (1:500; Invitrogen). Cell nuclei were stained with DAPI (1:100; Invitrogen). The fluorescence staining was photographed (Axio Imager M1; Carl Zeiss Meditec, Göttingen, Germany) and visualized with the system software (Axio Vision 4.7 software; Carl Zeiss Meditec).

RESULTS

Immunodepletion of High-Abundance Serum Proteins

For all samples, the depletion efficiency and consistency of the high-abundance serum proteins was controlled by quantification of three representative proteins (albumin, apolipoprotein A-I, and haptoglobin) which should have been depleted by the column (ProteomeLab IgY-12; Beckman Coulter). To determine the equal depletion (in percentage of initial albumin input) across samples, the albumin content of each sample was quantified before and after depletion on one-dimensional gels as band volume intensities according to the scanning software (LabScan ver. 5.0; GE Healthcare). A reduction of 45% ± 13% (SD) of the total quantity of albumin in serum was achieved in depleted sera samples. Apolipoprotein A1 was reduced approximately 53% in the depleted-sera samples. To rule out nonspecific binding of proteins to the resin, the column eluate was separated by 2-D DIGE, and all spots were identified by mass spectrometry (MALDI-TOF/TOF). The identified spots represented eight proteins, albumin, apolipoprotein-A1, haptoglobin, IgG 7 gc, IgM, α1-antitrypsin, α-2-macroglobulin, and serotransferrin. All of them are expected to be depleted by the column. Further, no (unspecific bound) proteins were identified in the column eluate (data not shown).

Differentially Expressed Spots Detected in Depleted Sera from ERU Animals Compared with Healthy Controls

A fluorescent overlay image (Fig. 1a) illustrates Cy3 labeled depleted control serum (Fig. 1a, blue) and Cy5-labeled de-
pleted serum of a uveitis case (Fig. 1a, green). With the image-analysis software, we detected between 1318 and 1669 spots per gel (Fig. 1b). A total of 34 significant \( (P \leq 0.05) \), differentially expressed spots were revealed by the BVA in the protein repertoire between the uveitic and healthy horses \( (n = 5; \text{Fig. 1b, red-encircled spots}) \). Twenty spots had lower expression in uveitic cases compared with healthy controls, whereas 14 spots were upregulated in the uveitic state (Fig. 1b).

**Mass Spectrometry Identification of Differentially Expressed Proteins in Uveitis and the Healthy Control**

Fifteen spots, representing 10 differentially expressed proteins, were unambiguously identified by tandem mass spectrometry (MALDI-TOF/TOF). Five of these proteins were downregulated in uveitic condition. These were albumin (corresponding to spots 5, 6, 12, and 15; Figs. 1b, 2), kininogen-1 (corresponding to spot 9, Figs. 1b, 2), apolipoprotein H (corresponding to spot 10; Figs. 1b, 2), apolipoprotein A-IV (corresponding to spot 11; Figs. 1b, 2) and immunoglobulin gamma 5 hc (corresponding to spot 13 and 14; Figs. 1b, 2). The five upregulated proteins in sera of horses with recurrent uveitis were identified as immunoglobulin M (corresponding to spot 1; Figs. 1b, 2), complement factor B (corresponding to spot 2, Figs. 1b, 2), Serotransferrin (corresponding to spot 3, Figs. 1b, 2), immunoglobulin gamma 4 hc (corresponding to spot 4, Figs. 1b, 2) and alpha 2HS glycoprotein (corresponding to spot 7 and 8, Figs. 1b, 2).

**Kininogen Expression Profile in Vitreous and Retina**

The expression pattern of kininogen was analyzed and quantified in the target tissue vitreous and retina by Western blot (Fig. 3). Of interest, kininogen showed a significant upregulation in both uveitic retina (Fig. 3a) and vitreous (Fig. 3b) compared with healthy control specimens.

**Validation of Kininogen by Immunohistochemistry**

The finding of downregulation of kininogen in uveitic sera (spot 9, Fig. 1b) and concurrent upregulation in vitreous and retina (Fig. 3) prompted us to investigate its expression pattern in the retina by immunohistochemistry. In healthy eyes, kininogen expression was not detected (Figs. 4a, 4b), whereas in uveitic eyes, kininogen was found in all retinal layers at inflamed sites (Fig. 4d, kininogen expression in red). In addition to kininogen expression (Fig. 4d, red), a marked expression of VEGF (Fig. 4d, green) was detected in the same regions of retinal inflammation. Healthy eyes were devoid of VEGF expression (Fig. 4b).

**DISCUSSION**

2-D DIGE presented a successful comprehensive approach to compare depleted serum proteomes of horses with ERU and healthy horses and has led to identification of 10 differentially expressed proteins that may be related to the disease. Only 22 high-abundance serum proteins make up 99% of the total protein mass, which leaves only 1% for the rest of potentially interesting proteins. Depletion of high-abundance proteins allows broader proteome coverage. Removal of high-abundance proteins from nonhuman serum samples presents a special challenge for several reasons. In this study, depletion of horse sera was performed with polyclonal IgY antibodies. The reason that chicken IgY antibodies are preferable over mammalian Ig antibodies is the evolutionary distance between chickens and mammals, which allows for greater immunogenicity against conserved mammalian proteins. Using polyclonal IgY antibodies for horse sera depletion, we could demonstrate a successful depletion of eight abundant serum proteins, whereas binding of nontarget proteins in the column eluate could not be discovered by mass spectrometry. Serum depletion allowed us to identify eight novel biomarker candidates, differing from the candidates found in complete sera.

IgG 5 hc isotype (spot 13, Figs. 1, 2; Table 1) had lower expression in uveitic sera. The principle of different IgG classes sharing individual effector functions during the immune response is generally conserved between mammalian species. Former studies also revealed a differential expression pattern of IgG isotypes in ERU. IgG 4 hc was found upregulated in sera and retina of ERU-diseased horses. It is known that the cytokine expression patterns of Th1 or Th2 immune responses are linked to production of specific IgG isotypes. The differential expression of IgG isotypes between control and diseased animals confirms the dysregulation of the immune response in ERU. Studies on dilatative cardio-myopathy and rheumatoid arthritis have demonstrated that certain IgG subclasses are particularly associated with disease severity and progression in humans. In the experimental model of horse uveitis, immunization with SAg induced S-antigen-specific antibodies with the isotype IgG4. These findings point to the necessity of determining the related immunologic function of the detected IgG isotypes in ERU.

In addition, we could observe an increase of IgM molecules (spot 1, Figs. 1, 2; Table 1). An upregulation of IgM indicates a novel immune response pattern originating from no longer than 4 weeks before sampling (reflecting acute response). The role of IgM in autoimmune disease is not clear yet. The key feature of recurrent uveitis is the remission and relapse of uveitic episodes. For spontaneous ERU and induced experimental horse uveitis, it is known that inter- and intramolecular epitope spreading occurs. Although it is speculative, the high levels of IgM may also point to intra- and intermolecular epitope spreading between various autoantigens, making it interesting to evaluate the specificity of these IgM antibodies.

A further high-abundance serum protein, serotransferrin (spot 3, Figs. 1, 2; Table 1) was found in higher amounts in ERU sera than in the control. Besides the main function of serotransferrin in iron transport it is known to play a role in T-cell differentiation. A recent study demonstrated in a hypotranferrinemic mouse model, that a deficiency of circulating transferrin or decreased expression of transferrin receptors results in a reduction of the total number of lymphocytes as well as in a severe reduction of the number of lymphocytes that differentiate. Since autoimmune uveitis is a T-cell-mediated disorder induced by activated T cells homing from the spleen to the eye, it is interesting to find a factor upregulated in ERU that is possibly involved in T-cell proliferation. Furthermore, an upregulation of serotransferrin had been detected in the retina, the target organ of uveitis. Although up to now, it is not clear to what extent serotransferrin is connected with T-cell proliferation in uveitis, this context merits further exploration.

Another differentially expressed protein in ERU sera is albumin (spots 5, 6, 12, 15, Figs. 1, 2; Table 1). The decreased expression of albumin was not observed for full-length albumin, but for several fragments with lower masses. The relevance of these fragments is unknown and so the meaning of their lowered expression remains unclear. An artifact created by the albumin depletion via the used IgY column is not likely, because the lower albumin content was also detected in the unprocessed sera. Further, we routinely alternated depletions between control and ERU samples to rule out a difference deriving from column efficiency from one group of sera to the other.
FIGURE 2. Differentially regulated candidates of the 2-D DIGE experiment in depleted sera of horses with spontaneous uveitis, according to image analysis. Each panel includes (a) an enlarged 2-D picture of respective spots in depleted ERU sera corresponding to the 2-D survey map in Figure 1b; the spot number corresponds to the mass spectrometry (Table 1); (b) corresponding spots in healthy control serum; (c) 3-D graph of spot volume in ERU; (d) 3-D graph of spot volume in healthy control serum. Graphs (right) show the difference in abundance of spot pairs in all gels. The standardized abundance is defined as the ratio of normalized spot volumes. Dark gray circles: the spot pair shown in (a–d); gray squares: internal standard.
We also observed two members of the apolipoprotein family significantly downregulated in ERU; apolipoprotein H (Apo H), which binds to various kinds of negatively charged substances (spot 10, Figs. 1, 2, Table 1) and apolipoprotein A-IV (Apo A-IV), which may have a role in chylomicron and VLDL catabolism (spot 11, Figs. 1, 2; Table 1). Although the altered expression of Apo H was not yet reported in autoimmune uveitis, another protein from this family, apolipoprotein I was decreased expressed in the vitreous proteome of uveitis cases.10 Of interest, lower expression of Apo A-IV was also found in plasma of patients with rheumatoid arthritis and in vitreous of patients with diabetic retinopathy.31 Therefore, the decreased expression levels of apolipoproteins H and A-IV in the pathologic condition may be directly related to a functional role in disease pathogenesis and should be evaluated more closely.

Another upregulated component in ERU sera was α2-HS glycoprotein (AHSG) (spots 7, 8; Figs. 1, 2; Table 1). AHSG promotes endocytosis, possesses opsonic properties, and is a negative acute-phase protein.32,33 The upregulated expression of AHSG in uveitic sera could be connected to the role of AHSG as a modulator of immune responses and could demonstrate a reaction to the inflammatory process and therefore deserves closer investigation.

An important finding of this study was the upregulation of complement factor B (spot 2, Figs. 1, 2) in uveitic sera. Complement factor B belongs to the alternative pathway of the complement system and catalyzes the amplification step of complement activation to initiate inflammatory responses, cell lysis, and phagocytosis.34-35 The complement system and its regulation is known to be involved in the pathologic activity of various ocular diseases such as age-related macular degeneration (AMD), keratitis, and uveitis.36 For experimental autoimmune uveitis (EAU) it was shown that complement deficient rats show a reduced incidence, duration, and severity of disease.37 Uregulation of complement factor B, belonging to positive acute-phase proteins, in uveitic sera, may reflect an acute-phase response because of ongoing inflammation in the eye. The involvement of the complement system in the disease process of uveitis is supported by the findings of upregulated C3 in vitreous of uveitic horses.10 The relevance of involvement of the complement system in the disease process, in particular in the target tissue, the retina, merits further examination.

An important and interesting finding is the as of yet unaddressed association of the high-molecular-weight kininogen 1 (HK; spot 9, Figs. 1, 2, Table 1) with spontaneous recurrent uveitis. HK is a multifunctional, multidomain protein with each HK domain having distinct functions.38 HK was significantly downregulated in all sera of the ERU group in comparison to the healthy control subjects, but showed an upregulation in uveitic retina and vitreous (Figs. 3a, 3b). This opposite expres-
A known promotion factor of angiogenesis in uveitic retina,10 uveitis, we investigated the expression of kininogen and VEGF, which play a significant role in the pathogenesis of diabetic retinopathy.50 Since it is known that neovascularization and inflammatory processes of disease contribute to the breakdown of the blood-retinal barrier. It is known that during the course of disease, the blood–retinal barrier breaks down allowing an accumulation of serum proteins in the eye.10,11 HK is a plasma protein that is cleaved by kallikrein to release bradykinin. The remaining portion of the molecule is designated cleaved kininogen or kininostatin (HKa), which physiological function is not clear yet.46,47 Of interest, we found complement factor B to be upregulated (spot 2; Figs. 1, 2) in sera of ERU cases. Ten differentially expressed proteins have been identified that reflect the ongoing immune response or are linked to inflammatory processes. Notably, the role of kininogen and its activation in the disease process merits further examination in our opinion.

**Table 1. Differentially Expressed Proteins in Depleted Sera of Horses with ERU**

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<tr>
<th>Spot ID</th>
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<th>Accession Number</th>
<th>Mascot Score</th>
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The expression pattern of the identified proteins in ERU is indicated with arrows (↑, upregulated, and ↓, downregulated in ERU sera). For statistical analysis Student’s *t*-test (*P* ≤ 0.05) was performed to validate the significance of the differences detected between spot volumes from depleted uveitic sera and depleted control sera.

The proangiogenic effect of HK was demonstrated by assay in the chicken chorioallantoic membrane (CAM).48 This is further supported by the finding that angiogenesis is suppressed in kininogen-deficient rats.49 A recent study demonstrated that intravitreal injections of plasma kallikrein increased retinal vascular permeability and inflammation in an animal model of diabetic retinopathy.50 Since it is known that neovascularization plays a significant role in the pathogenesis of uveitis, we investigated the expression of kininogen and VEGF, a known promotion factor of angiogenesis in uveitic retina.10 by immunohistochemistry. In the uveitic retina, focal kininogen and VEGF expression was demonstrated in areas that displayed retinal destruction due to ongoing inflammatory processes (Fig. 4c, d), whereas in healthy retinas, neither kininogen nor VEGF was detected (Fig. 4a, b). Kininogen expression appears to be distributed to the tissue rather than confined to specific cells. We postulate that the expression of kininogen throughout all retinal layers in diseased eyes contributes to the neovascularization and inflammatory processes of disease.

In conclusion, 2-D DIGE was a successful comprehensive approach to the detection of differentially expressed proteins when comparing depleted sera of ERU-diseased horses with those of healthy control animals. Ten differentially expressed proteins have been identified that reflect the ongoing immune response or are linked to inflammatory processes. Notably, the role of kininogen and its activation in the disease process merits further examination in our opinion.

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**References**


