Intravitreous levels of hepatocyte growth factor/scatter factor and vascular cell adhesion molecule-1 in the vitreous fluid of diabetic patients with proliferative retinopathy

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**Summary**

**Objective:** Hepatocyte growth factor, also known as the scatter factor (HGF/SF) has been involved in the etiopathogenesis of proliferative diabetic retinopathy (PDR). To further explore this issue we have determined the intravitreous levels of HGF/SF taking into account the problems that could lead to misinterpretation of the results when the vitreous fluid is used to indirectly explore the events that are taking place in the retina. In addition, the relationship between HGF/SF and vascular cell adhesion molecule 1 (VCAM-1) was also investigated.

**Patients and methods:** Serum and vitreous samples were obtained during vitrectomy from 22 diabetic patients with PDR and 25 non-diabetic control subjects. Patients in whom intravitreous hemoglobin was detectable were excluded. A correction for plasma levels of either HGF/SF and VCAM-1 and intravitreal proteins was performed.

**Results:** Vitreal levels of both HGF/SF and VCAM-1 were higher in patients with PDR in comparison with the control group (p < 0.001 and p = 0.003, respectively). However, after correcting for total vitreal proteins both HGF/SF and VCAM-1 (ng/mg of proteins) were lower in diabetic patients than in non-diabetic control subjects (p = 0.03 and p < 0.0001, respectively). No relationship between the vitreal levels of either HGF/SF or VCAM-1 with PDR activity was detected. Finally, a correlation between the vitreal levels of HGF/SF and VCAM-1 was observed in diabetic patients (r = 0.61, p = 0.005) but not in the control group.

**Conclusion:** Our results suggest that intracellular production of HGF/SF might be more important in mediating inflammatory and fibroproliferative processes rather than in angiogenesis itself.

**Key-words:** HGF/SF · VCAM-1 · Diabetic retinopathy · Vitreous fluid.

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**Résumé**

**Objectif :** L’hepatocyte growth factor, également connu comme scatter factor (HGF/SF) a été impliqué dans la pathogénie de la rétinopathie diabétique proliférante (RDP). Pour explorer cette problématique, nous avons déterminé les taux intravitréens de HGF/SF en prenant en compte les problèmes pouvant conduire à une interprétation biaisée des résultats lorsque le corps vitré est utilisé pour explorer indirectement les événements se déroulant dans la rétine. De plus, nous avons étudié les liens entre HGF/SF et VCAM-1 (vascular cell adhesion molecule 1).**

**Patients et méthodes :** Les échantillons sériques et vitrés ont été obtenus lors de vitrectomies chez 22 patients diabétiques avec RDP et 25 témoins non diabétiques. Les patients chez qui de l’hémoglobine intravitréenne était détectée ont été exclus. Une correction pour les taux plasmatiques de HGF/SF et VCAM-1 et pour les protéines intravitréennes a été appliquée.

**Résultats :** Les taux vitréens de HGF/SF et VCAM-1 sont plus élevés chez les patients avec RDP par comparaison avec le groupe contrôle (p < 0.001 et p = 0.003, respectivement). Cependant, après correction pour les protéines vitréennes totales, HGF/SF et VCAM-1 (ng/mg de protéines) étaient plus bas chez les patients diabétiques que chez les témoins (p = 0.03 et p < 0.0001, respectivement). Aucun lien entre taux vitréen de HGF/SF ou VCAM-1 avec l’activité de la RDP n’a été observé. Enfin, une corrélation entre taux vitréen HGF/SF et VCAM-1 a été notée chez les patients diabétiques (r = 0.61, p = 0.005) mais pas chez les témoins.

**Conclusion :** Nos résultats suggèrent que la production intracellulaire de HGF/SF pourrait être plus importante dans la médiation de processus inflammatoires et fibroprolifératifs que dans l’angiogenèse elle-même.

**Mots-clés :** HGF/SF · VCAM-1 · Rétinopathie diabétique · Corps vitré.
Proliferative diabetic retinopathy (PDR) remains the leading cause of new blindness among working-age individuals in developed countries [1]. Neovascularization is the hallmark of PDR and in recent years several growth factors have been involved in its etiopathogenesis. One such growth factor has been the hepatocyte growth factor, also known as the scatter factor (HGF/SF) due to its ability to promote the dissociation or scattering of formed colonies of cultured epithelium [2]. HGF/SF is expressed predominantly in cells of stromal origin, including fibroblasts, vascular smooth muscle cells and glial cells, and exhibits pleiotropic biological functions such as a mitogenic, motogenic and morphogenic factor in epithelial cells, and an angiogenic factor in endothelial cells [3, 4]. HGF/SF exerts its action through activation of a high-affinity tyrosine kinase receptor, c-met, which is expressed mainly by epithelial and endothelial cells [5, 6].

Although HGF/SF is a powerful angiogenic factor, its role in PDR is still controversial. Several authors [7-9] and ourselves [10] have detected higher intravitreous levels of HGF/SF in diabetic patients with PDR than in non-diabetic control subjects. However a normalization for intravitreous protein concentrations was not performed in these studies. This could lead to a misinterpretation of the results because the disruption of the blood-retinal barrier that occurs in diabetic retinopathy produces an increase of proteins in the vitreous fluid. Therefore, the enhancement of any protein (i.e. HGF/SF) within the vitreous fluid does not necessarily mean its intraocular production and may only reflect the non-specific increase of total vitreal proteins due to serum diffusion. Vitreous hemorrhage should also be taken into account when the vitreous fluid is used to indirectly explore the events that are taking place in the retina.

In the present study we have considered the methodological questions above mentioned to further explore the intravitreous levels of HGF/SF and its relationship with PDR activity. In addition, since HGF/SF is not only an angiogenic factor but also participates in wound healing we wanted to explore its relationship with vascular cell adhesion molecule 1 (VCAM-1), a member of the immunoglobulin supergene family of cellular adhesion molecules, that is also involved in angiogenesis and the processes of tissue regeneration and repair.

**Methods**

**Study population**

The study included 22 consecutive diabetic patients with PDR (8 type 1 and 14 type 2) on whom a vitrectomy had been performed. Twenty-five non-diabetic patients with other conditions requiring vitrectomy, but in which the retina was not directly affected by neovascularization, served as a control group. In the control group the diagnosis included macular hole (n = 10), rhegmatogenous retinal detachment without proliferative vitreoretinopathy (n = 8), and idiopathic epiretinal membrane (n = 7). Both venous blood and vitreous samples were collected at the time of vitrectomy surgery. Patients with previous vitreoretinal surgery, recent vitreous hemorrhage (less than 2 months), and those who had received photocoagulation in the preceding 3 months were excluded.

**Vitrectomy and collection of Specimens**

In all cases a classic three port pars plana vitrectomy was performed. For visualization of the vitreous cavity we used a wide-field system with a precorneal Volk lens of 130 and inversion image system Moeller-Wedel (Hamburg, Germany).

Retinopathy was graded intraoperatively in all eyes by two ophthalmologists using a method previously reported [11]. In summary, neovascularization was considered to be active when perfused preretinal capillaries existed, and to be quiescent if only non-perfused gliotic vessels or fibrosis were present.

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 (Alcon Model, Accurus 800xS4; Irvine, CA, USA) before starting the intravitreal infusion of a balanced salt solution. The vitreous samples were transferred to a sterile tube, placed immediately on ice and centrifuged at 16000 g for 5 minutes at 4°C. Supernatants were frozen at -80°C until assayed.

For serum determinations, blood samples were collected simultaneously with the vitrectomy, then centrifuged at 3000 g for 10 minutes at 4°C to obtain serum, aliquoted and stored at -80°C until assayed.

The protocol for sample collection was approved by the hospital ethics committee, and informed consent was obtained from patients.

**Laboratory assays**

**HGF/SF**

Concentrations of immunoreactive HGF/SF in undiluted vitreous and serum samples were measured by an enzyme linked immunosorbent assay (ELISA) for HGF/SF (R&D Systems, Abingdon). This assay employs the quantitative sandwich enzyme immunoassay technique. It uses a monoclonal antibody specific for HGF/SF precoated onto a microtitre plate and an enzyme linked polyclonal antibody specific for HGF/SF as second antibody. The intra-assay coefficient of variation (CV) was 3.6% and the inter-assay CV was 5.6%.

**VCAM-1**

Concentrations of VCAM-1 in undiluted vitreous and serum samples were determined by ELISA (Boehringer
Mannheim, Germany). The lower measurable concentration was 3 ng/ml. The intra-assay CV was 3.6%, and the inter-assay CV was 5.1%.

Protein assay

Vitreal proteins were measured by a previously validated micro-turbidimetric 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 method of Lowry. The lowest level of proteins detected was 0.02 mg/ml. Coefficients of variation intra and inter-assay were 2.9 and 3.7%, respectively.

Vitreous hemoglobin

Apart from excluding patients with recent vitreous hemorrhage, we also excluded those in which intravitreous hemoglobin was detected. For this purpose, vitreous hemoglobin levels were measured by spectrophotometry (Uvikon 860, Kontron Instruments, Zürich) using the classic method of Harboe for measuring plasma hemoglobin in micromolar concentration [12]. This method has been further validated [13], and in our studies the lowest limit of detection was 0.03 mg/ml.

Statistical analysis

Because of their skewed distribution, the statistical comparisons of HGF, VEGF and hemoglobin were performed using a nonparametric test (Mann-Whitney U-test). The Spearman rank correlation coefficient was used to examine correlations, and they have been graphically represented by means of Pearson’s correlation test. Levels of statistical significance were set at p < 0.05. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS-PC). The results are expressed as the median and range.

Results

The main clinical characteristics and the results of serum measurements performed in diabetic patients with PDR and in non-diabetic control subjects are summarized in Table I. We did not observe significant differences in serum HGF and VCAM-1 between diabetic patients with PDR and the control subjects included in the study. No correlation between serum HGF/SF and VCAM-1 was detected either in the diabetic patients or in the control group.

Vitreal levels of HGF/SF were higher in the diabetic patients with PDR in comparison with the control group. The ratio vitreous to serum for HGF/SF and VCAM-1 in each patient. The ratio vitreous to serum for HGF/SF remained higher in diabetic patients with PDR than in non-diabetic control subjects (26.4 ng/ml [11.8-106] vs. 7.92 ng/ml [7.9-24.2]; p = 0.0001). However, the ratio vitreous to serum for VCAM-1 was similar both in diabetic patients and in the non-diabetic control group (0.059 [0.032-0.23] vs. 0.051 [0.03-0.11]; p = n.s).

To explore the influence of the breakdown of the blood-retinal barrier and, in consequence, the increased serum diffusion that occurs in PDR patients, the levels of both HGF/SF and VCAM-1 were normalized for total vitreal protein concentration (Fig 2). We detected higher intravitreous protein concentrations in diabetic patients with PDR than in controls (3.97 mg/ml [1.5-13.8] vs. 0.65 mg/ml [0.2-2.6]; p < 0.0001). After correcting for total vitreous protein concentration [ratio vitreal HGF/SF or VCAM-1 (ng/ml)/vitreal proteins (mg/ml)], both HGF/SF and VCAM-1 were lower in diabetic patients with PDR than in the control group (p = 0.03 and p < 0.0001, respectively) (Fig 2). No relationship between the vitreous levels of either HGF/SF or VCAM-1 with PDR activity was detected (quiescent: 14.28 ng/ml [13.15-180] vs. 15.22 ng/ml [9.45-35.3]; p = n.s for HGF/SF, and quiescent: 25 ng/ml [19.52] vs. active: 26 ng/ml [19.5-2]; p = n.s for VCAM-1).

The intravitreous concentration of HGF/SF was significantly higher in vitreous than in serum diabetic patients with PDR (17.04 ng/ml [9.45-80] vs. 0.67 ng/ml [0.26-2.72]; p < 0.0001) and in the control group (5.7 ng/ml [2.5-17.3] vs. 0.71 ng/ml [0.26-1.64]; p < 0.0001). In contrast, VCAM-1 was higher in the serum than in the vitreous fluid in both diabetic patients (483 ng/ml [255-814] vs. 26 ng/ml [19-98]) and control subjects (447 ng/ml [265-881] vs. 22 ng/ml [19-47]). No correlation between serum and vitreous levels

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<th>Table I</th>
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<td>Main clinical features of subjects included in the study.</td>
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<th>Diabetic Patients</th>
<th>Control Group</th>
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<tr>
<td>Age (years)</td>
<td>50 ± 15</td>
<td>57 ± 19</td>
<td>n.s</td>
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<tr>
<td>Gender (M/F)</td>
<td>11/11</td>
<td>11/14</td>
<td>n.s</td>
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<tr>
<td>Duration of diabetes (years)</td>
<td>19 (5-34)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>8.4 ± 3.7</td>
<td>—</td>
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<tr>
<td>Serum HGF (ng/ml)</td>
<td>[0.26-2.72]</td>
<td>0.71</td>
<td>n.s</td>
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<tr>
<td>Serum VCAM-1 (ng/ml)</td>
<td>[255-814]</td>
<td>447</td>
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A correlation between the vitreal levels of HGF/SF and VCAM-1 was observed in diabetic patients (r = 0.61, p = 0.005) but not in the control group (r = -0.09, p = n.s.).

Discussion

Vascular endothelial cells, fibroblasts, glial cells and retinal pigment epithelial (RPE) cells have the ability to produce and release HGF/SF [14]. Moreover, HGF/SF receptor, c-met, is expressed in RPE cells, thus creating an autocrine loop within the retina [14]. Because HGF/SF is a potent angiogenic factor in vitro and in vivo [15, 16] it might be speculated that HGF/SF is involved in the etiopathogenesis of PDR. In this regard, high intravitreous levels of HGF/SF in patients with PDR have been found by several authors [7-10]. However, the confounding factors that could lead to a misinterpretation of the results (vitreous hemorrhage, intravitreal protein concentration, and serum HGF/SF levels) have not been fully considered previously. In the present study, after considering these confounding factors, a strikingly higher level of HGF/SF was observed within the vitreous fluid than in the serum of both diabetic patients with PDR and non-diabetic patients. This finding supports the idea that retinal production, not serum diffusion, is the main contributor to the high intravitreous HGF/SF, not only in PDR patients but also in non-diabetic subjects.

However, it should be noted that after correcting for intravitreous proteins we have found lower intravitreous HGF/SF (ng/mg of proteins) in PDR patients than in non-diabetic controls. Therefore, the vitreous fluid of diabetic patients contains less HGF/SF per mg of protein than that of control subjects, thus suggesting that an impairment of HGF/SF production by the retina could exist in diabetic patients. In this regard, it should be noted that high glucose concentration [17] and hypoxia [18] downregulate HGF expression in endothelial cells. In addition, it has been shown that HGF/SF acts as an anti-apoptotic factor for endothelial cells, thus preventing endothelial cell death being induced by either serum deprivation [19, 20], high glucose concentrations [21, 22], or hypoxia [23]. Furthermore, HGF/SF has an inhibitory action on endothelin-1 synthesis in endothelial cells [24]. Finally, intravitreous injection of recombinant human HGF/SF (rhHGF) has been shown to be neuroprotective in a rat model of retinal ischemia-reperfusion injury [25]. Taken together, these findings suggest that HGF/SF could be a survival growth factor physiologically synthesized by the retina that is impaired during PDR development.

VCAM-1 is expressed on endothelial cells as a result of VEGF stimulation, and it has been proposed as a surrogate marker of angiogenesis [26]. However, a lower VCAM-1 concentration was obtained after adjusting for intravitreal proteins in PDR patients in comparison with non-diabetic control subjects. In addition, no correlation between intravitreous VCAM-1 and PDR activity was found. These results extend and reinforce our previous observation on this issue [27] and, as suggested, it could be attributed to a quenching of VCAM-1 by the retina that impedes its passage into the vitreous fluid.
Previous studies have found higher HGF/SF concentrations in patients with active PDR than in those with quiescent PDR [7, 8]. However, in the present study, after carefully considering the main confounding factors, no relationship between PDR activity and intravitreous HGF/SF concentrations was observed. This result agrees with previous reports in which a relationship between intravitreous HGF/SF and active angiogenesis was not found in either PDR [28] or in retinopathy of prematurity [29]. Nevertheless, it should be noted that HGF/SF increases the expression of VEGF receptor flk-1 in human endothelial cells [30], and HGF/SF enhances VEGF-induced angiogenesis in vitro and in vivo [31]. Moreover, HGF/SF-induced expression of VEGF has been implicated in paracrine amplification of angiogenesis [32]. Therefore, although a direct correlation between intravitreous HGF/SF and PDR activity was not found, a possible effect of HGF on neovascularization by means of VEGF can not be excluded.

There is emerging evidence indicating that HGF/SF participates in the inflammatory and fibroproliferative processes through the increased expression of several adhesion molecules [33-36]. In addition, both VCAM-1 and HGF/SF are essential in wound healing [37, 38]. However, the relationship between VCAM-1 and c-met-HGF pathway has only been previously demonstrated within the lymphoid microenvironment [39]. In the present study we have found a relationship between HGF/SF and VCAM-1 within the vitreous fluid of PDR patients. This finding has not been previously reported and adds new information by which HGF could be involved in the inflammatory pathways (i.e. leukostasis) operating in diabetic retinopathy.

In conclusion our results suggest that intraocular production of HGF/SF might be more important in mediating inflammatory and fibroproliferative processes rather than in angiogenesis. The relationship between HGF/SF and VCAM-1 opens a new insight into the potential role of HGF in the etiopathogenesis of diabetic retinopathy. However, further studies are required to elucidate the specific role of HGF/SF in the retina not only of diabetic patients but also in non-diabetic healthy subjects.

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References


