The Challenging Equilibrium of Angiogenic Factors Leptin & Erythropoietin versus Antiangiogenic IL-12 in Pathogenesis of Proliferative Diabetic Retinopathy

Nirvana, A. Khalaf, Ayman Shouman and Leqaa, A. Moemen

Abstract: Diabetic retinopathy is a leading cause of visual disturbance in adults. In proliferative diabetic retinopathy (PDR). Ischemia – induced neovascularization often causes catastrophic loss of vision. Erythropoietin EPO and Leptin have been identified by its angiogenic properties. Interleukin 12 is a potent anti-angiogenic factor. This study tries to clarify the challenging equilibrium of angiogenic factors versus anti-angiogenic IL 12 in pathogenesis of PDR. This study conducted on 45 patients. Vitreous EPO, leptin and IL 12 were assayed by enzyme linked immunosorbent assay (ELISA). Erythropoietin, leptin and IL 12 levels in vitreous are strikingly higher in diabetics. Significant elevation of EPO, leptin and IL 12 were detected in PDR cases compared to non PDR cases. Active PDR patients have higher, EPO & IL 12 compared to quiescent PDR. This study concluded that the challenging balance between angiogenic-antiangiogenic factors need more research on wider range. Inhibition of molecular mechanism could be a new therapeutical strategy in halting or preventing pathological angiogenesis in diabetic retinopathy. The increase of intraocular synthesis of Epo that occurs in PDR can be contemplated as a compensatory mechanism to restore the damage induced by the diabetic milieu rather than a pathogenic contributor.

Key words: Proliferative diabetic retinopathy, Erythropoietin, leptin, interleukin 12, angiogenesis.

Introduction

Diabetic retinopathy (DR) has been classically considered to be a microcirculatory disease of the retina caused by the deleterious metabolic effects of hyperglycemia per se and the metabolic pathways triggered by hyperglycemia (Villarroel et al, 2010).

Retinal angiogenesis is a major cause of blindness in ischemic retinopathies including diabetic retinopathy (Yoshida et al, 2011). Photoreceptors and other cells of the retina consume large quantities of energy to efficiently convert light information into a neuronal signal understandable by the brain. The necessary energy is mainly provided by the oxygen-dependent generation of ATP in the numerous mitochondria of retinal cells. To secure the availability of sufficient oxygen for this process, the retina requires constant blood flow through the vasculature of the retina and the choroid. (Caprara & Grimm 2012). Inefficient supply of oxygen and nutrients, as it may occur in conditions of disturbed hemodynamics or vascular defects, results in tissue ischemia or hypoxia. This has profound consequences on retinal function and cell survival, requiring an adaptation response by cells to cope with the reduced oxygen tension. Central to this response are hypoxia inducible factors, transcription factors that accumulate under hypoxic conditions and drive the expression of a large variety of target genes involved in angiogenesis, cell survival and metabolism. Prominent among these factors are vascular endothelial growth factor and erythropoietin, which may contribute to normal angiogenesis during development, but may also cause neovascularization and vascular leakage under pathologically reduced oxygen levels. Since ischemia and hypoxia may have a role in various retinal diseases such as diabetic retinopathy, studying the cellular and molecular response to reduced tissue oxygenation is of high relevance. In addition, the concept of preconditioning with ischemia or hypoxia demonstrates the capacity of the retina to activate endogenous survival mechanisms, which may protect cells against a following noxious insult. Part of these mechanisms is the local production of protective factors such as erythropoietin (Caprara & Grimm 2012).

Erythropoietin, a 30-kDa glycoprotein hormone, is initially produced by the liver but shortly after birth its production is shifted to kidney (Hernández C, Simó R, 2011). EPO is upregulated in diabetic retinopathy. Its up regulation can prevent apoptosis and associated inflammation (Ghezzi P & Brines M. 2004).

Erythropoietin (EPO) is the principal regulator of erythropoesis by inhibiting apoptosis and by stimulating the proliferation and differentiation of erythroid precursor cells. However, EPO also performs extrarythropoietic actions of which the neuroprotective effects are among the most relevant. Apart from kidney and liver, EPO is also produced by the brain and the retina. In addition, EPO receptor (EPO-R) expression has also
been found in the brain and in the retina, thus suggesting an autocrine/paracrine action which seems essential for the physiological homeostasis of both brain and retina. (Hernández C & Simó R. 2011).

Effects in the retina including neuro and vaso-protective activities, erythropoietin has gained strong interest as potential therapeutic factor for retinal degenerative diseases (Caprara & Grimm 2012). Meanwhile (McVicar et al, 2011). Detected erythropoietin (EPO) may be protective for early stage diabetic retinopathy, although there are concerns that it could exacerbate retinal angiogenesis and thrombosis. A peptide based on the EPO helix-B domain (helix B-surface peptide [pHBSP]) is nonerythrogenic but retains tissue-protective properties.

Nevertheless, when retinal hypoxia is a predominant event and high levels of VEGF exist, EPO could enhance effect of VEGF, thus contributing to neovascularization, in consequence worsening PDR (Grant et al, 2008). EPO might act as a double-edged sword in pathogenesis of diabetic retinopathy. Intravitreal EPO has shown a short term positive effect in diabetic macular edema (Li et al, 2010), Meanwhile (Garci-arumi et al, 2009 & Pat et al, 2008 and Katsura et al, 2005) found high levels of intravitreal EPO in patients with diabetic macular oedema suggesting a link between retinal angiogenesis and pathogenesis of diabetic retinal complication, Wang et al, (2010), hypothesize that EPO reduces the loss of retinal pericytes and therefore can be used as a novel therapeutic agent for early form of DR, which is based on its antioxidant, anti-inflammatory, and neuroprotective properties.

Leptin is an anti-obesity hormone, 167 of amino acids protein synthesized in the pituitary gland, adipose tissue. Serum leptin concentrations are proportional to the body adipose mass. Leptin induces lipid metabolism, neuroprotective properties.

Interleukin 12 (IL-12) is a disulfide linked heterodimer composed two subunits with molecular masses of 35 and 40 KD, is a multifunctional cytokine produced by macrophage, B cell lines. IL-12 was reported to inhibit angiogenesis in a mouse model of corneal neovascularization induced by basic fibroblast growth factor (bFGF), (Cao et al., 1995). It has been generally believed that angiogenesis is the result of vigorously maintained equilibrium between negative and positive regulator. Thus this study tries to clarify the challenged equilibrium between angiogenic factors erythropoietin and leptin versus antiangiogenic IL-12 in pathogenesis of PDR.

**Material and Methods**

This study was conducted on 45 patients performing pars plana vitrectomy in Research Institute of Ophthalmology. Eight age matched controls were included in this study, they were performing vitrectomy because of macular hole or giant retinal tear as long as there was no vitreoretinal proliferation. Patients and controls are age matched (ranged 57-65 years), and with duration of diabetes 10 years. All diabetics are controlled by oral hypoglycemic drugs. Full ophthalmologic examination and medical history was taken for each subject including:

- Intraocular pressure measurement with Goldman applanation tonometry.
- Slit lamp examination.
- Fundus examination by a binocular indirect ophthalmoscope and a slit lamp biomicroscopic examination with a 90 D lens to evaluate the grade of vitreous proliferation and detect the presence and nature of macular oedema.
- Fundus fluorescein angiography was done using Topcon fundus camera TRC .50 EX on image –net, 5 ml of 10% sodium fluorescein was injected in antecubital vein and photography was carried out.
- Preoperative findings were clarified and clinical severity was assayed; according to presence and extent of fibrovascular tissues, vitreous hemorrhage, tractional retinal detachment.
- Recent vitreous hemorrhage was excluded by clinical examination to avoid affecting the vitreous samples.
- Undiluted vitreous fluid samples were harvested at the start of vitrectomy after informed consent was obtained from each subject following an explanation of the purpose and potential adverse effects of the
Vitrectomy was performed on the 30 patients with PDR, seven diabetic patients without retinopathy included four with macular hole and three with epiretinal membrane, which may threaten visual acuity if not treated properly, and eight non diabetics with macular hole or giant retinal tear ocular diseases as controls.

- Exclusion criteria for this study were:
  1. Previous ocular surgery.
  2. History of ocular inflammation.
- Angiography was performed to differentiate proliferative and non proliferative retinopathy.
- PDR was classified as active (20) eyes if there were new preretinal capillaries and as quiescent (10 eyes) if the Vasoproliferation only consisted of large vessels within the membrane at the time of surgery (Moravski et al., 2000).
- Pars plana vitrectomy was done by a standard technique using three pars plana sclerotomy incisions. The undiluted samples of vitreous fluid (0.2 – 0.5 ml) were aspirated under standardized conditions directly from the mid-vitreous at the beginning of surgery and were immediately transferred to sterile tubes.
- Vitreous samples were centrifuged at 10,000 rpm for 5 minutes to remove contaminating cells. The supernatants were frozen at -80°C until assay.
- Blood samples were collected in two tubes part on EDTA, the other part centrifuged and serum was separated and stored at -80°C until assay.
- Routine laboratory investigations were performed including estimation of fasting blood glucose, creatinine, and complete lipid profile using commercial available kits.
- Estimation of vitreal levels of erythropoietin mU/ml (Caro & Erslev, 1988) and leptin pg/ml by enzyme linked immunosorbent assay (ELISA) (Leroy et al., 1996) with minimal detectable level of 7.8 pg/ml for leptin. Detection of vitreal IL 12 was measured also by ELISA assay (Winkler et al., 1998) Quantikine high sensitivity human by (R&D), system Minneapolis USA. The minimal detection levels of IL12 are less than 5 pg/ml.

**Statistical Analysis**

Analysis of data was done via SPSS package version 9 (statistical package social science). Different tests were applied. Mean and standard deviation were used for data description. ANOVA (analysis of variance) was done to compare mean ranks of different parameters for more than two groups. P<0.05 is considered significant. Pearson's correlation was done to detect association correlation coefficient (r) where p< 0.05 was considered significant.

**Results:**

The results of this study are illustrated in table (1).

<table>
<thead>
<tr>
<th>Table 1: The mean levels ± SD of erythropoietin, leptin and interleukin 12 vitreous of all studied groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No</strong></td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>EPO (mU/ml)</td>
</tr>
<tr>
<td>Leptin pg/ml</td>
</tr>
<tr>
<td>IL 12 pg/ml</td>
</tr>
</tbody>
</table>

P<0.001 highly significant

- The mean vitreous levels of erythropoietin were significantly elevated in all diabetic groups compared to controls and this elevation is more pronounced in PDR groups compared to diabetics without retinopathy P<0.001. The active stage of PDR show higher significant vitreous erythropoietin levels compared to quiescent stage P<0.05.
- The mean vitreous leptin levels were significantly elevated in all diabetic groups compared to controls, this elevation is more evident in PDR groups compared to those without retinopathy P<0.05. The mean vitreous leptin level is higher in active PDR compared to quiescent PDR but this elevation in not significant. P>0.05.
- The mean vitreous interleukin 12 levels were higher all diabetic groups compared to controls P<0.001. Also, the elevation of IL 12 levels were significantly evident in PDR patients compared to diabetic without retinopathy cases P<0.01. Active PDR patients have significantly higher IL 12 levels compared to quiescent patients P<0.01.
- A significant positive correlation was detected between vitreous erythropoietin and leptin levels in all studied groups (controls, diabetic without retinopathy, quiescent and active PDR r=0.61, P<0.01, r= 0.62, P<0.01, r= 0.65, P<0.01, r=0.67, P<0.01 respectively.
- Also significant positive correlations were detected between EPO levels in quiescent and active stage of PDR and IL 12 levels, r=0.65, P<0.01 & r=0.66, P<0.01 respectively.
Diabetic retinopathy is a leading cause of visual disturbance in adults. In proliferative diabetic retinopathy, ischemia-induced pathologic growth of new blood vessels often causes catastrophic loss of vision. Besides VEGF, the existence of another potent ischemia-induced angiogenic factor is postulated. Since ischemia-inducible erythropoietin (Epo) has recently been identified its angiogenic properties, we investigated its potential role during retinal angiogenesis in proliferative diabetic retinopathy (PDR) Takagi et al., (2007), moreover they found that erythropoietin is an ischemia-induced angiogenic factor that acts independently and as potently as VEGF in proliferative diabetic retinopathy (PDR).

In this study, the mean vitreous levels of Epo were significantly elevated in diabetics compared to controls where as active stage PDR is the more evident elevation. This is in agreement with the elevated concentration of Epo in vitreous of diabetic patients (~ 30-fold- higher than plasma and ~ 10- fold higher than in non-diabetic subjects). The retina is the most metabolically active tissue in the human body. Therefore, is highly sensitive to reduction of oxygen tension. Hypoxia is a major stimulus for both systemic and intraocular Epo production. In fact, high intravitreous levels of Epo have recently been reported in ischemic retinal diseases such as proliferative DR (PDR) (Hernández. et al, 2006; Katsura et al, 2005; Watanabe et al, 2005; Asensio-Sánchez et al, 2008 and Watanabe, 2007).

There are several reasons for pointing to Epo as a significant factor in the physiological homeostasis of the retina. First, it has been demonstrated using an in vitro model of the bovine blood–brain barrier (BBB) that Epo protects against vascular endothelial growth factor (VEGF)-induced permeability of the BBB and restores the tight junction proteins. Epo treatment also prevents an increase in BBB permeability in a rat model of induced seizures. Because the blood–retinal barrier (BRB) is structurally and functionally similar to the BBB, it is possible that Epo acts as an ant permeability factor in the retina. In fact, Epo was able to improve diabetic macular edema when it was administered for treatment of anemia in diabetic patients with renal failure.

Second, there is growing evidence that Epo is a neurotrophic factor not only in the brain but also in the retina. Third, Epo exerts an anti-inflammatory effect on the brain, and this action might also be extrapolated to the diabetic retina. Fourth, it has been reported that the retinal expressions of Epo and its receptor (Epo-R), as well as Janus kinase 2 phosphorylation, are each tightly linked to a specific duration after oxidative stress in anticipation of daily light onset. This is consistent with physiological protection against daily light induced, oxidatively mediated retinal apoptosis. Finally, Epo is a potent physiological stimulus for the mobilization of endothelial progenitor cells, and, therefore, it could play a relevant role in regulating the traffic of circulating endothelial progenitor cells toward injured retinal sites (Herna´ndez & Simo, 2011).

Epo exerts its actions through Epo-R. Epo-R belongs to the cytokine receptor type I super family and is activated via homodimerization. When Epo binds Epo-R, it causes dimerization of the receptor, autophosphorylation, and activation of Janus kinase 2 (JAK2), which can initiate multiple signal transduction pathways associated with cell survival. Of particular interest is the activation of the protein kinase B (PKB)/AKT pathway, which has been shown to stimulate anti-apoptotic signals that facilitate the inhibition of mitochondrial cytochrome c release and to help maintain mitochondrial membrane potential. Epo is also well known to activate phosphatidylinositol- 3-kinase [PI (3)K], and STAT1, STAT3, STAT5A, and STAT 5B, especially in cytokine-induced signaling pathways. Finally, the stem cell factor (SCF) or c-kit is also known to interact with Epo-R (Herna´ndez & Simo, 2011).

Apart from hypoxia, other factors could regulate Epo expression (Watanabe et al, 2005) observed an increase in the vitreous Epo levels in patients with inflammatory eye diseases. Given that inflammation has been involved in the pathogenesis of DR, this might be a contributing factor to the high levels of Epo observed in diabetic patients. Hyperglycemia could be another factor that induces Epo production. Although there are no studies evaluating the effect of glucose on Epo expression in retinal cells, a direct relationship has been shown between glucose and Epo concentrations in a Chinese hamster ovary cell line (Sun & Zhang, 2001). A reduction in Epo catabolism could also contribute to the higher Epo levels detected in the retina and the vitreous fluid from diabetic donors. In this regard, the glycosylation of Epo reduces its affinity for Epo-R (Darling et al, 2002). Because Epo is degraded only by Epo-R-expressing cells and their receptor binding determines the rate of intracellular degradation (Gross et al, 2006) it is possible that a higher degree of Epo glycosylation is associated with lower clearance of Epo.

Epo also protects Retinal pigment epithelium (RPE) cells against the increase of permeability induced by diabetic conditions, and this effect is mainly mediated by the downstream signaling of JAK2 and PI3/AKT pathways. Moreover, Epo treatment leads to an increase of intracellular free Ca2+ in RPE cells by inducing the influx from the extracellular space. This effect could contribute to the protective effects of Epo on the barrier function of RPE cells and is also mediated by the JAK2 and PI3/AKT pathways (Carcia – Ramirez et al, 2011).

Apart from its ant permeability, anti-inflammatory, and neuroprotective actions, Epo protects against high-glucose induced apoptosis as well as the deleterious effect of free radicals. Finally, as previously mentioned, Epo is a potent physiological stimulus for the mobilization of endothelial progenitor cells (EPCs) and, therefore,
it could play a significant role in regulating the traffic of circulating EPCs toward injured retinal sites (Hu et al., 2011). For all these reasons, the increase of intraocular synthesis of Epo that occurs in DR can be contemplated as a compensatory mechanism to restore the damage induced by the diabetic milieu rather than a pathogenic contributor.

In this study, elevated mean vitreous leptin levels were detected in all diabetics, the elevation is more evident in PDP patients. These results corroborate with the finding of (Hamdi, et al., 2005 & Maberly et al., 2000). Hypoxia is an important factor for the expression of leptin. Therefore, leptin expression is strongly associated with vascular disorders including diabetes mellitus. Gariano et al (2000) was the first to our knowledge suggesting an involvement of leptin in retinal diseases. Hamdi et al., (2004) suggested that elevated vitreous leptin in PDR cases due to breakdown of blood-ocular barriers. Also, leptin is involved in the development of angiogenesis and microvascular and proliferative complication of diabetes. Leptin might be autocrine or paracrine and play a modulator role in vasoproliferative diseases.

Maberley et al, (2006) suggested that leptin reach the vitreous a) Via the extravasation of blood from active retinal neovascularization. This suggestion goes with our result of higher leptin levels in active PDR compared to Quiescent PDR. b) By the expression of leptin by fibroblast within diabetic neovascular membrane (Maberley et al., 2006). On the other hand, Hernández c; et al, (2004) concluded that intraocular production of leptin is not critically involved in etiopathogenesis of PDR and serum diffusion is a relevant source of leptin vitreous. However, leptin stimulate angiogenesis through Jak – STAT pathway (Sierra-Honigmann et al, 1998) in an animal model, and also modulates angiogenic response included by VEGF, (Cao et al, 2001). Gariario et al, (2000) detected not only leptin but also its receptors in fibrovascular membranes in diabetics. Thus, leptin have a possible involvement in microvascular and vasoproliferative retinal diseases.

In this study, the mean IL 12 levels in vitreous were higher in all diabetics compared to controls. This elevation is more evident in PDR cases especially active PDR. These results are in agreement with Winkler et al, (1998), they reported that elevation of IL 12 could be a consequence of retinopathic complication of diabetes as part of consecutive immunological mechanism. To the contrary of theoretically proposed that lower of IL 12 levels might be a predisposing factor for diabetic retinopathy. To our knowledge IL 12 not measured in vitreous of PDR cases. Instead, El-Shabrawi et al, (1998) reported elevated IL 12 in aqueous and vitreous humor of patients with uveitis suggest its role in susceptibility to ocular inflammation. IL 12 has been considered as a strong anti-angiogenic cytokines, this effect is mediated by interferon \( \gamma \). Chorostowska-Wynimko, et al, (2005). The interferon \( \gamma \) in turn regulate production of secondary chemokines by inducing interferon inducible protein 10 which is considered most important mediator of IL 12 dependent activation of anti-angiogenic process (Maier et al, 2008). Also, IL 12 was reported to inhibit angiogenesis in vivo model of corneal vascularization. Sgardari et al, (1996) documented the importance of interferon inducible protein 10 as mediator of angiogenesis inhibition by IL 12.

Several proinflammatory cytokines are involved in maintenance of equilibrium between positive and negative regulators. Meanwhile, neuroprotective properties of Epo, its effect in preventing microvascular damage in the diabetic retina as well as its capacity to protect the barrier function of retinal pigment epithelium cell are solid reasons for processing Epo or Epo-receptor agonist as a new therapeutic agent in treatment of early stage of DR (Hernández & Simó, 2011).

However, to the best of our knowledge, there are no experimental or clinical studies using eye drops of Epo for treatment of DR. systemic administration of Epo-derived peptides without capacity to increase hematocrit or exacerbate neovascularization but treating tissue protective properties against vascular insult could be a new approach for preventing or arresting microvascular complications in diabetics. The challenging equilibrium between angiogenic-antiangiogenic factors need more research on wide range. Inhibition of molecular mechanism could be a new therapeutical strategy in halting or preventing pathological angiogenesis in diabetic retinopathy. The increase of intraocular synthesis of Epo that occurs in PDR can be contemplated as a compensatory mechanism to restore the damage induced by the diabetic milieu rather than a pathogenic contributor.

References


