Vitreous Levels of Vascular Cell Adhesion Molecule and Vascular Endothelial Growth Factor in Patients With Proliferative Diabetic Retinopathy

A case-control study

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OBJECTIVE — To evaluate the intravitreal concentration of vascular cell adhesion molecule (VCAM-1) in diabetic patients with proliferative diabetic retinopathy (PDR) and the relationship of VCAM-1 with vascular endothelial growth factor (VEGF).

RESEARCH DESIGN AND METHODS — Serum and vitreous fluid samples were obtained simultaneously at the onset of vitrectomy from 20 diabetic patients with PDR and 20 nondiabetic control subjects with nonproliferative ocular disease. Both groups were matched by serum levels of VCAM-1 and VEGF. VCAM-1 and VEGF were determined by enzyme-linked immunosorbent assay. Statistics were determined using the Mann-Whitney U test and Spearman’s rank correlation test.

RESULTS — The intravitreal concentration of VCAM-1 was significantly elevated in diabetic patients with PDR compared with control subjects (26 ng/ml [19–118] vs. 22 ng/ml [20–47], P < 0.05). A direct correlation between VCAM-1 and total vitreous proteins was detected in diabetic patients (r = 0.64, P = 0.003), but not in control subjects. After adjusting for total vitreous proteins, VCAM-1 was significantly lower in diabetic patients with PDR than in control subjects (8.2 ng/ml [4–31.4] vs. 43.1 ng/ml [9.7–100], P < 0.001). Intravitreal VEGF concentrations were higher in patients with PDR than in control subjects in absolute terms (1.34 ng/ml [0.16–6.22] vs. 0.009 ng/ml [0.009–0.044], P < 0.0001) and after correcting for total vitreal proteins (0.33 ng/ml [0.01–2.3] vs. 0.013 ng/ml [0.003–0.035], P = 0.0001). Finally, the vitreous ratio of VCAM-1 to proteins correlated with the vitreous ratio of VEGF to proteins in both diabetic patients (r = 0.74, P = 0.001) and control subjects (r = 0.84, P = 0.005).

CONCLUSIONS — The low proportion of VCAM-1 in relation to total vitreal proteins observed in diabetic patients with PDR suggests that VCAM-1 is quenched by diabetic retina. In addition, the direct correlation detected between VCAM-1 and VEGF suggests that cellular adhesion and neovascularization may be linked processes.


Proliferative diabetic retinopathy (PDR) is characterized by extensive neovascularization and vessel intrusion into the vitreous body, with subsequent bleeding and scarring surrounding new vessels, leading to severe visual impairment. Ischemic areas of the retina seem to be essential stimuli for the angiogenic process mediated by specific growth factors. This includes the expression of vascular endothelial growth factor (VEGF) by retinal glial cells (1) and vascular endothelial cells (2). It is thought that capillary occlusion by increased adhesion of leukocytes and macrophages to the endothelium has a crucial role in the process of retinal ischemia (3). In addition, monocytes exposed to glycated collagen show increased adhesiveness (4), and several studies indicate that circulating leukocytes are activated in diabetic patients (5,6).

Vascular cell adhesion molecule (VCAM)-1, a member of the immunoglobulin supergene family of cellular adhesion molecules, is involved in the recruitment of leukocytes, their adhesion to vascular endothelium, and their subsequent migration into surrounding tissue. Interestingly, the expression of VCAM-1 has been found in epiretinal membranes from diabetic patients with PDR (7,8). In addition, it has been demonstrated that VCAM-1 promotes angiogenesis both in vitro and in vivo (9,10), but its relationship with VEGF has not been reported.

Olson et al. (11) detected increased serum levels of VCAM-1 in diabetic patients with PDR. Moreover, circulating levels of various adhesion molecules increase in patients with progressively worsening retinopathy, presumably as a result of shedding from both activated leukocytes and injured epithelium. However, systemic levels of VCAM-1 do not reflect the local production of VCAM-1 by the retina. Vitrectomy fluid samples ob-
tained from diabetic patients with PDR are currently being used to explore indirectly the retinal synthesis of several proteins, including growth factors, cytokines, and adhesion molecules. Two previous studies demonstrated that soluble VCAM-1 is increased in the vitreous cavity of diabetic patients with PDR compared with the vitreous of patients undergoing macular hole repair (12) or from cadaveric eyes (13). However, neither study considered the serum levels of VCAM-1 or a correction for total vitreous proteins produced. In the present study, we determined intravitreal levels of VCAM-1 and accounted for these confounding factors. In addition, we investigated the relationship between vitreal VCAM-1 and VEGF.

RESEARCH DESIGN AND METHODS — The study included 20 consecutive diabetic patients with PDR (6 type 1 and 14 type 2) in whom a classic three-port pars plana vitrectomy was performed. Also, 20 nondiabetic patients with other conditions requiring vitrectomy, but in whom the retina was not directly affected by neovascularization, were included as control subjects. Both groups were matched by serum levels of VCAM-1 and VEGF (Table 1). In the control group, the diagnoses included rhegmatogenous retinal detachment (n = 7), epiretinal membrane (n = 6), macular hole (n = 5), and macular edema not caused by retinal vascular obstruction (n = 2). Both venous blood and vitreous samples were collected at the time of the vitrectomia. In all cases, patients who had a recent (<2 months) vitreous hemorrhage were not included. We also excluded patients who had a history of vitreoretinal surgery before sampling.

Retinopathy was graded intraoperatively in all of the eyes by the same ophthalmologist, using a method previously reported (14). In summary, neovascularization was considered to be active when perfused preretinal capillaries were found, and it was considered to be quiescent if only nonperfused gliotic vessels or fibrosis were present.

Sample collection
Undiluted vitreous samples (0.5–1 ml) were obtained at the onset of vitrectomy by aspiration into a 1-ml syringe attached to the vitreous cutter (Ten-Thousand Systems, Abingdon, U.K.). The CV intraassay was 3 ng/ml with an intra-assay coefficient of variation (CV) of 3.6% and an interassay CV of 4.6%.

VEGF method assay. VEGF concentrations (ng/ml) in vitreous and serum samples were measured by ELISA (R & D Systems, Abingdon, U.K.). The CV intraassay was 3.8%, and the CV interassay was 5.1%. For data processing, we allocated the minimum value detected by ELISA (0.009 ng/ml) to all samples with concentrations below the detection threshold.

Protein method assay. Vitreal proteins (mg/ml) were measured by a previously validated microturbidimetric method with an autoanalyzer (Hitachi 917; Boehringer Mannheim). This method, based on the benzetonium chloride reaction, is a highly specific method for the detection of proteins and has a higher sensitivity and reproducibility than the classic Lowry method. The lowest level of proteins detected was 0.02 ng/ml. The intra-assay and interassay CVs were 2.9 and 3.7%, respectively.

Correction for vitreous hemorrhage. Although the patients with a recent vitreous hemorrhage were not included in the study, the possibility that intravitreal 
Hb could significantly influence the results of total vitreal proteins could not be excluded. For this reason, the vitreous protein levels were adjusted by vitreous 
Hb using (15) the following:

$$[X]_{corrected} = ([X]_{measured} \times [Hb]_{blood}) - ([X]_{blood} \times [Hb]_{vitreous} / [Hb]_{blood}) - ([Hb]_{vitreous})$$

Table 1 — Age and levels of VCAM-1, VEGF, and proteins in diabetic patients with PDR compared with control subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients</th>
<th>Control subjects</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48 ± 10</td>
<td>56 ± 19</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCAM-1 (ng/ml)</td>
<td>477 (260–814)</td>
<td>472 (265–881)</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF (ng/ml)</td>
<td>0.177 (0.057–490)</td>
<td>0.170 (0.066–0.517)</td>
<td>NS</td>
</tr>
<tr>
<td>Vitreous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCAM-1 (ng/ml)</td>
<td>26 (19–118)</td>
<td>22 (20–47)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VEGF (ng/ml)</td>
<td>1.34 (0.16–6.22)</td>
<td>0.009 (0.009–0.044)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Proteins (mg/ml)</td>
<td>3.29 (0.61–9.27)</td>
<td>0.58 (0.27–2.61)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hb (mg/ml)</td>
<td>0 (0–0.4)</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Proteins corrected by Hb (mg/ml)</td>
<td>3.18 (0.61–9.27)</td>
<td>0.58 (0.27–2.61)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are means ± SD or median (range).
where \( X \) is the concentration of vitreal proteins. Corrected vitreous protein levels were used to perform all of the statistical analyses. Vitreous Hb levels were measured by spectrophotometry (Unikon 860; Kontron Instruments, Zurich, Switzerland), using the classic method of Harboe (16) for measuring plasma Hb in micromolar concentration. This method has recently been validated (17), and we found the lower limit of detection to be 3 mg/dl.

**Statistical analysis**

VCAM-1, VEGF, and total intravitreal proteins were displayed as median (range), because of their skewed distribution. A nonparametric test (Mann-Whitney \( U \) test) was used to compare VCAM-1 and VEGF concentrations between diabetic patients and control subjects. To examine correlations, Spearman's rank correlation test was used, and correlations have been graphically represented by means of Pearson's correlation test. Levels of statistical significance were set at \( P < 0.05 \).

**RESULTS** — Results of all measurements made in serum and vitreous fluid of diabetic patients with PDR and control subjects are summarized in Table 1.

Intravitreal protein levels were significantly higher in patients with PDR than in control subjects (3.29 mg/ml [0.61–9.27] vs. 0.58 mg/ml [0.27–2.61], \( P < 0.001 \)). Vitreous Hb was detectable in nine diabetic patients (45%). The median concentration of vitreous Hb was 0.15 mg/ml (0.06–0.40) in those diabetic patients with detectable Hb, and it was below the limit of detection in all of the control subjects. Intravitreous Hb represents only 5% of total vitreal proteins in the diabetic patients with PDR who had detectable Hb, thus making negligible the influence of vitreous hemorrhage on the enhancement of total vitreal proteins detected in the diabetic patients with PDR who were included in the study.

Intravitreous concentration of VCAM-1 (median and range) was significantly higher in the diabetic patients with PDR than in the control subjects (26 ng/ml [19–118] vs. 22 ng/ml [20–47], \( P < 0.05 \)). Furthermore, a direct correlation between VCAM-1 and vitreous proteins was detected in diabetic patients, but not in control subjects (Fig. 1). After adjusting for total intravitreous protein concentration (ratio of vitreal VCAM-1 [ng/ml] to vitreal protein [mg/ml]), VCAM-1 was significantly lower in patients with PDR than in control subjects (8.2 [4.5–31.4] vs. 43.1 [9.4–90.3], \( P < 0.001 \)) (Fig. 2A).

The vitreous VEGF concentrations were significantly higher in diabetic patients with PDR than in control subjects (1.34 ng/ml [0.16–6.22] vs. 0.009 ng/ml [0.009–0.044], \( P = 0.0002 \)), but it was not higher in control subjects (0.009 ng/ml [0.009–0.044] vs. 0.170 ng/ml [0.066–0.517], \( P < 0.001 \)). In contrast, serum concentrations of VEGF were higher than intravitreous levels in diabetic patients and in control subjects (477 ng/ml [260–814] vs. 26 ng/ml [19–118] and 472 ng/ml [265–881] vs. 22 ng/ml [20–47], respectively).

We detected an association between vitreous levels of VEGF and retinopathy activity (quiescent PDR 0.7 ng/ml [0.16–1.26] vs. active PDR 1.94 ng/ml [0.22–4.64], \( P < 0.05 \)), but there was no association with vitreous levels of VCAM-1 (quiescent PDR 35 ng/ml [19–52] vs. active PDR 26 ng/ml [23–118], NS).

Finally, after adjusting for intravitreous proteins, a correlation between intra-
Vitreous levels of VCAM-1 and VEGF were observed in diabetic patients with PDR ($r = 0.74$, $P = 0.001$), as well as in control subjects ($r = 0.84$, $P = 0.005$) (Fig. 3).

**CONCLUSIONS**— Vitreous fluid obtained from diabetic patients with PDR, submitted to vitrectomy, is a unique material for indirectly exploring the retinal synthesis of several proteins, such as growth factors and soluble adhesion molecules. However, the intravitreal increase of a particular protein does not necessarily signal intraocular production; it could reflect only the unspecific increase of total vitreal proteins observed in these patients caused by the disruption of the blood-retinal barrier. In this regard, we previously reported concentrations of vitreous proteins that were higher in diabetic patients with PDR than in control subjects (18,19). Therefore, to investigate the contribution of the intraocular synthesis in the enhancement of a specific protein into the vitreous cavity in diabetic patients with PDR, the result in absolute terms should be adjusted by total vitreal proteins. Previously, we applied this approach to other proteins, such as IGF-1, IGFBP-1, and IGFBP-3, and showed that the increase observed in absolute terms in the vitreous fluid of diabetic patients was lost or dramatically reduced after the correction by total vitreal proteins, thus suggesting that the elevated intravitreous concentration was mainly caused by serum diffusion (19). Another caveat that should be kept in mind is that serum levels could influence the intravitreal concentration of a particular protein. To circumvent this problem, in the present study, diabetic patients and control subjects were matched by serum levels of both VCAM-1 and VEGF. Finally, to exclude the possible influence of vitreous hemorrhage on the elevation of total vitreal proteins detected in diabetic patients with PDR, a correction by vitreous Hb was performed. After correcting by vitreous Hb concentration, total vitreal proteins were only slightly lower than those obtained in absolute terms, thus indicating that the significant elevation of total vitreal proteins observed in diabetic patients with PDR was not due to vitreous hemorrhage.

In the present study, intravitreous VCAM-1 levels were increased in diabetic patients in absolute terms. However, the proportion of VCAM-1 in relation to total vitreal proteins was notably lower in diabetic patients with PDR than in control subjects. Without a doubt, the vitreous fluid of diabetic patients contains less VCAM-1 per milligram of protein than that of control subjects, i.e., there is a relative deficit of VCAM-1 in the vitreous fluid of diabetic patients. In fact, considering the ratio of VCAM-1 to total vitreal proteins detected in control subjects, it was expected that the median intravitreous concentration of VCAM-1 in diabetic patients would be 109 ng/ml, instead of the 26 ng/ml actually obtained. Certainly, it was an unexpected result and has not been previously reported. We believe that this finding cannot be attributed to the molecular size of VCAM-1, because serum diffusion is the primary reason for the intravitreous enhancement of a bigger molecule, such as IGF-1 (19,20). Although our results do not suggest intraocular production of VCAM-1 in diabetic patients with PDR, there is relevant information arguing the contrary (7,8). To reconcile these opposing data, it is tempting to hypothesize that VCAM-1 is quenched by the retina in the passage from the blood stream to the vitreous fluid. In addition, locally produced VCAM-1 might also remain in the retina by virtue of its adhesive properties. Therefore, both serum-derived and intraocular-synthesized VCAM-1 might be partly retained in the retina, thus developing its pathogenic action. This phenomenon could explain the lack of relationship between intravitreous VCAM-1 levels and the activity of retinopathy observed in our study.

VEGF is a potent stimulus of vascular permeability and plays a major role in the intraocular neovascularization in PDR. We found higher intravitreous concentrations of VEGF in diabetic patients with PDR than in control subjects, not only in absolute terms, but also after adjusting for total vitreal proteins. In addition, vitreous levels of VEGF in patients with PDR were strikingly higher than serum VEGF concentrations. Thus, our results reinforce the concept that intraocular synthesis of VEGF, not serum diffusion, is the primary contributing factor to the high VEGF concentration observed in vitreous fluid from patients with PDR.

We detected a direct correlation between VCAM-1 and VEGF concentrations after adjusting for vitreous proteins in both patients with PDR and control subjects. The relationship between VEGF and VCAM-1 has not previously been directly investigated in vivo, and there are few in vitro studies with divergent results (21,22). However, there is growing evidence in favor of the direct participation of soluble adhesion molecules in the angiogenic process (9,10). In this context, our results are not surprising, but they deserve confirmative studies. It must be noted that there are common pathways, i.e., hyperglycemia, advanced glycosylation end products, and hypoxia involved in the synthesis of both VEGF and VCAM-1 (23–27). In addition, hypoxia...
has been shown to increase leukocyte adhesion to the endothelium (28). Recently, it has been suggested that inflammatory cells, such as macrophages and lymphocytes, may be a relevant source of both VEGF (29) and VCAM-1 (30), particularly when activated. These exogenous cells may be the main source of both VEGF and VCAM-1 within the ischemic retina. Certainly, macrophages and lymphocytes have been identified in epiretinal membranes from patients with PDR (31–33), and activated macrophages have been detected in a model of human retinal angiogenesis (34). Altogether, these observations support the concept that cellular adhesion and angiogenesis may be linked processes.

In conclusion, the lower proportion of VCAM-1 in relation to total vitreal proteins detected in diabetic patients with PDR in comparison with that of control subjects suggests that VCAM-1 is quenched by diabetic retina. In addition, the direct correlation observed between VCAM-1 and VEGF after adjusting for total vitreal proteins further supports the concept that cellular adhesion and angiogenesis may be linked processes.

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