Macrophage migration inhibitory factor levels in the vitreous of patients with proliferative diabetic retinopathy

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Abstract

Aims—To assess the potential role of macrophage migration inhibitory factor (MIF) in the pathogenesis of proliferative diabetic retinopathy (PDR).

Methods—MIF levels were assayed in the vitreous and paired serum samples of 73 consecutive patients with PDR (32 eyes) and macular hole or idiopathic epiretinal membrane (controls, 41 eyes). An enzyme linked immunosorbent assay technique was used to determine the concentrations of MIF.

Results—The median vitreous level of MIF was 11.93 ng/ml (range 4.16–103.85) in the patients with PDR, and 1.79 ng/ml (undetectable–8.93) in the controls. Vitreous levels in eyes with PDR were significantly greater than those in the controls (p<0.0001). Vitreous levels were significantly higher than serum levels in eyes with PDR (p=0.0026). MIF levels were significantly higher in the vitreous of PDR patients with severe fibrous proliferation than in those with slight proliferation (p<0.05).

Conclusion—The results indicate increased levels of MIF in the vitreous of patients with PDR and a significant association between MIF levels and grades of fibrous proliferation, suggesting the possibility that MIF may play a part in the development of the proliferative phase of PDR.

(Br J Ophthalmol 2000;84:636–639)

Materials and methods

PATIENTS

This study was carried out with approval from the institutional review committee. Informed consent was obtained from each patient to the collection of samples.

The study included 73 consecutive eyes of 73 patients who underwent pars plana vitrectomy in Toho University Sakura Hospital. Undiluted vitreous samples and paired serum samples were collected from 32 patients with PDR (13 females and 19 males ranging in age from 23 to 71 years (mean 50.1)) and 41 patients with macular hole (29 patients) or idiopathic epimacular membrane (12 patients) (controls; 26 females and 15 males ranging in age from 23 to 77 years (mean 63.3)).

It has been reported that MIF is secreted by T cells, macrophages, monocytes, eosinophils, and other blood elements, so we assumed that the blood contains highly concentrated MIF. Before this study, we investigated venous blood and serum samples of 16 patients (PDR; 12 patients, controls; four patients; these patients were included in this study) for the presence of MIF. The MIF levels of the venous blood were 1481–6843 ng/ml (mean 3159 (SD 1326)), and serum MIF levels were 3.97–12.28 ng/ml (6.54 (2.72)), suggesting that blood cells contain highly concentrated MIF. This result indicates the possibility that vitreous MIF levels may be affected.

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Accepted for publication 28 January 2000
by vitreous haemorrhage. Therefore, only the PDR cases with no or little vitreous haemorrhage (90% or more area of the fundus was clearly visible) were included in this study. We also excluded patients who had a history of vitreoretinal surgery before sampling.

Of the 32 PDR cases, 30 had tractional retinal detachment and two had macular oedema. Six of the 32 PDR cases had no vitreous haemorrhage, and the others had slight vitreous haemorrhage. Three of the 32 PDR patients had rubecitic glaucoma.

The clinical ocular findings were graded at the time of vitrectomy with respect to the amount of fibrous proliferation and preoperative retinal photocoagulation. The degree of fibrous proliferation was graded as follows: no apparent fibrous proliferation = 0, fibrous proliferation around the disc only = 1, fibrous proliferation around the vascular arcade = 2, and fibrous proliferation throughout the posterior pole = 3. The extent of retinal photocoagulation before vitrectomy was graded as follows: no retinal photocoagulation = 0, focal retinal photocoagulation = 1, panretinal photocoagulation (PRP) = 2, supplemental retinal photocoagulation after the initial PRP = 3.

SAMPLE COLLECTION
The vitreous samples were obtained during pars plana vitrectomy. Before intraocular infusion, the vitreous cutter was used to remove the vitreous, but ensuring that it was not mixed with blood. Special attention was paid with PDR cases: the vitreous gel of the superior vitreous cavity was aspirated to prevent the mixture of vitreous haemorrhage which usually exists in the inferior vitreous cavity. The vitreous humour was collected undiluted and stored in a syringe at −80°C until the assay was performed.

The serum samples from each patient were collected before the operation.

PREPARATION OF RABBIT POLYCLONAL ANTIBODIES AGAINST HUMAN MIF
Polyclonal anti-human MIF serum was generated by immunising New Zealand White rabbits with purified recombinant human MIF. Human recombinant MIF was expressed in E. coli and purified to homogeneity as previously described. In brief, the rabbits were inoculated intradermally with 100 µg of MIF diluted in complete Freund adjuvant at weeks 1 and 2, and with 50 µg of MIF diluted in incomplete Freund adjuvant at week 4. Serum was collected 1 week after the last inoculation. The IgG fraction was prepared using Protein A Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer’s protocol.

MIF ANALYSIS
We employed the quantitative “sandwich” enzyme linked immunosorbent assay (ELISA) technique in this study. Briefly, the anti-human MIF antibody was added to each well of a 96 well microtitre plate and left for 1 hour at room temperature. All wells were filled with PBS containing bovine serum albumin (1%) for blocking and left for 1 hour at room temperature. After removal of blocking solution and washing, 10 µl of samples were added in duplicate to individual wells and incubated for 1 hour at room temperature. After washing, 50 µl of biotin-conjugated anti-human MIF antibody was added to each well. Following incubation for 1 hour at room temperature, avidin-conjugated horseradish peroxidase (Pierce, Rockford, IL, USA) was added to individual wells and incubated for 1 hour at room temperature. A volume of 50 µl of substrate solution containing o-phenylenediamine (Wako, Osaka, Japan) and hydrogen peroxide in citrate phosphate buffer (pH 5.0) were added. After incubation for 20 minutes at room temperature, the reaction was terminated with 1 N sulphuric acid. The absorbance at 492 nm was measured using an ELISA plate reader (Bio-Rad, Model 3550). Duplicate readings were averaged.

This ELISA method consistently detected MIF concentrations of higher than 1 ng/ml. For statistical calculations, samples with undetectable levels were entered as 1 ng/ml.

PROTEIN AND HAEMOGLOBIN CONCENTRATIONS OF THE VITREOUS
Protein in all the vitreous samples was determined using a Pierce protein assay kit (Pierce, Rockford, IL, USA).

In all cases, vitreous haemoglobin levels were investigated to confirm that no blood was mixed into the vitreous samples. Vitreous haemoglobin levels were determined using spectrophotometry (Sysmex, NE-8000). The detection limit was defined as 0.1 g/dl. (Three PDR patients in which vitreous haemoglobin was detected have been excluded from this study.)

STATISTICAL ANALYSIS
In view of the skewed distribution, all values are displayed as the median value and range, unless otherwise indicated. Data were statistically analysed with the Mann–Whitney U tests for comparison of the two groups and with the Kruskal–Wallis test for multiple groups in the vitreous concentrations of MIF and protein. Comparisons between serum and vitreous levels of MIF were examined by Wilcoxon signed ranks test. Levels of statistical significance were set at p<0.05.

Results
There was no significant difference in intravitreous protein levels between the patients with PDR (median 5.65 mg/ml, range 1.28–27.30 mg/ml) and the controls (4.70 mg/ml, 1.04–7.54 mg/ml) (p=0.0869, Mann–Whitney U test).

The vitreous MIF levels were undetectable in only 10 of 41 control samples. The MIF concentrations of all subject eyes (73 eyes) ranged from undetectable to 103.85 ng/ml.

The median vitreous levels of MIF were 11.93 ng/ml (range 4.16–103.85) in eyes with PDR and 1.79 ng/ml (undetectable–8.93 ng/ml) in the controls (Fig 1). The vitreous MIF levels in the PDR eyes were significantly greater than those in the controls (p<0.0001,
Mann–Whitney U test). The MIF concentrations in the serum were 7.17 ng/ml (3.98–23.95) in eyes with PDR and 5.76 ng/ml (3.42–27.43) in the controls. Vitreous MIF levels were significantly higher than serum MIF levels in PDR (p=0.0026), but significantly lower than serum levels in the controls (p<0.0001) (Wilcoxon signed ranks test).

In eyes with PDR, fibrous proliferation ranged from grade 1 to grade 3. The MIF level was 6.96 ng/ml (4.16–11.66) in the eyes with grade 1 (four eyes), 11.19 ng/ml (4.65–31.71) in grade 2 (19 eyes), and 30.24 ng/ml (5.77–103.85) in grade 3 (nine eyes) (Fig 2, Table 1). Significant differences were found among these three groups (p=0.0199, Kruskal–Wallis test). When the MIF levels of the three groups were compared independently with each of the other groups, the levels were significantly greater in grade 3 than in grades 1 or 2 (p=0.0076, p=0.0009, respectively, Fisher’s PLSD).

In the PDR cases, the degree of preoperative retinal photocoagulation ranged from grades 0 to 3. The MIF level was 12.82 ng/ml (5.07–31.71) in grade 0 (four eyes), 42.18 ng/ml (6.83–103.85) in grade 1 (four eyes), 11.66 ng/ml (4.65–44.52) in grade 2 (21 eyes), and 5.77 ng/ml (4.16–6.76) in grade 3 (three eyes) (Table 1). No significant differences were found among these four groups (p=0.0585, Kruskal–Wallis test), but there was a tendency towards lower levels in eyes with grade 3.

As for vitreous haemorrhage, the MIF concentration was 12.99 ng/ml (6.76–40.55) in eyes without vitreous haemorrhage (six eyes) and 11.93 ng/ml (4.16–103.85) in eyes with slight vitreous haemorrhage (26 eyes) (Table 2). No significant difference was found between these two groups (p=0.0217, Mann–Whitney U test).

In the three eyes with rubeotic glaucoma, the MIF level was 30.24 ng/ml (24.71–103.85). In the other 29 eyes without glaucoma, the level was 11.19 ng/ml (4.16–70.15) (Table 2). MIF levels were significantly greater in eyes with rubeotic glaucoma than in eyes without it (p=0.0217, Mann–Whitney U test).

Discussion
MIF was the lymphokine reported to prevent random migration of macrophages and to recruit macrophages at inflammatory loci. MIF also enhances macrophage adherence, motility, and phagocytosis. These MIF actions are similar to the actions of chemokines, such as monocyte chemotactic protein-1 (MCP-1) or macrophage colony stimulating factor. It was reported that the vitreous levels of MCP-1

![Figure 1](http://bjo.bmj.com/) Macrophage migration inhibitory factor (MIF) levels in the vitreous and paired serum samples from eyes with macular hole or idiopathic epiretinal membrane (controls: n=41) and proliferative diabetic retinopathy (PDR: n=32). Open circles represent vitreous levels and open squares represent serum levels. The horizontal lines indicate the median concentration in each group. The vitreous MIF levels in PDR were significantly greater than levels in the controls (p<0.0001). Vitreous MIF levels were significantly higher than serum MIF levels in PDR (p=0.0026), but significantly lower than serum levels in the controls (p<0.0001).

![Figure 2](http://bjo.bmj.com/) Macrophage migration inhibitory factor (MIF) levels in the vitreous samples from eyes with grade 1 (n=4), grade 2 (n=19), and grade 3 (n=9) of fibrous proliferation in proliferative diabetic retinopathy (PDR). The horizontal lines indicate the median concentration in each group. Vitreous MIF levels were significantly greater in grade 3 than in grades 1 or 2 (p=0.00076, p=0.0009, respectively).

### Table 1 Vitreous MIF levels* and clinical findings (1)

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<th>Grade 0</th>
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<th>Grade 3</th>
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<tr>
<td>(n=0)</td>
<td>(n=4)</td>
<td>(n=19)</td>
<td>(n=9)</td>
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<td>Preoperative photocoagulation</td>
<td>12.82 (5.07–31.71)‡</td>
<td>42.18 (6.83–103.85)‡</td>
<td>11.66 (4.65–44.52)‡</td>
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<tr>
<td>(n=0)</td>
<td>(n=4)</td>
<td>(n=21)</td>
<td>(n=3)</td>
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</table>

*All values indicate vitreous MIF levels given as the median (range) ng/ml.
†p<0.05. Significant differences were found among 3 groups (p=0.0199).
‡NS = no significant differences were found among 4 groups (p=0.0585), although there was a tendency toward lower levels in eyes with grade 3.
Table 2  Vitreous MIF levels* and clinical findings (2)

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<td></td>
<td>(n=26)</td>
<td>(n=6)</td>
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<tr>
<td>Vitreous haemorrhage</td>
<td>11.93 (4.16–103.85)†</td>
<td>12.99 (6.76–40.55)†</td>
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<tr>
<td></td>
<td>(n=3)</td>
<td>(n=29)</td>
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<tr>
<td>Rubeotic glaucoma</td>
<td>30.24 (24.71–103.85)‡</td>
<td>11.19 (4.16–70.15)‡</td>
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</tbody>
</table>

*All values indicate vitreous MIF levels given as the median (range) ng/ml.
†NS = no significant difference was found (p=0.4991).
‡p<0.05. MIF levels were significantly greater in eyes with rubeotic glaucoma than in eyes without it (p=0.0217).

MIF in PDR eyes with moderate or more vitreous haemorrhage are needed.

In conclusion, the results indicate increased levels of MIF in the vitreous of patients with PDR and a significant association between MIF levels and grades of fibrous proliferation, suggesting the possibility that MIF may play a part in the development of the proliferative phase of PDR. However, we cannot distinguish whether the increased MIF levels are the cause or the effect of PDR in this study, thus further investigations of the mechanism of MIF action are needed to understand the precise role of MIF in PDR.

This study was supported in part by the Japan National Society for the Prevention of Blindness, Tokyo.