Australian Journal of Basic and Applied Sciences, 2(4): 1320-1324, 2008
ISSN 1991-8178

Erythropoietin Is a Potent Ischemia Induced Angiogenic Factor in Proliferative Diabetic Retinopathy

[1]Soher A. Ismail MD, [2]Mostafa M. Salah Eldine MD and [2]Ahmed A. Zaki MD

[1]Biochemistry Department, Research Institute of Ophthalmology, Cairo, Egypt.
[2]Ophthalmology Department, Research Institute of Ophthalmology, Cairo, Egypt.

Abstract: Purpose: Erythropoietin (FPO) has been recently found to be increased in the vitreous fluid from ischemic retinal diseases such as proliferative diabetic retinopathy (PDR). The aim of this study was to measure erythropoietin levels in the vitreous fluid and plasma in patients with PDR, also to investigate whether vitreal EPO levels were related to the presence of systemic anaemia and to the levels of vitreal and plasma vascular endothelial growth factor (VEGF). Patients and Methods: This study included 40 eyes of diabetic patients (25 males and 15 females) with severe PDR who were undergoing vitrectomy , their ages ranged from 45-55 years, in addition 20 eyes of non diabetic subjects, their ages ranged from 47-60 years, undergoing conventionl retinal detachment (RD) surgery serving as controls. PDR group of patients was subdivided according to the presence of anaemia into: 18 anaemic diabetics (Hb<13g/dl in males and Hb<12g/dl in females) and 22 non-anaemic diabetics. All diabetic and non-diabetic subjects were subjected to complete ophthalmological examination. Fundus fluorescein angiography was done for PDR patients to assess the ischaemic status of the retina. Vitreous samples were taken during vitretomy for PDR patients , while for RD control subjects, samples were taken from evacuation of subretinal fluid of detached retina. Blood and vitreal samples were taken at the time of surgery after an overnight fast. Blood samples were collected in evacuated tubes containing EDTA and were centrifuged. Kidney function tests, urine proteins, complete lipid profile, post-prandial blood sugar , glycosylated haemoglobin, blood haemoglobin were estimated in controls and diabetic patients using Bechman autoanalyser and ion exchange chromatography. Estimation of vitreal and plasma levels of EPO (ml U/ml) and VEGF (pg/ml) were done using enzyme linked immunosorbent assays (ELISA). Results: In this work, a statistically high significant increase of vitreal EPO and VEGF levels in PDR patients compared to controls was shown, but the increase was more in vitreal EPO levels. A statistically high significant increase of plasma VEGF levels in PDR patients compared to controls was found. A statistically high significant decrease of plasma EPO levels in PDR patients compared to controls was shown. No correlations were present between plasma nor vitreal EPO and VEGF levels in PDR patients. No difference in plasma nor in intravitreal EPO concentrations between anaemic and non anaemic patients could be found. Conclusion: This study revealed that EPO is a potent ischemia- induced angiogenic factor that acts indepently on VEGF or anaemia during retinal angiogenesis in PDR. There is a clear need for further studies to elucidate the role of this important factor in diabetic retinopathy.

Key words: Ischemic retinal diseases, proliferative diabetic retinopathy PDR, Angiogenesis, Vascular endothelial growth factor VEGV, Erythropoieten EPO

INTRODUCTION

Diabetic retinopathy is characterized by increased vascular permeability, ischemia and neovscularization that often result in catastrophic loss of vision which is often considered the most serious complication of human disease, other than death.

Ischemia in the retina can induce neovascularization via induction of vascular endothelial growth factor (VEGF) expression by the transcription factor hypoxia-inducible factor-1 (Forsythe *et al,* 1996). VEGF levels are elevated in the vitreous fluid of patients with PDR and induces proliferation in vascular endothelial cells *in vitro* (Aiello *et al,* 1994).

Corresponding Author: Mostafa M. Salah, Ophthalmology Department, Research Institute of Ophthalmology-- Cairo, Egypt.
Email: mostafams65@yahoo.com Mobile:+ 0122422501

Although inhibition of VEGF reduces retinal neovascularization (Bainbridge *et al,* 2002), it does not completely inhibit ischemia-driven retinal neovascularization. Thus, other angiogenic factors seem to be involved in this process.

The glycoprotein erythropoietin (EPO), the major growth factor for erythropoiesis, stimulates the formation of red cells by enhancing both their proliferation and their differentiation and by preventing apoptotic death of erythropoietin-responsive erythroid precurscor cells (Youssoufian *et al,* 1993). Hypoxia is the major signal that stimulate EPO gene expression.It has been reported that EPO is expressed at a number of sites including kidney, liver, brain,uterus (Chikuma *et al,* 2000) and the adult mamalian retina, which also expresses EPO receptors (Grimm *et al,* 2002). The brain has a paracrine system involving EPO and EPO receptors, suggesting that EPO contributes to the survival of neurons by protecting them from ischemic damage (Morishita *et al,* 1997). Furthermore, EPO shows angiogenic activity in vascular endothelial cells, stimulating proliferation, migration and angiogenesis in vitro, probably by means of EPO receptor expressed in those cells (Yamaji *et al,* 1996). Such angiogenic activity involves several transduction cascades such as extracellular signal regulated kinase, Janus kinase2 (JAK2) and signal transducer and activator of transcription 5 (STAT5) (Fuste *et al,* 2002). Therefore, the inhibition of EPO by soluble EPO receptor abrogates angiogenesis *in vivo*.

Since EPO is an ischemia-induced paracrine factor that promats angiogenesis, so, its potential role during retinal angiogenesis in PDR should be clarified.

The aim of this study were to measure EPO level in the vitreous fluid and plasma of patients with PDR and to investigate whether vitreal EPO levels were related to the presence of systemic anaemia and to the level of vitreal and plasma VEGF.

MATERIALS AND METHODS

This study included 40 eyes of diabetic patients (25 males and 15 females) with severe PDR who were undergoing vitrectomy, their ages ranged from 45-55 years, in addition 20 eyes of non diabetic subjects, their ages ranged from 47-60 years undergoing conventional retinal detachment (RD) surgery serving as controls.

Complete ophthalmological examination was done for each subject including: V.A, slit lamp examination, IOP, and fundus fluorescein angiography (for PDR patients to assess the ischemic status of the retina).

PDR group of patients was subdivided according to the presence of anaemia into 18 anaemic diabetics (Hb <13g/dl in males and Hb <12g/dl in females) and 22 non-anaemic diabetics.

Vitreous samples were taken during vitrectomy for PDR patient, while for control patients, samples were taken from evacuation of subretinal fluid of detached retina.

- N.B.: Blood and vitreal samples were taken at the time of surgery after an overnight fast. Blood samples were collected in evacuated tubes comtaining EDTA and were centrifuged.Plasma and vitreous samples were stored at –70°C till analysis.

Routine Laboratory Investigations Were Done Including:

- Kidney function tests (Ann, 1975).
- Detection of protein in 24 hours urine was assessed qualitatively.
- Complete lipid profile: triacylglycerol (TAG) (Young *et al,* 1975), cholesterol (Fruat, 1975), low-density lipoprotein cholesterol (LDL-C) (Fruchart, 1982) and high density lipoprotein cholesterol (HDL-c) (Burstein, 1970) were assessed colorimetrically using Becman autonalyser.
- Post-prandial blood sugar was assessed enzymatically (Barham *et al,* 1972).
- Glycosylated haemoglobin (HbA_1C) was assessed by the kit provided by BIO-MIDI (Blisse and Abraham, 1985) using ion exchange chromatography.
- Blood haemoglobin (Hb) was determined by cyanmethaemoglobin method (Betke and Savelsberg, 1950).

Estimation of vitreal and plasma levels of EPO (mIU/ml) and VEGF (pg/ml) were done using enzyme linked imunosorbent assays [ELISA techniques] (Caro and Erslev, 1988; Kondo, 1994).

Exclusion Criteria Included:

- Any other major systemic disease like hypertension or any cardiac disease.
- Nephropathy or neuropathy.

- Any other ocular disease.
- History of previous inraocular surgery.

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. T-test for dependent and independent variables. Kruskal Walis ANOVA (analysis of variance) was done to compare mean ranks of different parameters for more than two groups, followed by LSD test for significance (P-value < 0.05 is considered significant). Person's correlation was done to detect association. Correlation coefficient "r" ranges from-1 to +1 (Armitage and Berry, 1989).

RESULTS AND DISCUSSION

Results:

Results of this study are illustrated in table (1):

Table 1: Comparison of EPO and VEGF in plasma and aqueous humor in the 2 studied groups

	Study groups				
	Controls (n=20)		PDR (n=40)		
	Mean	+SD Deviation	Mean	+SD Deviation	*P value
Plasma EPO (mIU/ml)	21.08	1.16	18.54	.42	<0.001
Vitreous humor EPO (mIU/ml)	37.81	1.97	472.17	23.27	<0.001
Plasma VEGF (pg/ml)	93.70	17.54	172.68	46.78	<0.001
Vitreous humor VEGF (pg/ml)	16.16	.73	237.47	36.95	<0.001

* p is significant <0.05

The Results Showed:

- A statistically high signficant increase of vitreal EPO and VEGF levels in PDR patients compared to controls (P<0.001) but the increase was more in vitreal EPO levels.
- A statistically high significant increase of plasma VEGF levels in PDR patients compared to controls (P<0.001).
- A statistically high significant decrease of plasma EPO levels in PDR patients compared to controls (P<0.001).
- No correlations were present between plasma nor vitreal EPO and VEGF levels in PDR patients.
- To examine whether EPO concentrations were related to anaemia , the PDR group of patients was subdivided according to the presence of anaemia into : 18 anaemic diabetics (Hb<13g/dl in males and Hb<12g/dl in women) and 22 no anaemic diabetics. However, there was no difference in plasma EPO concentrations between anaemic (18.26 mlU/ml $\pm$ 0.22) and non anaemic patients (18.12 mlU/ml $\pm$ 0.42) nor in intravitreal EPO concentrations between anaemic (448.6 mlU/ml $\pm$ 19.22) and nonanaemic patients (452.3 mlU/ml $\pm$ 20.27).

Discussion:

In this study, vitreal EPO concentrations were markdly higher in PDR patients than in control subjects who had no retinopathy. These results are consistent with those of Inomata *et al.,* 2004 who reported that vitreal EPO concentration are higher in PDR than in other ocular diseases. They also found, in contrast to the current observations, significant and strong correlation between EPO and VEGF concentrations in vitreous fluid. This difference may be explained by the fact that they examined all patients with all eye diseases, wheres the present study only included diabetic patients who had markedly increased intravitreous EPO levels in our analysis.

Diasuke *et al.,* 2005 have been found no correlation between the vitreous and plasma levels of EPO in PDR patients and this was in agree with the results of the current study suggesting that increased EPO levels in vitreous fluid are probably due to local production in the retina.

The presence of EPO in the vitreous is probably not due to the breakdown of the blood –brain barrier. This is probably may be due to the fact that a stimulant other than ischemia, such as high glucose levels,

oxidative stress or presence of other cytokines, may also differentially affect EPO expression. Cristina *et al.,* 2006 have been found that EPO is expressed in human retina and it is upregulated in diabetic patients even without retinopathy and these findings suggest that other factors, apart from ischemia are involved in the overexpression of EPO in PDR.

Because systemic hypoxia may aggravate diabetic retinopathy, so, the relation of EPO and anaemia was studied in this work. Anaemia induces systemic hypoxia and may increase hypoxia in retina, but it is poor prognostic factor for PDR (Ando *et al,* 1993). However, in the current study no significant difference was found between the vitreous EPO concentrations of anaemic patients and those of non anaemic patients, suggesting that vitreous EPO is not induced by systemic anaemia.

Stephen *et al.,* 2003 have been suggested that the anaemia associated with the kidney disease in PDR patients responds to supplemental synthetic EPO injection in conjunction with iron supplementation.

The role of EPO in the vitreous fluid of diabetic patients can be explained by the fact that EPO promotes the proliferation and differentiation of erythroid precursors through the induction of antiapoptotic proteins (Silva *et al,* 1996) and inhibition of apoptosis (Tilbrook and Klinken, 1999). Lioyd, 2005 reported that EPO has the same angiogenic potential on endothelial cells as VEGF. Some diabetic patients with severe renal anaemia may be treated with recombinant human EPO (rh EPO) and subsequently, EPO can induce intraocular angiogenesis as the therapeutic use of rh EPO can cross the blood-retinal barrier (Jaquet *et al,* 2002). However, one study has actually reported that EPO treatment has a favorable impact on retinopathy in diabetic subjects (Friedman *et al,* 2003), suggesting that the increased blood Hb level induced by rhEPO imporved oxygen carriage to retinal tissue and ameliorated diabetic retinopathy. Recently, EPO has been shown to be neuroprotective following ischaemic and hypoxic stress in the nervous system (Genc *et al,* 2004). EPO also protects retinal ganglion cells from ischaemia and reperfusion injury through an antiapoptotic mechanism (Junk *et al,* 2002).

Retinas of diabetic patients have significantly more apoptotic neurons than control subjects (Barber *et al,* 1998), and apoptosis plays a role in diabetic retinopathy.

Therefore, while EPO levels are elevated in the eyes of patients with PDR, there is evidence to suggest that treatment with EPO may benefit patients with retinal diseases, and therefore, further studies are needed to elucidate the role of this important factor in diabetic retinopathy.

In conclusion, this study revealed that EPO is a potent ischemia-induced angiogenic factor that acts indepently on VEGF during retinal angiogenesis in PDR.

REFERENCES

Aiello, L.P., R.L. Avery and P.S. Arrigg, *et al.* 1994. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N. Engl. Med., 331: 1480-7.

Ando, F., T. Nagasaka and K. Sasano, *et al.* 1993. Factors influencing surgical results in proliferative diabetic retinopathy. Ger. J. Ophthalmol., 2: 155-160.

Ann. Clin. Biochem., 1975. 12: 265.

Armitage, P. and G. Berry, 1989. Statistical methods in medical research. Oxford: Block-well Scientific Publications.

Bainbridge, J.W., A. Mistry and M. Alwis, *et al.* 2002. Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptors sFlt-1. Gene Ther., 9: 320-6.

Barber, A.J., E. Lieth and S.A. Khin, *et al.* 1998. Neural apoptosis in the retina during experimental and human diabetes: early onset and effect of insulin. J. Clin. Invest., 102: 783-791.

Barham, D. and P. Trinder, 1972. Analyst 97.

Betke, K. and W. Savelsberg, 1950. Stufenphotometrische haemoglobin- bestimmung mittels cyanhaemoglobin.z. Biochem, 320-431.

Blisse, E. and E. Abraham, 1985. CJ. Chromatog, 344: 81-91.

Burstein, M., 1970. Lipid Rees., 11: 583.

Caro, J. and A. Erslev, 1988. Erythropoietin assays and their use in the study of anemias. Contrib Nephrol, 66: 54-62.

Chikuma, M., S. Masuda and T. Kobayashi, *et al.* 2000. Tissue-specific regulation of erythropoietin production in the murine kidney, brain, and uterus. Am. J. Physiol. Endocrinol. Metab., 279: 1242-1248.

Cristina Hernandez, Alex Fonollosa and Marta Garcia- Ramirez, *et al.* 2006. Erythropoietin is expressed in the human retina and its is highly elevated in the vitreous fluid of patients with diabetic macular edema. Diabetes Care, 29: 2028-2033.

Diasuke Watanabe, Kiyoshi Suzuma and Shigeyuk: Matsui, *et al.*, 2005. Eryropoietin as a retinal angigenic factor in proliferative diabetic retinopathy. N. Engl. Med., 353: 782-92.

Forsythe, J.A., B.H. Jaing and N.V. Lyer, *et al.,* 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia- inducible factor, Mol. Cell. Boil., 16: 4604-4613.

Friedman, E.A., F.A. L'Esperance and C.D. Brown, *et al.,* 2003. Treating azotemia-induced anemia with erythropoietin improves diabetic eye disease. Kidney Int (suppl), 557-563.

Fruat, 1975. Ann. Clin. Biochem., 12: 265.

Fruchart, J.C., 1982. Rev. Fn. Des Laboratories, 103: 7-10.

Fuste, B., M. Serradell and G. Escolar, *et al.*, 2002. Erythropoietin triggers a signaling pathway in endothelial cells and increase the thrombogenicity of their extracellular matrices in vitro. Thromb Haemost, 88: 678-85.

Genc, S., T.F. Koroghi and K. Genck, 2004. Erythropoietin and the nervous system. Brain Res., 1000: 19-31.

Grimm, C., A. Wenzel and M. Groszer, *et al.*, 2002. HIF-1 induced erythropoietin in the hypoxic retina protects against light- induced retinal degeneration. Nat. Med., 8: 718-724.

Inomata, Y., A. Hirata and E. Takahashi, *et al.*, 2004. Elevated erythropoietin in vitreous with ischemic retinal diseases. Neuroreport, 15: 877-879.

Jaquet, K., K. Krause, M. Tawakol-Khodai, *et al.*, 2002. Erythropoietin and VEGF exhibit equal angiogenic potential. Microvasc. Res., 64: 326-333.

Junk A.K., A. Mammis, S.I. Savitz, *et al.*, 2002. Erythropoietin administration protects retinal neurons from acute ischemia reperfusion injury. Proc. Natl. Acad. Sci. USA., 99: 10659-10664.

Kondo, S., 1994. Growth factors. Biochem. Biophys. Acta., 221:211.

Lioyd Paul Aiello, 2005. Angiogenic pathways in diabetic retinopathy. NEJM, 353:839-841.

Morishita, E., S. Masuda and M. Nagao, *et al.* 1997. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience, 76: 105-16.

Silva, M., D. Grillot and A. Benito, *et al.*, 1996. Erythropoietin can promate erythroid progenitor survival by repressing apoptosis through Bcl-2. Blood., 88: 1576-1582.

Stephen, H, D. Cherie and Adera Levin, 2003. Treatment of anemia in the diabetic patient with retinopathy and kidney disease. Am. J. Ophthalmol., 135(5): 740-743.

Tilbrook, P.A. and S.P. Klinken, 1999. Erythropoietin and erythropoietin receptor. Growth Factors, 17: 25-35.

Yamaji, R., T. Okada and M. Moriya, *et al.,* 1996. Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. Eur. J. Biochem., 239: 494-500.

Young, R.J, D.F. Bum and W.G. Biggs, 1975. Clin. Chem., 25: 273.

Youssoufian, H., G. Longmore and D. Neumann, *et al.,* 1993. Structure, function, and activation of the erythropoietin receptor. Blood, 81: 2223-36.