Plasminogen in proliferative vitreoretinal disorders

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Abstract
Objective—Intravitreal fibrin formation is a frequent observation after vitrectomy performed for a variety of vitreoretinal disorders including proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR), and endophthalmitis. Plasminogen activators (PA) have been used for the management of this postoperative complication. This approach requires the presence of plasminogen, the substrate for PA mediated fibrinolysis, in the vitreous cavity.

Methods—Quantification of plasminogen in the vitreous of 60 patients with PVR, PDR, and macular pucker was performed by streptokinase mediated activation using a chromogenic substrate. The presence of immunoreactive plasminogen was confirmed by immunoblot analysis of vitreal proteins and immunocytochemistry of surgically removed epiretinal membranes.

Results—Plasminogen levels were dramatically increased in the vitreous of PVR and PDR patients compared with macular pucker patients and normal controls. Staining for plasminogen in epiretinal membranes was confined to the extracellular matrix. Predominant staining of perivascular areas in PDR specimens indicated that breakdown of the blood-retinal barrier is an important source of intravitreal plasminogen in that condition.

Conclusion—Plasminogen may play a role in traction membrane formation in PVR and PDR. Our biochemical analysis of presurgical vitreous indicates that there may be abundant substrate for PA mediated fibrinolysis in the vitreous cavity after vitrectomy.

Intravitreal fibrin formation is a frequent complication after vitrectomy performed for a variety of vitreoretinal disorders including proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR), and endophthalmitis. Numerous studies have focused on the use of tissue plasminogen activator (tPA) for the treatment of this postoperative complication. However, the use of intravitreal tPA in the treatment of vitreous haemorrhage has been advocated. The fibrinolytic activity of tPA requires the presence of the β-globulin, plasminogen, in the vitreous and its conversion to the active enzyme, plasmin. There is only little information on the availability of plasminogen in normal and pathological vitreous. Here, we report that the levels of streptokinase activable plasminogen are dramatically increased in the vitreous of PVR and PDR patients compared with macular pucker patients and normal controls.

Materials and methods

SAMPLIES
Vitreous samples were obtained from patients suffering from traumatic PVR (n=10), idiopathic PVR (PVR after rhegmatogenous retinal detachment) (n=10), PDR (diabetes type I, n=10; diabetes type II, non-insulin dependent, n=10; diabetes type II insulin dependent, n=10), and macular pucker (n=10) by aspirating 100 μl of vitreous from the centre of the vitreal cavity before vitrectomy. Patients with intraocular bleeding at the time of surgery were excluded. Control samples (n=10) were taken from keratoplasty donor eyes within 6 hours post mortem. Epiretinal membranes were obtained from patients undergoing vitrectomy for traumatic PVR (n=5) and PDR (n=5). The membranes were immediately frozen at −70°C. Sections of 6 μm were prepared on a cryostat and fixed for 10 minutes in acetone at −20°C.

PLASMINOGEN ASSAY
Quantification of plasminogen was performed by streptokinase mediated activation using a chromogenic substrate. The use of this synthetic chromogenic substrate for the determination of plasminogen in biological fluid samples has been shown to be a simple and reliable method that correlates well with immunological assays and caseinolytic methods. Vitreous samples and commercially available plasminogen standards (Kabi, Mölndal, Sweden: 0.03, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 mg/ml) were diluted 1:40 in TRIS-HCl (0.05 mM, pH 7.4, 37°C). Streptokinase (10 000 U/ml) was added and incubated for 10 minutes followed by addition of the chromogenic substrate solution S-2251 (Kabi). Photometric readings were obtained every 10 minutes for 1 hour using an ELISA reader (Dynatek, MR-5000). The levels of plasminogen in the vitreous were calculated by plotting absorbance curves (AA/min) of samples against standards. Statistical analysis was performed by the Mann-Whitney U test. SPSS Software (SPSS Inc, Chicago, IL, USA) was used. Protein levels were measured by the Lowry method.

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Vitreal protein was determined using the Lowry method. Vitreal protein was measured enzymatically as described in Methods. Horizontal bars in the boxplots represent the median. The boxplots show upper and lower quartiles. Error bars indicate ranges of values (* = 0.02; ** p < 0.01 by Mann–Whitney U test compared with control vitreous).

**IMMUNOBLOT ANALYSIS**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Multiphor II Electrophoresis Unit, Pharmacia, Uppsala, Sweden) with subsequent blotting after protein transfer to nitrocellulose was performed as previously described. Vitreous and plasma samples were diluted 1:10 and 1:30 in loading buffer (Tris-HAc, 0.5 M, pH 7.5, SDS 10 g/l, dithiothreitol (DTT) 5 mM, respectively. Vitreal proteins (13 ml/lane) were separated on polyacrylamide gels (gradient 8–18%). Immunoreactive plasminogen was detected using a specific rabbit derived polyclonal antibody (Dako, Hamburg, Germany) and followed by addition of anti-rabbit IgG-F(ab)2 alkaline phosphatase (Dako). Naphthol AS-MX phosphate and fast red TR salt (Sigma, St Louis, MO, USA) were used as substrate.

**IMMUNOHISTOCHEMISTRY**

The general immunostaining procedures were performed as described. The membrane sections were thawed and refixed for 10 minutes in ice cold acetone. Antibodies were diluted in phosphate buffered saline (PBS, pH 7.4) containing 0.5% bovine serum albumin. The rabbit derived primary antibody bound to plasminogen (Dako, 1:100) was labelled by alkaline phosphatase conjugated anti-rabbit IgG-F(ab)2 (Dako, 1:200). Alkaline phosphatase activity was visualised by the fast red substrate system (Dako). All antibodies were incubated in a moist chamber at room temperature for 2 hours followed by rinsing with PBS. Negative controls were performed by exchanging the primary antibody with non-immune rabbit IgG (Sigma). Counterstaining was obtained with haematoxylin and eosin.

**Results**

**ELEVATED PLASMINOGEN LEVELS IN THE VITREOUS OF PDR AND PVR PATIENTS**

The levels of plasminogen in normal and pathological vitreous were measured by an assay that is based on the activation of plasminogen to plasmin by streptokinase and subsequent determination of plasmin activity using a chromogenic substrate. Plasminogen levels in normal vitreous samples amounted to 0.015 (SD 0.005) mg/ml. There was a highly significant (p < 0.01) increase in plasminogen in most proliferative vitreoretinal disorders compared with normal human postmortem eyes except in macular pucker (Fig 1). In insulin dependent diabetes mellitus type II, plasminogen values in the vitreous reached normal serum levels which are in the range of 0.2 mg/ml.

Total vitreal protein levels were elevated in most of the investigated vitreoretinal disorders (Table 1). This increase proved to be statistically significant (p ≤ 0.01) when tested against the control group. However, in the macular pucker group this increase was only minor (p=0.2). This is consistent with earlier reports from our laboratory where the low levels of total vitreal protein in macular pucker were thought to reflect the minimal breakdown of the blood-retinal barrier in that condition. Very high levels of total vitreal protein (≥ 8 mg/ml) content were detected in PVR and PDR (insulin dependent diabetes type II). The concentration of a vitreal protein is influenced by local synthesis and plasma filtration or exudation, the latter especially in vitreoretinal disorders which are characterised by disturbances of blood-ocular barriers. Therefore, we correlated levels of vitreal plasminogen with levels of total vitreal protein, both in the single patients and in the disease entities pooled.

We noted that the levels of plasminogen in normal vitreous, relative to those of total protein, exceed those in plasma almost 10-fold. Nevertheless, we observed a good correlation between plasminogen and protein levels in all of the investigated diseases (r=0.68–0.92, Table 1). This correlation was significant at a level of p ≤ 0.05 in macular pucker, PDR type I and PDR type II (non-insulin-dependent) and highly significant (p ≤ 0.03) in all other groups. The ratio between plasminogen and total protein content decreased (0.12–0.13)...

![Figure 1](http://bjo.bmj.com/)

**Table 1** Levels (SD) of plasminogen and total vitreal protein in the vitreous and plasminogen/protein ratios in normal controls and in vitreoretinal disorders. Plasminogen was measured enzymatically as described in Methods.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasminogen (mg/ml)</th>
<th>Total protein (mg/ml)</th>
<th>Correlation r</th>
<th>p Value</th>
<th>Ratio Plasminogen/total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.016 (0.005)</td>
<td>0.85 (0.36)</td>
<td>0.92</td>
<td>0.001</td>
<td>0.019</td>
</tr>
<tr>
<td>Macular pucker</td>
<td>0.027 (0.012)</td>
<td>*1.37 (0.65)</td>
<td>ns</td>
<td>0.79</td>
<td>0.048</td>
</tr>
<tr>
<td>Idiopathic PVR</td>
<td>0.108 (0.039)</td>
<td>**8.24 (1.86)</td>
<td>**0.81</td>
<td>0.009</td>
<td>0.013</td>
</tr>
<tr>
<td>Traumatic PVR</td>
<td>0.109 (0.040)</td>
<td>**8.87 (3.91)</td>
<td>**0.73</td>
<td>0.015</td>
<td>0.012</td>
</tr>
<tr>
<td>PDR (diabetes type I)</td>
<td>0.079 (0.041)</td>
<td>**3.25 (0.97)</td>
<td>**0.68</td>
<td>0.039</td>
<td>0.024</td>
</tr>
<tr>
<td>PDR (diabetes type II)</td>
<td>0.112 (0.031)</td>
<td>**3.94 (1.12)</td>
<td>**0.73</td>
<td>0.036</td>
<td>0.028</td>
</tr>
<tr>
<td>PDR (diabetes type II + insulin)</td>
<td>0.174 (0.054)</td>
<td>**8.57 (2.98)</td>
<td>**0.84</td>
<td>0.018</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Vitreal protein was determined using the Lowry method. Ratios were derived from these data (*p = 0.02; **p < 0.01 by Mann–Whitney U test compared with control vitreous).
in PVR compared with control and macular pucker (0.020) but was comparable with or higher than control in diabetic patients (0.020–0.028).

The presence of plasminogen in the vitreous was confirmed by immunoblot analysis (Fig 2).

Vitreous samples of human postmortem eyes (lane 2) showed the predicted immunoreactive band at 85–90 kDa for plasminogen. Similarly, all samples from patients with macular pucker, traumatic PVR, and PDR were positive. Human plasma served as a positive control.

DETECTION OF IMMUNOREACTIVE PLASMINOGEN IN EPITRINAL MEMBRANES OF PVR AND PDR PATIENTS

We also examined whether immunoreactive plasminogen was deposited in epiretinal membranes that are formed adjacent to pathological vitreous (Fig 3). All of 10 specimens of different disease aetiology showed strong immunoreactivity for plasminogen. This was abolished when non-immune IgG was substituted for the specific antibody (Fig 3B). Homogeneous staining of the extracellular matrix was observed in PVR (Fig 3A) as well as in PDR (Fig 3C) specimens. The intensity of staining was similar in all membranes. Strong immunoreactivity for plasminogen was observed in areas adjacent to small vessels in PDR membranes (Fig 3D). In contrast, cell rich areas did not display strong immunoreactivity in either PVR or PDR.

Discussion

Plasminogen is the central protein of fibrinolysis. Its activation to plasmin involves limited proteolysis and is physiologically catalysed by
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tPA or urokinase-like PA (uPA). In addition to fibrinolysis and thrombolysis, numerous biological processes are affected by plasminogen activation. These include cell migration, tissue remodelling, wound healing, and angiogenesis processes that are key features in PVR and PDR. Several components of the coagulation cascade have been identified in epiretinal membranes or vitreous, including fibrinogen, von Willebrand factor, plasma transglutaminase (blood coagulation factor XIII), fibronectin, thrombospondin, and plasmin.

Our special attention was drawn to plasminogen because its activation to plasmin may enhance release of cells from the RPE cell layer by degrading extracellular matrix and thus contribute to the development of PVR. In posterior vitreous detachment, plasmin is used to induce plaque formation.

Ophthalmic surgeons use tPA or uPA to dissolve postoperative vascular fibrin clots or to treat vitreous haemorrhage. The need for fibrinolytic agents in the treatment of postoperative intraocular fibrin formation is generally accepted since experimental subretinal fibrin deposition is associated with tearing of photoreceptor segment sheets and retinal degeneration.

Yet, little is known about the availability of plasminogen in the vitreal cavity. The quantification of vitreal plasminogen in physiological and pathological states was the first goal of this study. Interestingly, plasminogen levels in the intact physiological vitreous are higher than in serum in relative terms. Plasminogen is synthesised in many tissues, including kidney, eosinophils, or liver, the latter being the main source. There is no report on the source of intraocular plasminogen.

Plasminogen levels were uniformly elevated in all vitreoretinal disorders included in this study (Fig 1, Table 1). This is not surprising since there is blood-vitreous barrier dysfunction and elevated total vitreal protein in all these conditions. Direct evidence for blood derived plasminogen comes from the immunohistochemical studies which showed intense perivascular plasminogen staining in PDR (Fig 3D). In contrast, cell rich areas of the epiretinal membranes were largely devoid of plasminogen. If simple plasma exudation was the major factor determining plasminogen levels in the pathological vitreous, plasminogen protein ratios would be predicted to fall with increasing protein levels, given the lower plasminogen protein ratio in plasma compared with vitreous. However, the ratios of plasminogen levels and total vitreal protein content did not yield a uniform picture in the different conditions. The increase of total protein was associated with a relative decrease of plasminogen in both types of PVR. This finding could indicate enhanced turnover of plasminogen in PVR and is consistent with high levels of tPA in the vitreous of PVR patients. Likely sources of intraocular synthesis of tPA are retinal pigment epithelial cells which are the dominant cell type in PVR. Yet, these cells may also produce an inhibitor of PA, PAI-1, at least in vitro.

In contrast with PVR, the relative levels of plasminogen in two of three PDR groups were even higher than in controls, suggesting enhanced intraocular synthesis or accumulation—for example, as a consequence of decreased turnover of plasminogen. The latter is consistent with low PA levels in plasma of diabetic subjects with late complications such as PDR and reduced levels of retinal tPA immunoreactivity in eyes from insulin dependent diabetics. Diabetes mellitus is associated with increased fibrinogen turnover and high risk of thromboembolism not only due to angiopathy but also to changes in coagulation factors. Alteration of coagulation factors, especially increased levels of antithrombin III, have been reported. We have previously reported the presence of vitronectin in epiretinal membranes and elevated plasma levels of this protein in diabetic patients. Vitronec tin inhibits heparin catalysed inhibition of thrombin by antithrombin III, stabilises plasminogen activator inhibitor-1 (PAI-1), and mediates PAI-1 binding to extracellular matrix.

Thus, there are probably disease specific alterations in plasminogen metabolism in the pathological vitreous that correspond with different disease processes in PVR and PDR. Although we have analysed presurgical but not postsurgical vitreous, our findings indicate the presence of rich ocular and non-ocular sources of plasminogen which may be the precondition for successful application of PA for the prevention of postoperative fibrin clot formation and the management of intraocular bleeding.

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