Original article

Serum and intraocular concentrations of erythropoietin and vascular endothelial growth factor in patients with type 2 diabetes and proliferative retinopathy

F. Semeraro\textsuperscript{a}, A. Cancarini\textsuperscript{a,\ast}, F. Morescalchi\textsuperscript{a}, M.R. Romano\textsuperscript{b}, R. dell’Omo\textsuperscript{b}, G. Ruggeri\textsuperscript{c}, L. Agnifili\textsuperscript{d}, C. Costagliola\textsuperscript{b}

\textsuperscript{a} Department of Medical and Surgical Specialties, Radiological Sciences and Public Health, University of Brescia, P.le Spedali Civili 1, 25123 Brescia, Italy
\textsuperscript{b} Department of Medicine and Health Sciences, University of Molise, Campobasso, Italy
\textsuperscript{c} Department of Laboratory Medicine, A.O. Spedali Civili, Brescia, Italy
\textsuperscript{d} Department of Ophthalmology, University of Chieti-Pescara, Chieti, Italy

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Abstract

Aim. – This study compared systemic and intraocular concentrations of erythropoietin (EPO) and vascular endothelial growth factor (VEGF) in patients with type 2 diabetes (T2D) and proliferative diabetic retinopathy (PDR) with levels in patients without diabetes, and looked for possible correlations between the concentrations found and other variables analyzed.

Methods. – Concentrations of EPO and VEGF were measured in the aqueous and vitreous humours and serum of patients undergoing vitrectomy for PDR (33 patients) or for macular holes or puckers (20 control patients). EPO was assayed by radioimmunoassay, with a lower limit of detection (LOD) of 1.0 mIU/mL. VEGF was assayed using enzyme-linked immunosorbent assay (ELISA), with a lower LOD of 10.0 pg/mL.

Results. – EPO concentrations in serum did not differ significantly between the two groups, whereas EPO in vitreous and aqueous were higher in diabetic than in non-diabetic patients. VEGF in serum was lower in diabetic patients than in non-diabetics; conversely, VEGF concentrations in vitreous were significantly higher in diabetic patients. A direct correlation was found between vitreous and aqueous EPO concentrations, and between vitreous EPO and blood glucose concentrations. A significant, negative correlation between vitreous EPO concentration and age was also recorded.

Conclusion. – High EPO concentrations in the vitreous of patients with PDR and its correlation with blood glucose suggest that EPO could play a role in the pathogenesis of PDR. All possible factors affecting serum and ocular concentrations of EPO and VEGF should be determined to identify compounds able to prevent and control this serious microvascular complication of diabetes.

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Keywords: Diabetes mellitus; Diabetic retinopathy; Erythropoiesis stimulating agents; Erythropoietin; Vascular endothelial growth factor

1. Introduction

Diabetes mellitus is the most prevalent metabolic disease in all countries. According to the International Diabetes Federation’s \textit{IDF Diabetes Atlas}, 382 million people worldwide have diabetes and that number is expected to grow to 592 million by the year 2035 [1].

The increased blood glucose concentrations in patients with diabetes put them at risk of both microvascular (retinopathy, nephropathy and neuropathy) and macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), which can severely impair quality of life and reduce life expectancy. Ito et al. [2] reported that the incidence of diabetic retinopathy (DR) in people with newly diagnosed diabetes increased substantially only in patients with 2-h plasma glucose concentrations $>11.1$ mmol/L.

While much is known of the risk factors and pathogenesis of proliferative diabetic retinopathy (PDR), many aspects still...
need to be clarified [3,4]. Hyperglycaemia in humans affects the concentration of plasma cytokines, which are implicated in insulin resistance (tumour necrosis factor (TNF)-α, interleukin (IL)-6), plaque destabilization (IL-18) and cardiovascular events (IL-6) [5]. In addition, erythropoietin (EPO) and vascular endothelial growth factor (VEGF) production are also stimulated by high glucose concentrations [6,7].

VEGF plays a key role in the development of choroidal neovascularization; in fact, vitreous concentrations of VEGF are significantly higher in patients with choroidal neovascularization compared with those recorded in healthy controls [8,9]. As VEGF plays a key role in the pathogenesis of choroidal neovascularization, targeting VEGF has been an attractive strategy in the treatment of this complication and the focus of extensive research in recent years [10]. Anti-VEGF therapy blocks choroidal neangiogenesis, thereby reducing vascular permeability, which frequently is the main cause of visual acuity deterioration. These findings provide the rationale for anti-VEGF therapy in retinal vascular diseases associated with neovascularization such as PDR [11]. However, as inhibition of VEGF is not associated with total regression of retinal neovessels [10], angiogenic factors other than VEGF may play a role in this process. The following are involved in the pathogenesis of DR: insulin-like growth factor; angiopoietins; adiponectin; stromal cell-derived factor-1; basic fibroblast growth factor-2; hepatocyte growth factor; TNF; IL-6; EPO; and pigment epithelium-derived factor. Although the precise role of EPO in the retina is yet to be elucidated, there is evidence to support the possibility of a physiological role under normal conditions and in cases of DR. Intravitreal concentrations of EPO are 3.5 times higher than those found in plasma, and it appears to act as a potent neuroprotective agent in the eye. Moreover, EPO is involved in directing circulating endothelial progenitor cells to injured retinal sites [12]. In vivo evidence suggests that EPO in the vitreous binds to EPO in PDR membranes, which subsequently leads to the proliferation of new retinal vessels [13]. The main known effect of EPO is to stimulate erythropoiesis by enhancing the proliferation and differentiation of erythroid precursors [14]; hypoxia is a powerful signal that stimulates its production. The effects of EPO appear to be independent of VEGF despite having an identical effect on PDR, namely, stimulation of ischaemia-induced retinal angiogenesis [15].

The present study evaluated systemic and intraocular concentrations of EPO and VEGF in type 2 diabetes (T2D) patients with PDR and in non-diabetic control patients to determine the role of these factors in the pathogenesis of DR.

2. Methods

This study enrolled patients with diabetes and PDR who had to undergo vitrectomy between May 2011 and January 2012 at the Unit of Ophthalmology, A.O. Civil Hospital, and University of Brescia. Inclusion criteria were T2D, age ≥ 18 years and PDR. Exclusion criteria were age < 18 years, type 1 diabetes, initial DR, patients with PDR not requiring surgery, and previous vitrectomy in the eye under examination, or other ophthalmic surgery or laser therapy within the previous 3 months. Non-diabetic patients who underwent vitrectomy for macular holes or puckers constituted the control group. Their inclusion criteria were age > 18 years and the presence of macular puckers or macular holes requiring vitrectomy. Exclusion criteria were age < 18 years, diagnosis of diabetes, and previous vitrectomy in the eye under examination, or other ophthalmic surgery or laser therapy within the past 3 months.

Fifty-three patients (53 eyes) fulfilled the inclusion criteria: 33 were T2D patients with PDR; and 20 were non-diabetic patients who served as controls. Informed consent was obtained from all patients after a detailed description of the aims and procedures of the study. The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the ethics committee of the Civil Hospital of Brescia. The trial is registered on the ClinicalTrials.gov website (available online at www.clinicaltrials.gov, number NCT01871207).

All patients (diabetics and controls) underwent complete ophthalmological examination (visual acuity, slit lamp, tonometry, fluorescein retinal angiography and optical coherence tomography). All patients also underwent a 23- or 25-gauge pars plana vitrectomy, with intravitreal injection of bevacizumab 10–15 days before the vitrectomy. Each patient’s demographic, clinical, ocular and biochemical data were also recorded.

The primary outcome of the study was the measurement of EPO and VEGF concentrations in serum and in the vitreous and aqueous humours. Blood samples taken before surgery were centrifuged at 3000 rpm for 10 min to separate the serum fraction, which was stored at −80°C. Aqueous and vitreous samples were taken during the surgery and immediately frozen at −80°C. Both EPO and VEGF concentrations were measured; however, owing to the small amount of sample, only EPO concentrations were determined in the aqueous humour. EPO was assayed by radioimmunoassay (Immulite EPO 200, Siemens Healthcare Diagnostics), with a lower limit of detection (LOD) of 1.0 mIU/mL, while VEGF was assayed by ELISA (Human VEGF Immunoassay, R&D Systems Europe, Abingdon, Oxon, UK), with a lower LOD of 10.0 pg/mL. VEGF concentrations below the lower LOD were set as 5 pg/mL for statistical analysis. VEGF concentrations > 2000 pg/mL were set at 2500 pg/mL; statistical tests were also performed after deleting any data > 2000 pg/mL. Laboratory analyses were carried out at the third laboratory of the Spedali Civili of Brescia.

Statistical methods: data were reported as means ± standard deviation (SD) or medians and interquartile ranges according to their distribution. For quantitative variables, comparisons between the two groups were by Student’s t-test or Mann–Whitney U test according to their distribution, and by Chi² test for qualitative variables.

Concentrations of EPO and VEGF in serum and in the vitreous and aqueous humours were compared between the diabetic and non-diabetic patients after their logarithmic transformation by means of factorial analysis of covariance (ANCOVA), considering the patient groups and hypertension as two factors, and serum creatinine together with metabolic serum variables [high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol and triglycerides] as covariates. Correlations
between concentrations of EPO and VEGF in serum and in the vitreous and aqueous were done after adjustment by means of Spearman’s partial correlation coefficients.

Multiple regression analysis was carried out to define the set of independent variables associated with log-transformed EPO concentrations in the vitreous and aqueous humour; all variables (log-transformed) with \( P < 0.10 \) by univariate analysis were included in the first model, and the final set of independent variables was obtained using a backward procedure.

With a sample size of 20 in each group, it is possible to demonstrate with a power of around 0.80 an effect size of about 1 with the Mann–Whitney U test, carried out at a level of statistical significance of 0.05 (two-tailed); in addition, it is possible to demonstrate with a power of around 0.80 a difference of about 40–45% with a Chi\(^2\) test performed at a statistical significance level of 0.05 (two-tailed). With 50 subjects, it is possible to demonstrate with a power of about 0.80 an association of around 0.40 with a correlation analysis carried out at a significance level of 0.05 (two-tailed).

The level of statistical significance was set at \( P < 0.05 \). All statistical analyses were performed using Statgraphics software.

3. Results

The main characteristics of the 53 patients (33 T2D patients with PDR and 20 non-diabetic control patients) enrolled in the study are summarized in Table 1. The cases and controls showed no significant differences in age, gender and smoking habits. There were, however, significant differences with regard to the presence of arterial hypertension and systemic therapy: 20 (71%) of the diabetic patients were taking angiotensin-converting enzyme (ACE) inhibitors, seven (25%) were taking angiotensin II receptor blockers (ARBs) and one (4%) was taking both; and 17 (52%) were taking HMG-CoA reductase inhibitors, 22 (67%) were taking antplatelet drugs and two (6%) an oral anticoagulant. Among the controls, five (25%) were taking ACE inhibitors, two (10%) were taking ARBs and two (10%) were taking both, while four (20%) were taking HMG-CoA reductase inhibitors, none were taking antplatelet drugs and three (15%) were taking an oral anticoagulant.

All of the diabetic patients had been treated with laser therapy for DR and were suffering from diabetic macular oedema; 18 (54%) had vitreous haemorrhages at the time of surgery. Twelve patients underwent cataract surgery during the vitrectomy and 13 patients were pseudophakic. The average macular retinal thickness was 0.49 mm (range: 0.14–1.20 mm) as measured by optical coherence tomography. The 20 control patients had all undergone vitrectomy: 11 (52%) for macular puckers and nine (48%) for macular holes; four were pseudophakic; 10 had undergone cataract surgery at the time of vitrectomy; and six had no cataracts.

Table 2 presents the concentrations (medians and ranges) of EPO and VEGF in serum and in the vitreous and aqueous humour of our diabetic and non-diabetic patients. No significant difference in serum EPO concentration was recorded between the two groups (\( P = 0.914 \)). In ocular fluids, EPO concentrations were significantly higher in diabetics vs. controls for both aqueous [36 (range: 2–98) vs. 4 (range: 1–42); \( P < 0.001 \)] and vitreous [144 (range: 8–509) vs. 24 (range: 3–114); \( P < 0.001 \)] humour. Serum concentrations of VEGF were lower in the diabetic vs. control patients [74 (range: 5–414) vs. 362 (range: 51–1166); \( P < 0.001 \)], whereas vitreous concentrations of VEGF were not statistically significantly different in diabetic vs. non-diabetic patients [41 (range: 5–1056) vs. 14 (range: 10–52); \( P = 0.179 \)].

In the diabetic group, EPO concentrations were significantly higher in the aqueous and vitreous humour than in serum (\( P < 0.001 \)). Moreover, vitreous concentrations of EPO were significantly higher than those in the aqueous (\( P < 0.001 \)).

As shown on Fig. 1A, EPO concentrations in the vitreous and aqueous humour were highly correlated (Spearman’s correlation coefficient of 0.71).
correlation $r = 0.75; P < 0.001$), whereas no statistical correlation was found between either vitreous or aqueous EPO and serum EPO concentrations. As expected, serum concentrations of EPO were negatively correlated with haemoglobin values ($r = -0.40; P = 0.003$). Also, an increase in blood glucose was associated with higher concentrations of EPO in both the vitreous ($r = 0.66; P < 0.001$) and aqueous ($r = 0.63; P < 0.001$; Fig. 1B). In all patients (with or without diabetes), vitreous concentrations of EPO diminished with age ($r = -0.38; P = 0.0056$); however, this relationship was highly significant only in the diabetic group ($r = -0.49; P = 0.0038$; Fig. 1C). Age had no effect on EPO serum concentrations. VEGF serum concentrations did not correlate with those in the vitreous, which in turn showed no statistically significant correlations with EPO concentrations in serum or in the vitreous or aqueous humours (Fig. 1D). Serum VEGF concentration was negatively correlated with EPO concentrations in the aqueous ($r = -0.43; P = 0.0013$; Fig. 1E) and vitreous ($r = -0.38; P = 0.0052$; Fig. 1F) humours. No statistically significant correlation was found between EPO concentrations in the vitreous and serum. Furthermore, no statistically significant association was found between EPO and VEGF values and heart disease, kidney disease, neuropathy, smoking, vitreous haemorrhage, pseudophakia and cataract surgery (yes or no), with the exception of arterial hypertension. Median values for aqueous EPO in normotensive and hypertensive patients were 5.5 mIU/mL and 26.0 mIU/mL, respectively ($P = 0.0298$), whereas values for vitreous EPO were 28.0 mIU/mL and 81.0 mIU/mL, respectively ($P = 0.0222$).

Glycated haemoglobin levels showed a marginally non-significant trend ($P = 0.08$) towards a direct association with EPO concentrations in vitreous humour.

Patients taking antiplatelet drugs had lower concentrations of VEGF in serum (median: 72.5 pg/mL vs. 206.0 pg/mL; $P = 0.0184$) and higher concentrations in the vitreous (median: 43.0 pg/mL vs. 14.0 pg/mL; $P = 0.0253$); no other variables studied were associated with VEGF.

Tables 3A–3C shows the results of the multivariate regression analysis to obtain the independent set of variables predictive of aqueous EPO, vitreous EPO and serum VEGF. The log of EPO concentration in the aqueous humour increased by 1.861 and 0.670 for each unit increase of the natural logarithm for blood glucose and creatinine, respectively; in contrast, it decreased by 1.859 for each unit increase of the natural logarithm for

**Table 3A**

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Regression coefficient</th>
<th>95% CI</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (ln)</td>
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<td>1.179, 2.544</td>
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<tr>
<td>Creatinine (ln)</td>
<td>0.670</td>
<td>−0.105, 1.445</td>
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<tr>
<td>Cholesterol (ln)</td>
<td>−1.859</td>
<td>3.294, −0.423</td>
<td>0.0122</td>
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**Table 3B**

<table>
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<th>Independent variables</th>
<th>Regression coefficient</th>
<th>95% CI</th>
<th>$P$</th>
</tr>
</thead>
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<td>Age</td>
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<td>−0.044, −0.005</td>
<td>0.0158</td>
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<td>Blood glucose (ln)</td>
<td>1.992</td>
<td>1.372, 2.612</td>
<td>&lt;0.0001</td>
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<tr>
<td>LDL cholesterol (ln)</td>
<td>2.302</td>
<td>0.397, 4.207</td>
<td>0.0189</td>
</tr>
<tr>
<td>Cholesterol (ln)</td>
<td>−4.132</td>
<td>−6.885, −1.380</td>
<td>0.0041</td>
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</table>

LDL: low-density lipoprotein.
serum cholesterol. The log of EPO concentration in the vitreous humour increased by 1.992, 0.024 and 2.302 for each unit increase for age and the natural logarithm for blood glucose and LDL, respectively; in contrast, it decreased by 4.132 for each unit increase of the natural logarithm for serum cholesterol. However, the results of this multiple regression disappeared when group variables (either diabetic or non-diabetic) were introduced. The log of VEGF concentration in plasma increased by 1.247 and 3.236 for each unit increase of the natural logarithm for serum haemoglobin and triglycerides, respectively; in contrast, it decreased by 1.713 for each unit increase of the natural logarithm for blood glucose. Also in this case, the introduction of group variables deleted the effects of this particular variable.

4. Discussion

Our present findings are similar to those of other researchers [8,9,15–19]: EPO and VEGF concentrations in ocular fluids of patients with PDR were significantly higher than in control patients. Recently, Cheung et al. [20] demonstrated a progressive increase of VEGF concentrations from controls to diabetic patients without DR to diabetic patients with PDR, further supporting the significant role of VEGF in the pathogenesis of DR. Hypoxia is a major stimulus of systemic and intraocular VEGF and EPO production. EPO has an angiogenic potential equivalent to that of VEGF; thus, EPO could be an important factor involved in stimulating retinal angiogenesis in PDR [12]. In Takagi et al. [21], multivariate logistic regression analyses indicated that EPO and VEGF equally and independently contribute to retinal neovascularization in the pathogenesis of PDR. They also found that EPO is more strongly associated with PDR than is VEGF. Moreover, exogenous EPO administration by intravitreal or intraperitoneal injection in early diabetes prevented structural, vascular and neural damage in experimental animal models [22], and injections of EPO into eyes with severe, chronic diabetic macular oedema resulted in a short-term positive response in visual acuity [23]. Contrary to the findings of Mohan et al. [15] and Asensio-Sánchez et al. [24], however, serum concentrations of EPO in our study population did not significantly differ between diabetic patients and controls, and were inversely related to haemoglobin concentration. Nevertheless, our results are in agreement with those previously reported by Inomata et al. [25], who demonstrated that, despite significant rises in vitreous EPO concentrations in patients with PDR compared with those detected in patients with macular holes, no differences in EPO serum concentrations were reported. This means that anaemia—the main physiological driver of EPO concentrations in serum—overcomes the effect of other possible factors.

Our present study found no correlation between haemoglobin and intraocular EPO concentrations (in both vitreous and aqueous humours). Katsura et al. [15] also recorded no differences in vitreous concentrations of EPO between anaemic patients and those with normal levels of haemoglobin, suggesting that intravitreal EPO is not affected by anaemia. Concentrations of EPO in vitreous and aqueous humours were not correlated with serum concentrations, although diabetics with normal concentrations of systemic EPO may become anaemic. EPO-dependent anaemia is a possible complication of diabetic neuropathy; the condition may arise in diabetics who have autonomic neuropathy in conjunction with modest renal impairment [26].

The finding of higher EPO concentrations in the vitreous and aqueous humours of both diabetic and non-diabetic patients supports intraocular production of EPO, probably by retinal cells, as suggested by Watanabe et al. [17]. Recent experimental evidence suggests that EPO has a potent protective effect on the retina; as retinal degeneration is an early event in DR, higher EPO production may represent an important factor in counteracting neurodegeneration [18]. The hypothesis of intraocular production is further supported by the increased expression of the EPO gene and EPO mRNA concentrations in human and animal retinas, and the presence of EPO receptors in the ganglion cells, amacrine cells and astrocytes of the mammalian retina [27,28]. Other factors responsible for increased EPO production are inflammation, hyperglycaemia and reduced catabolism of glycated EPO [29,30]. In our study population, the effect of hyperglycaemia was confirmed by the significant correlation between blood glucose and EPO concentrations in both the vitreous ($P < 0.001$; Fig. 1B) and aqueous ($P < 0.001$) humours. EPO glycation is critical for reducing the rate of in vivo clearance, which is mainly mediated by its receptor. The reduced affinity for the receptor delays catabolism, thereby increasing circulating concentrations of EPO [29]. This, however, does not explain the correlation found between EPO and intraocular glucose concentrations, but not between EPO and blood glucose. Such a lack of correlation might be due to the intravitreal injection of bevacizumab causing a reduction in EPO blood concentrations (the effect lasted from 4.4 ± 2.2 days to 34.8 ± 33.7 days after injection) [31].

In diabetic patients, glycated haemoglobin values showed a marginally non-significant trend ($P = 0.08$) towards a direct association with concentrations of EPO in the vitreous. Recently, Hämäläinen et al. [32] demonstrated the presence of high serum EPO concentrations in diabetic patients, with a significant association between EPO and abdominal obesity. This was perhaps due to the adipose tissue hypoxaemia typically found in T2D. Nevertheless, the mechanism responsible for increased EPO concentrations in the vitreous of patients with PDR has still not been completely elucidated, given the numerous agents involved in its upregulation in the diabetic eye. Clinical and experimental evidence has so far been insufficient to clarify whether the actual role of EPO in diabetes is protective or pathogenic; however, most of the available information points to a protective rather than pathogenic effect, at least in the early stages of DR [12]. In fact, several studies have demonstrated that EPO exerts a neuroprotective effect on the brain and retina [33,34], counteracting

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</tr>
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<tr>
<td>Blood glucose (ln)</td>
<td>−1.713</td>
<td>−2.677, −0.748</td>
<td>0.0008</td>
</tr>
<tr>
<td>Haemoglobin (ln)</td>
<td>3.236</td>
<td>0.562, 5.909</td>
<td>0.0187</td>
</tr>
<tr>
<td>Triglycerides (ln)</td>
<td>1.247</td>
<td>0.349, 2.144</td>
<td>0.0075</td>
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neurological insults such as hypoxia and ischaemia [35], photo-oxidation [36] and increased intraocular pressure [37]. The very high range of EPO concentrations in both the aqueous and vitreous of patients with PDR may be a reflection of either a different degree of retinal blood–barrier involvement in such patients or a previously unrecognized vitreous haemorrhage [38].

Hyperglycaemia appears to play a key role in the aetiology of DR; however, recent attention has been directed more towards the molecular basis of the disease, and several biochemical factors other than hyperglycaemia have been considered. Such mechanisms affect cellular metabolites and subsequently induce the release of cytokines; of these, VEGF is the most representative, and its role in angiogenesis and microvascular permeability is well known [39]. In our study population, concentrations of intravitreal VEGF tended to be higher in diabetic patients, albeit not significantly. The small degree of intravitreal VEGF elevation together with its low concentration in serum could be explained by the fact that all diabetic patients received an intravitreal injection of bevacizumab before vitrectomy. In fact, it has been demonstrated that, despite the presence of the blood–ocular barrier, anti-VEGF agents may be detected in the plasma of treated patients once injected into the vitreous humour [40]. Moreover, the blood–ocular barrier is often damaged in neovascular diseases, which facilitates the absorption of anti-VEGF drugs into the systemic circulation, where they suppress serum concentrations of VEGF [40,41]. The ratio of vitreous to plasma concentrations of VEGF is significantly higher in diabetic patients, thereby supporting the hypothesis of local production of VEGF in the retina, as several studies have shown [42,43].

The action of EPO appears to be independent of VEGF, although both stimulate neovascularization [12]. Yet, our study found no correlation between concentrations of EPO and VEGF in the vitreous, which is inconsistent with findings in the literature [16,17]. Both serum and vitreous concentrations of VEGF did not correlate significantly with EPO concentrations in either serum or the vitreous or aqueous humours, nor were any correlations found between VEGF in serum and the vitreous and other variables examined (continuous and discrete).

The results of multivariate regression analysis are difficult to understand; diabetes is the most important factor, as it affects serum concentrations of glucose, cholesterol and triglycerides.

In conclusion, our results confirm that VEGF contributes to the pathogenesis of PDR, although the mechanism underlying the increase in VEGF production is not unique. As the inhibition of VEGF is not associated with total regression of retinal neovascularization, it is important to consider the other factors that may play a role in the process in PDR patients. Insulin-like growth factor, angiopoietins, stromal cell-derived factor-1, basic fibroblast growth factor-2, hepatocyte growth factor, TNF, IL-6, EPO and pigment epithelium-derived factor have all been identified as novel factors in the pathogenesis of DR. Among them, as recently suggested by Mohan and coworkers [15], EPO appears to act synergistically with VEGF in the pathogenesis of PDR. This implies that therapy for DR needs to counteract the effects of other cytokines in addition to VEGF to achieve more effective results.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Appendix A. Supplementary data

Supplementary data (French abstract) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.diabet.2014.04.005.

References


