

# Hepatocyte growth factor in vitreous and serum from patients with proliferative diabetic retinopathy

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## Abstract

**Background**—Hepatocyte growth factor (HGF) is an endothelium specific growth factor that has been implicated in angiogenesis, a crucial event for the development of proliferative diabetic retinopathy (PDR). The aim of the study is to determine the intravitreal concentrations of HGF in diabetic patients with PDR, and to investigate whether its serum levels could contribute to its intravitreal concentration.

**Methods**—17 diabetic patients and seven non-diabetic patients in whom a vitrectomy was performed were studied. Both groups were matched by serum levels of HGF. Venous blood and vitreous samples were collected simultaneously at the time of vitreoretinal surgery. Vitreous and serum HGF were determined by ELISA.

**Results**—Intravitreal concentrations of HGF (median and range) were higher in diabetic patients (17.04 ng/ml (9.98–80)) in comparison with non-diabetic patients (5.88 ng/ml (2.57–14.20);  $p=0.003$ ). Intravitreal HGF concentrations were strikingly higher than serum HGF concentrations both in diabetic patients (17.04 ng/ml (9.98–80) *v* 0.66 ng/ml (0.26–1.26);  $p<0.001$ ) and in the control group (5.88 ng/ml (2.57–14.20) *v* 0.68 ng/ml (0.49–0.96);  $p=0.003$ ). No correlation was found between serum and vitreous levels of HGF in both groups (diabetic patients,  $r=-0.31$ ;  $p=0.5$  and control subjects  $r=-0.15$ ;  $p=0.5$ ).

**Conclusion**—The high vitreous levels of HGF observed in diabetic patients with PDR cannot be attributed to serum diffusion across the blood-retinal barrier. Therefore, intraocular synthesis appears to be the main contributing factor for the high vitreous HGF concentrations in diabetic patients, a cytokine that seems to be directly involved in the pathogenesis of PDR.

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Ocular neovascularisation, which is strongly associated with retinal ischaemia, is the hallmark of proliferative diabetic retinopathy (PDR). Growth factors play an important part in neovascularisation associated with PDR.<sup>1</sup> Hepatocyte growth factor (HGF), a potent in vitro mitogen for hepatocytes,<sup>2</sup> is an endothelium specific growth factor<sup>3</sup> which regulates cell growth, cell motility, and morphogenesis of various types of cells.<sup>4</sup> Interestingly, HGF has

been implicated in angiogenesis, a crucial event for the development of PDR, inducing the formation of capillary-like tubules.<sup>5</sup> The HGF receptor has been identified as the protein product of the *c-met* proto-oncogene<sup>6</sup> which encodes a transmembrane tyrosine kinase,<sup>7</sup> and it has been demonstrated that large and microvessel derived endothelial cells express the *c-met* receptor and respond to HGF.<sup>8</sup> Therefore, it seems logical that HGF might induce the proliferation of some intraocular endothelial cells during the angiogenic response that occurs in PDR.

Recently, elevated levels of HGF have been found in serum<sup>9,10</sup> and in vitreous<sup>11</sup> from patients with PDR. However, there are no studies in which both serum and vitreous fluid have been analysed simultaneously for HGF. This is an important point because the breakdown of the blood-retinal barrier that occurs in PDR facilitates the extracapillary leakage of serum proteins and their passage from the blood stream to the vitreous fluid, thus enabling the serum to have an effect on intravitreal protein levels. Moreover, a wide dispersion in serum levels of HGF has been found both in control subjects and diabetic patients.<sup>9,10</sup> Therefore, serum concentrations of HGF must be considered for the accurate interpretation of the results obtained in vitreous fluid. In addition, HGF itself is a positive regulator of local HGF production.<sup>12</sup> On the other hand, high intravitreal levels of a particular protein in PDR diabetic patients do not necessarily mean its intraocular production. In fact, we have observed that intravitreal total protein concentrations are elevated threefold in diabetic patients with PDR in relation to controls.<sup>13</sup> The comparison of intravitreal levels with a simultaneous serum determination could help to prevent this unspecific increase in protein levels in vitreous of diabetic patients. It must be underlined that the intravitreal protein concentration is at least 20-fold less than in serum.<sup>13</sup> Thus, the higher intravitreal concentration of a particular protein in relation to its serum levels strongly supports its intraocular production.

On this basis, we studied a group of PDR diabetic patients and control subjects, matched by serum HGF levels, in order to compare HGF levels in vitreous fluid and to evaluate its potential source.

## Materials and methods

Both venous blood and vitreous samples were collected at the time of vitreoretinal surgery from 17 patients with PDR (13 with non-

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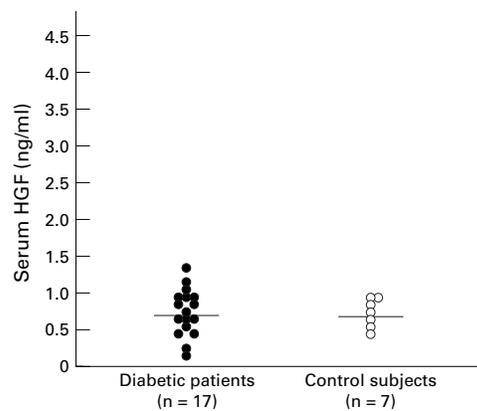


Figure 1 Diabetic and non-diabetic subjects matched by serum levels of hepatocyte growth factor (HGF). Serum HGF levels in diabetic patients and control subjects were 0.66 ng/ml (0.26–1.26) and 0.68 ng/ml (0.49–0.96), respectively;  $p=0.45$ . The horizontal line represents the median.

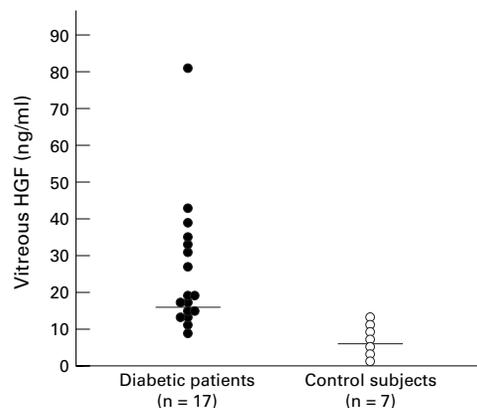


Figure 2 Vitreous concentrations of hepatocyte growth factor (HGF) in diabetic patients and control subjects with non-diabetic ocular disease. Differences between groups were statistically significant ( $p=0.003$ ). The horizontal line represents the median.

insulin dependent diabetes mellitus (NIDDM), and four with insulin dependent diabetes mellitus (IDDM) (mean age 49 (SD 11) years) and seven control non-diabetic patients with ocular diseases without intraretinal proliferation requiring vitrectomy (mean age 66 (13) years), matched by serum levels of HGF (Fig 1). The non-diabetic patients included one with macular oedema (not due to retinal vascular obstruction), one with macular hole, one with rhegmatogenous retinal detachment, and four with subretinal membranes, disorders in which the retina is not directly affected by neovascularisation. In order to avoid the influx of serum HGF into the vitreous, recent vitreous haemorrhage was excluded (less than 1 month). In both groups, 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 (Alcoon Model, Ten-Thousand Ocutome; Irvine, CA, USA) before starting the intravitreal infusion of balanced salt solution. The vitreous samples were transferred to a tube, placed immediately on ice and centrifuged at 16 000  $g$  for 5 minutes at 4°C. Supernatants were frozen at  $-80^{\circ}\text{C}$  until assayed.

For serum HGF determinations, blood samples were centrifuged at 3000  $g$  for 10 minutes at 4°C to obtain serum, then aliquoted and stored at  $-80^{\circ}\text{C}$  until assayed.

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

#### HGF METHOD ASSAY

Concentrations of immunoreactive HGF in vitreous and serum samples were measured by an enzyme linked immunosorbent assay (ELISA) for HGF (R & D Systems, Abingdon). This assay employs the quantitative sandwich enzyme immunoassay technique. It uses a monoclonal antibody specific for HGF precoated onto a microtitre plate and an enzyme linked polyclonal antibody specific for HGF as second antibody. The intra-assay coefficient of variation (CV), calculated from the differences of 54 duplicates was 3.5% for a mean concentration of 14.76 ng/ml. The inter-assay CV was 5.6%.

#### STATISTICAL ANALYSIS

The Mann–Whitney U test was used to compare serum and intravitreal concentrations of HGF. The results are expressed as median and range. Levels of statistical significance were set at  $p<0.05$ . The correlation between vitreous and serum HGF concentrations was examined by Spearman's rank correlation test.

#### Results

Intravitreal concentrations of HGF (median and range) were significantly elevated in diabetic patients with PDR (17.04 ng/ml (9.98–80)) compared with non-diabetic patients (5.88 ng/ml (2.57–14.20);  $p<0.003$ ) (Fig 2). Intravitreal HGF concentrations were strikingly higher than serum HGF concentrations both in diabetic patients (17.04 ng/ml (9.98–80) *v* 0.66 ng/ml (0.26–1.26);  $p<0.001$ ) and in the control group (5.88 ng/ml (2.57–14.20) *v* 0.68 ng/ml (0.49–0.96);  $p=0.003$ ). Finally, no correlation was found between serum and vitreous levels of HGF when all subjects were included in the analysis (Fig 3), as well as when both groups were analysed separately (diabetic patients,  $r = -0.31$ ;  $p=0.5$  and control subjects,  $r = -0.15$ ;  $p=0.5$ ).

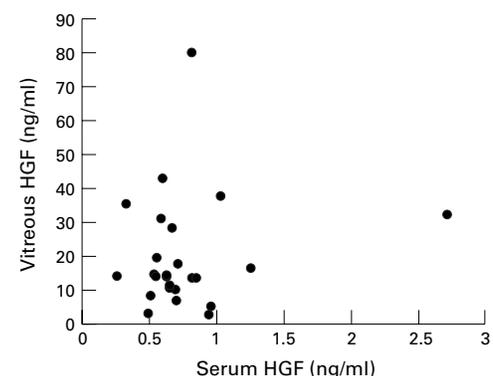


Figure 3 Lack of relation between serum and vitreous levels of hepatocyte growth factor (HGF) including both diabetic patients and control subjects.

## Discussion

HGF, originally isolated from rat platelets,<sup>14</sup> was described as a scatter factor because it induced the dissociation of colonies of epithelial cells.<sup>15</sup> This growth factor is produced mainly in the liver, but it has been found in several tissues such as lung, skin, spleen, brain, bone marrow, kidney, placenta, and even in intraocular structures such as the cornea, the lens, and the retina.<sup>16–20</sup> Interestingly, it has been shown that corneal cells, the retinal pigment epithelium, and epiretinal membranes in proliferative vitreoretinopathy express the *c-met* receptor.<sup>19, 20, 21</sup> In relation to its biological actions, it must be noted that HGF is a potent angiogenic factor *in vivo*<sup>22</sup> and its mitogenic activity is the most potent compared with that of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin-1 and 6.<sup>3</sup> As a powerful inducer of angiogenesis, HGF seems to contribute to the genesis of acquired immunodeficiency syndrome associated Kaposi's sarcoma, a cytokine dependent neoplasm characterised by a major component of neovascularisation.<sup>23</sup> However, there is a paucity of information about the relation between HGF and PDR, a process characterised by active neovascularisation.

There are few reports regarding the relation between serum HGF and diabetic retinopathy. Nishimura *et al*<sup>10</sup> found high serum levels of HGF only in diabetic subjects with PDR who had not undergone photocoagulation, but they did not observe differences among diabetic subjects with background retinopathy, preproliferative retinopathy, and PDR who had undergone photocoagulation. Kulseng *et al*<sup>9</sup> reported that type 1 diabetic patients have increased serum HGF levels, but they could not find significant differences in serum HGF between patients with or without PDR. Although prospective studies are needed, it seems that the contribution of systemic circulating HGF to the pathogenesis of diabetic retinopathy is not outstanding. However, local production of HGF could play a crucial part in the aetiopathogenesis of diabetic retinopathy.

Vitreotomy fluid samples obtained from diabetic patients with PDR are currently being used to explore indirectly the synthesis of growth factors by the retina. Katsura *et al*<sup>11</sup> found that levels of HGF in vitreous fluid of PDR patients were significantly higher than in non-diabetic patients, but they did not show serum HGF data. Nishimura *et al*<sup>24</sup> confirmed these results, showing again higher levels of HGF in vitreous fluid of PDR compared with controls. In addition, these authors compared vitreous HGF concentrations with their previously published results obtained in serum. The mean vitreous HGF concentrations seen in this study were 27-fold higher than the reported mean serum HGF levels in PDR subjects. In the present study, we compare for the first time serum and vitreous HGF concentrations in samples obtained simultaneously (at the time of vitreoretinal surgery), in the same group of patients, and the mean of vitreous HGF levels was 25-fold higher than serum concentrations. This ratio clearly exceeds our previous results

on VEGF, a demonstrated intraocular angiogenic factor,<sup>25</sup> which was 10-fold higher in vitreous fluid in relation to serum.<sup>13</sup> In addition, we did not find any relation between serum and vitreous HGF concentrations. This lack of correlation between serum HGF levels and HGF intraocular concentrations was also recently reported by Shinoda *et al*<sup>26</sup> studying aqueous humour from the anterior chamber. Taken together, these data suggest that intraocular synthesis of HGF but not serum diffusion is the main contributing factor to the high HGF concentrations observed in patients with PDR. Although specific studies in terms of mRNA and protein expression levels in patients with PDR are needed, the potential sites of HGF production could be the retinal pigment epithelial cells,<sup>20</sup> epiretinal membranes,<sup>21</sup> and macrophages.<sup>27</sup> Another possible mechanism that could contribute to the elevated concentrations of HGF in vitreous fluid is the ability of the hyaluronic acid and heparan sulphate, very abundant molecules in the vitreous body, to sequester HGF by means of recognised binding sites.<sup>28</sup> In this regard, it has been demonstrated that administration of heparin in patients with coronary disease caused significant increases in plasma HGF.<sup>29</sup> The serum collected after heparin administration had more prominent angiogenic properties than the serum collected before heparin administration, thus suggesting that HGF could play a significant part in the angiogenic effect of heparin. On the other hand, it has been reported that heparan sulphate glycosaminoglycans protect other growth factors such as bFGF from proteolytic degradation by extracellular proteinases.<sup>30</sup> Thus, it is tempting to speculate that these mechanisms could be involved in the PDR neovascularisation process induced by HGF.

Interestingly, we also observed higher HGF concentrations in vitreous fluid than in serum in the control group. This finding has not previously been reported and it probably reflects the HGF involvement in tissue regeneration and repair<sup>9</sup> in ocular diseases without intraretinal neovascularisation. Further investigations based on a large number of eyes without ocular neovascularisation will be necessary to confirm this hypothesis.

Recently, Shinoda *et al*<sup>26</sup> reported that HGF in aqueous fluid increased with the stage of diabetic retinopathy. However, intravitreous HGF levels observed in our patients were remarkably higher than the values reported by these authors in aqueous humour. This finding has been also previously described for VEGF,<sup>25</sup> probably reflecting the anterior-posterior gradients in the eye and/or the rapid clearance of these growth factors from anterior chamber.<sup>25</sup>

In summary, our present study provides *in vivo* evidence for increased intravitreous concentration of HGF in PDR, and suggests that intraocular synthesis of HGF, but not serum diffusion, is directly involved in the neovascularisation process. Further studies are needed to determine whether suppression of HGF actions, either by specific antibodies or other

mechanisms, could prevent or improve PDR and its consequences.

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