Identification, Quantitation, and Purification of a 36 kDa Circulating Protein Associated With Active Pars Planitis

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Purpose. To establish a correlation between the presence of a 36 kDa protein in the blood of patients with pars planitis and to characterize and purify this protein.

Methods. Blood samples were obtained from patients with pars planitis and other types of uveitis and from various controls. Samples were treated with polyethylene glycol and protein A and were analyzed on 10% SDS-PAGE for the presence of a 36 kDa protein. Quantitative estimation of the level of this protein was determined by densitometric tracing of the stained gels. Polyclonal antibodies were raised by immunizing New Zealand White rabbits with a mixture of the gel fragment containing the 36 kDa protein (p-36) and complete Freund's adjuvant. These antibodies were used in the immunoaffinity purification of this protein.

Results. The levels of p-36 were sixfold to eightfold higher in 81% of the patients with active pars planitis than in controls (P < 0.05). Furthermore, the levels of this protein correlated with disease activity. A partial amino terminal sequence analysis revealed that p-36 may be a novel protein. It has been purified from the patient's blood using affinity chromatography.

Conclusions. A 36 kDa protein (p-36) is found in elevated concentrations in the blood of many patients with active pars planitis. Its putative role in the etiopathogenesis of pars planitis is unknown. Invest Ophthalmol Vis Sci. 1996;37:1870–1876.

A leading cause of blindness in the United States is uveitis.¹ Pars planitis is a relatively common, clinically well-defined form of intermediate uveitis of unknown etiology. It is a chronic inflammatory disease of generally insidious onset.² Primarily, adolescents and young adults are affected, and they frequently complain of floaters in one or both eyes. Vision is decreased as a result of macular edema or a posterior capsular cataract. The prognosis for vision in the majority of patients is favorable.³ However, oral or periocular corticosteroids must be used on a chronic basis to prevent visually disabling complications.

We previously identified increased levels of a 36 kDa protein (p-36) in the blood of patients with pars planitis.⁴ The current studies were undertaken to explore further the unique association of p-36 with pars planitis and to characterize the protein.

MATERIALS AND METHODS

Reagents

CNBr-activated sepharose 4B beads, PEG-8000, and Ponceau S were obtained from Sigma Chemical (St. Louis, MO), complete Freund’s adjuvant was obtained from Difco Laboratories (Detroit, MI), I²¹⁵I protein A was obtained from Amersham Life Sciences (Arlington Heights, IL), and Econo-Pac Serum IgG Purification Kit was obtained from Bio-Rad (Richmond, CA).

Clinical Identification and Characterization of Patients

Blood (plasma or serum) samples from patients with different subsets of uveitis were collected during the active phase of the disease at the Washington University Eye Center (St. Louis, MO). Patients were evaluated by history, clinical examination, and diagnostic testing as previously described.⁵ Three groups of patients with uveitis were included in this study, those with acute idiopathic anterior uveitis (HLA-B27+ and B27–), chronic idiopathic panuveitis, and pars planitis (i.e., vitritis with snowbanking). For the purposes of this study, patients with uveitis with vitritis (snowballs) without pars plana deposits were not classified.
A 36 kDa Protein in Pars Planitis

Patients with pars planitis were considered to be in the active phase of the disease if they had moderate or severe vitritis (with pars plana snowbanks). Frequently, they had floaters or blurred vision and cystoid macular edema confirmed by biomicroscopy or fluorescein angiography, or they had progression of a posterior subcapsular cataract. They were considered in the inactive phase of the disease if they were visually asymptomatic or had no or mild vitritis, even in the presence of pars plana snowbanks.

Samples from patients with uveitis also were obtained from Dr. James Rosenbaum (University of Oregon Health Sciences Center, Portland, OR), and these samples were analyzed in a masked fashion.

Blood samples were obtained from patients with proliferative diabetic retinopathy after panretinal laser photocoagulation and patients with glaucoma who underwent cyclodestructive procedures. Samples from patients with acute rheumatoid arthritis and systemic lupus erythematosus were kindly provided by Dr. John P. Atkinson (Professor and Chairman of the Department of Medicine, Washington University), and samples from patients with multiple sclerosis were kindly provided by Dr. John Trotter (Department of Neurology, Washington University). Normal healthy subjects (students and staff of the Department of Ophthalmology and Visual Sciences) with no history of eye disease formed the control group. Serum or plasma samples were used immediately or stored at -80°C.

All human studies were performed following the guidelines of the Declaration of Helsinki and were approved by the institutional review board. Informed written consent was obtained from all patients before inclusion in the study.

**Identification and Quantitation of 36 kDa Protein**

As either serum or plasma, 250 µl of the sample was treated with polyethylene glycol-8000 (2.25% final concentration) at 4°C overnight. The resultant precipitate was resuspended in 100 µl of borate-buffered saline, pH 8, and was incubated at 37°C for 30 minutes, and then it was incubated with protein A beads at 4°C for 2 to 4 hours. The beads were washed twice with borate-buffered saline, and bound proteins were eluted by heating at 100°C in 0.25 M Tris, 2% SDS, 10% glycerin, pH 6.8. Before they were loaded on the gel, the samples were treated with 5% 2-ME and heated at 100°C for 10 minutes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described using a 10% slab gel, and gels were silver stained. Forty micrograms of the total protein was loaded in each lane. Molecular weight markers are indicated (kDa) on the right margin. The arrow indicates the 36 kDa protein, p36.

**Amino Terminal Sequence Analysis**

For amino terminal sequence determination, proteins from the polyacrylamide gels were transferred by electrophoretically to a polyvinylidene difluoride membrane. A membrane slice containing the unique protein was cut out, and filter-bound protein was subjected to NH2-terminal sequence analysis using an Applied Biosystems (Foster City, CA) 477 sequenator. The resultant NH2-
TABLE 1. Presence of p-36 in Blood as Detected by SDS–PAGE Analysis

<table>
<thead>
<tr>
<th>Disease</th>
<th>Disease Activity</th>
<th>Number of Patients</th>
<th>High p-36 Levels</th>
<th>Low p-36 Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pars planitis</td>
<td>Active</td>
<td>43</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>Pars planitis</td>
<td>Inactive</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Acute idiopathic anterior uveitis</td>
<td>Active</td>
<td>13</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Chronic idiopathic pan uveitis</td>
<td>Active</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Glaucoma patient after cyclodestructive procedure</td>
<td>NA</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Diabetic retinopathy after panretinal photocoagulation</td>
<td>NA</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Active</td>
<td>15</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Active</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>Active</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Normal controls</td>
<td>NA</td>
<td>19</td>
<td>0</td>
<td>19</td>
</tr>
</tbody>
</table>

High and low levels of p-36 were determined by subjective visual assessment of silver-stained SDS–PAGE. NA = not applicable.

Preparation of Polyclonal Antibodies

Polyclonal antibodies against 36 kDa protein were raised in New Zealand White rabbits. Gel fragments containing this protein were cut out and mixed with complete Freund’s adjuvant. This mixture, containing 100 to 200 ng of 36 kDa protein, was injected into a rabbit every 10 days. Three days after the sixth injection, blood was withdrawn and serum was collected. The specificity and titer of the antibody were determined by immunodiffusion and immunoblot analysis as described below.

Immunofluorescence Purification of the 36 kDa Protein

Affinity columns were prepared by coupling polyclonal antibodies raised against 36 kDa protein to CNBr-activated sepharose 4B beads following the standard methods. Before coupling, the antisera was partially purified using Econo-Pac Serum IgG Purification kit (Bio-Rad). Any plasma or serum testing positive for this 36 kDa protein was loaded on the affinity columns, and the bound proteins were eluted using the salt gradient (100 to 400 mM NaCl). The eluate was dialyzed extensively against water, lyophilized, and analyzed on 10% SDS–PAGE.

Immunoblot Analysis

Proteins separated by SDS–PAGE were transferred to nitrocellulose membranes essentially as previously described, and the transfer efficiency was monitored by staining the nitrocellulose with Ponceau S. The unbound sites on the nitrocellulose were blocked with 1% ovalbumin or 3% nonfat milk. Blots were incubated with 1:1000 and 1:2000 dilutions of polyclonal antibodies raised against 36 kDa protein for 3 hours at room temperature. Detection was performed with 125I-labeled protein A, and bound antibodies were visualized by the exposure of air-dried nitrocellulose to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) at −80°C for 2 to 3 days.

RESULTS

Blood Levels of p-36 Are Elevated in Active Pars Planitis

Blood samples from patients with pars planitis and various controls were treated with polyethylene glycol terminal sequences were compared with sequences stored in a National Biomedical Research Foundation Protein Identification Resource (release 28.0) database.

TABLE 2. Quantitative Estimation of p-36 in the Blood

<table>
<thead>
<tr>
<th>Disease</th>
<th>Disease Activity</th>
<th>Number of Patients</th>
<th>p-36 Levels (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pars planitis</td>
<td>Active</td>
<td>35</td>
<td>9.5 ± 0.9*</td>
</tr>
<tr>
<td>Pars planitis</td>
<td>Active</td>
<td>8</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Acute idiopathic anterior uveitis</td>
<td>Active</td>
<td>11</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>Acute idiopathic anterior uveitis</td>
<td>Active</td>
<td>2</td>
<td>9.0 ± 0.7*</td>
</tr>
<tr>
<td>Chronic idiopathic pan uveitis</td>
<td>Active</td>
<td>8</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Glaucoma patients after cyclodestructive procedure</td>
<td>NA</td>
<td>4</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>Normal control</td>
<td>NA</td>
<td>19</td>
<td>1.1 ± 0.7</td>
</tr>
</tbody>
</table>

p-36 bands were scanned densitometrically, and total peak area was determined. p-36 levels are given as mean of total peak area ± SD. Paired or unpaired Student’s t-test was used for statistical analysis.

NA = not applicable.

* P < 0.05 (two-sided).
FIGURE 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (10%) analysis of human plasma treated with PEG and protein A. (lane 1) Patient with inactive pars planitis. (lane 2) Same patient in the active phase of the disease. Each lane had 40 μg of the total protein. Molecular weight markers are indicated (kDa) on the left margin. The arrow indicates the protein, p-36. The 10% SDS–PAGE is used to resolve protein bands between 30 to 50 kDa. Proteins outside this range are not accurately resolved. Spurious bands, such as the 95 kDa and 25 kDa bands in lane 2 and the >97 kDa band in lane 1, occasionally were observed but were not consistently reproducible.

A 36 kDa Protein in Pars Planitis

By visual analysis of SDS–PAGE, we observed a protein of 36 kDa molecular weight in all the samples mentioned. However, this protein was present in much higher concentrations in the samples of patients with pars planitis (Fig. 1). The levels of p-36 were only high in 35 of 43 patients with active pars planitis and in 2 of 13 patients with acute idiopathic anterior uveitis (Table 1).

Ten patients with active pars planitis and high p-36 levels, who then entered an inactive phase, were reanalyzed on SDS–PAGE. A marked decrease in the levels of p-36 was observed (Fig. 2), suggesting a correlation of this protein with disease activity. No difference in the level of p-36 was noted between plasma and serum.

Quantitative estimation of the levels of p-36 in patients with pars planitis, as well as of all the controls, was determined to confirm the visual results of SDS–PAGE. Quantitation was performed by densitometric tracing of the stained gels (Fig. 3). We observed that in the 35 patients with active pars planitis who had high levels of p-36, there was a sixfold to eightfold increase in this protein compared to inactive pars planitis or controls (Table 2; P < 0.05). The levels of p-36 in the eight patients with active pars planitis, which visually appeared low, were similar to those observed in normals and controls. The levels of p-36 in the 10 patients with active pars planitis with active disease who then entered into the inactive phase of the disease were also similar to those of the controls (Table 2). In the two patients with active acute idiopathic anterior uveitis, high levels of p-36 also were observed on densitometric tracing (Table 2).

Fifteen samples of blood from patients with active pars planitis were obtained from another uveitis center in a different part of the country. They were analyzed in a masked fashion for the presence of p-36. Statistically significant high levels of this protein were observed in 65% of these samples (data not shown).

p-36 Is a Novel Protein

A partial amino terminal sequence of the 36 kDa protein was determined and is presented in Table 3. A search of the National Biomedical Research Foundation Protein Identification Resource computer database revealed this sequence to be unique.

Purification of p-36 by Affinity Chromatography

Polyclonal antibodies against p-36 were raised in New Zealand White rabbits. These antibodies recognized the 36 kDa protein by an immunodiffusion technique (not shown) and by immunoblot analysis (Fig. 4). Re-
results presented in Figure 4 show that these antibodies recognized the high levels of this protein observed in active pars planitis, as well as the barely visible levels noted in normals. However, the intensity of the hybridizing band was stronger in pars planitis.

These antibodies were used to purify the protein by affinity chromatography. Blood containing high levels of p-36 (determined by SDS–PAGE analysis) was used for purification. Bound proteins were eluted using a salt gradient. Fractions eluted at 100, 200, 300, and 400 mM NaCl were analyzed on SDS–PAGE for the presence of p-36. Figure 5A shows that the fractions eluted with 400 mM NaCl contain three protein bands, one strong band (approximately 36 kDa) and two weak bands (approximately 25 and 51 kDa). Western blot analysis was used to verify the authenticity of the purified 36 kDa protein band. Data presented in Figure 5B reveals reactivity of the polyclonal rabbit antibody raised against p-36 with the affinity purified protein at 36 kDa.

**DISCUSSION**

Pars planitis is a chronic inflammatory disease of unknown etiology. This article describes the identification of elevated levels of a 36 kDa protein in the blood of patients with pars planitis. Blood levels of this protein were higher (sixfold to eightfold; \( P < 0.05 \)) in 81% of the patients with active pars planitis than in various controls. The level of the p-36 correlated with disease activity and became low with resolution of the disease. No difference in the level of this protein was noted between plasma and serum samples.

Sixty-five percent of active pars planitis blood samples obtained from another uveitis center had statistically significant high levels of circulating p-36. Low levels of this protein can be explained by any combination of the following: a difference in the criteria to establish a diagnosis of active pars planitis; a difference in the etiology of the disease in different parts of the country; inaccurate assessment of disease activity; repeated freeze-thawing of the specimen.

A partial amino terminal sequence was determined for p-36. Search of the National Biomedical Research Foundation Protein Identification Resource database revealed this sequence to be novel. The amino terminal sequence of this 36 kDa novel protein did not match any known amino terminal sequence.

**TABLE 3. Amino Terminal Sequence of p-36**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Mass</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-36</td>
<td>36,000</td>
<td>1 X X Q F V D F P K I N X 12</td>
</tr>
</tbody>
</table>

Conventional one letter symbols are used for amino acids (X = not determined).

* Ambiguous.
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FIGURE 4. Immunoblot analysis using p-36 polyclonal antibodies. Normal human serum (lane 1) and serum obtained from a patient with active pars planitis (lane 2) were analyzed on 10% SDS-PAGE. Forty micrograms of the protein was loaded in each lane. The proteins, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were transferred to a nitrocellulose membrane, and the resultant blot was incubated with 1:1000 dilution of the p-36 polyclonal antibody. Bands were detected with 125I labeled protein A. The arrow indicates p-36, and the molecular weight markers (kDa) are indicated on the left margin.

36 kDa, which reacts with the polyclonal antibodies on Western blot analysis. Two faint bands at 25 and 51 kDa were detected, probably representing the light and heavy chains of human serum IgG, respectively.

The biologic function of this 36 kDa circulating protein is unknown. It is possible that high levels of this protein in active pars planitis simply might be the consequence of inflammation and tissue damage and may not serve a pathobiologic role. This is unlikely because patients with ocular tissue damage (e.g., those with proliferative diabetic retinopathy after panretinal photocoagulation or glaucoma after a cyclodestructive procedure) do not have high levels of this protein. Thus, this 36 kDa circulating protein may play a role in the etiopathogenesis of pars planitis. Quantitative assessment of the levels of p-36 in the patient's blood may be of value in monitoring disease activity and in guiding treatment.

Key Words
blood, etiopathogenesis, pars planitis, 36 kDa protein, uveitis

Acknowledgments
The authors thank Dr. Julian B. Fleischman for critical discussions.

References


