0031-3998/08/6304-0394
PEDIATRIC RESEARCH

Vol. 63, No. 4, 2008
Printed in U.S.A.

Elevated Erythropoietin mRNA and Protein Concentrations in the Developing Human Eye

SHRENA PATEL, MICHAEL J. ROWE, SALLY A. WINTERS, AND ROBIN K. OHLS

Department of Pediatrics [S.P.], University of Utah, Salt Lake City, UT 84132; Department of Pediatrics [M.J.R., S.A.W., R.K.O.], University of New Mexico, Albuquerque, NM 87131

ABSTRACT: Erythropoietin (Epo) is an erythropoietic, neurotropic, and angiogenic factor, and may be involved in retinal development. Studies in adult diabetic retinopathy patients reveal significantly elevated vitreal Epo concentrations. It is unknown whether Epo plays a similar role in retinopathy of prematurity. We sought to determine whether Epo is present in the normally developing human eye. Fetal serum and vitreous samples were obtained from 12 to 24 wk gestation. RNA was extracted from isolated retina for Epo mRNA and hypoxia inducible factor-1α (HIF) mRNA determination by real-time polymerase chain reaction. Fetal serum was isolated from the umbilical cord. Serum and vitreous samples were analyzed for Epo protein by enzyme-linked immunosorbent serologic assay. In fetal retina, Epo mRNA increased with increasing gestational age, while HIF mRNA remained constant. Epo protein increased with increasing gestation in both vitreous and serum. At each gestational group measured (12–14, 15–17, 18–20, and 21–24 wk), Epo concentrations were significantly greater in vitreous than in serum ($p < 0.05$). Epo mRNA and protein concentrations increase with increasing gestational age and are greater in the vitreous than serum. We speculate that changes in Epo production following preterm delivery might affect retinal vascular development. **(*Pediatr Res* 63: 394–397, 2008)**

Infants born at the limits of viability are susceptible to specific morbidities. One such morbidity involves the abnormal growth and maturation of the vascular retina, termed retinopathy of prematurity (ROP). Early studies in preterm infants evaluating preventive strategies for ROP involved limitations in light exposure and the addition of a variety of nutrients to the preterm infant's diet; however, no strategy other than prevention of premature birth has been shown to be effective in preventing ROP (1–4). The severity of the disease can be attenuated by preventing hyperoxia in the first weeks of life, as well as preventing episodes of hyperoxia and hypoxia (5–7). Despite significant focus on decreasing the incidence of ROP in extremely low birth weight infants, the disease remains prevalent (1–4).

Watanabe and colleagues reported elevations in vitreal erythropoietin (Epo) concentrations in patients with proliferative diabetic retinopathy (8), a disease with possibly similar mechanisms of vascular injury and neovascularization as seen in ROP. Those authors suggested that Epo is a potent ischemia-induced retinal angiogenic factor. In that study, blockage of Epo in a mouse model of hypoxia-induced retinopathy was shown to inhibit retinal neovascularization and endothelial cell proliferation *in vivo*.

The role of Epo in the developing eye and its involvement in the development of retinal vascular disease in preterm infants has not been examined. If Epo concentrations are negligible in the developing eye, then Epo administration after preterm delivery might abnormally influence growth after delivery. We hypothesized that Epo mRNA and protein are present in the mid-gestation human fetal eye, and that Epo mRNA and protein concentrations increase with increasing gestation. Our objectives were to quantify Epo mRNA and protein in the serum and vitreous during fetal development between 12 and 24 wk gestation. In addition to Epo mRNA concentrations, we sought to quantify hypoxia inducible factor 1α (HIF) mRNA concentrations to determine whether, similar to other fetal tissues, retinal concentrations remain relatively unchanged throughout the gestations tested and could be used to normalize target mRNA data. In fetal liver and kidney, HIF can be used as an endogenous control. HIF activity is post-transcriptionally controlled through an oxygen dependent ubiquitin proteosome degradation pathway (9,10); therefore, concentrations remain stable and do not fluctuate under hypoxic conditions (11).

METHODS

A total of 52 samples were processed for measurement of Epo mRNA and protein. Not all samples had obtainable protein or tissue. Gender could not be determined on most samples (12) and that information was not collected. Samples were collected between 12 wk and 24 wk gestation, for a total of 36 matched vitreous and serum samples (8 samples from 12 to 14 wk, 10 samples from 15 to 18 wk, 10 samples from 19 to 21 wk, and 8 samples from 22 to 24 wk). The aqueous and vitreous were collected from both fetal eyes and combined for each sample. Serum was obtained from all samples when available by collecting blood still present in the umbilical cord arteries and vein. Serum and vitreous were stored at −20°C until analyzed by enzyme-linked immunosorbent serologic assay (R&D Systems, Minneapolis,[1] MN).

The retina was removed from both eyes, and total RNA was extracted from 45 samples using TriZol (Invitrogen, Carlsbad, CA). Total RNA was quantified by spectrophotometer. The RNA was reverse transcribed using the cDNA Archive kit according to manufacture's instruction (Applied Biosystems Inc. [ABI] CA). Primers and probe specific for Epo (ABI) were used to determine mRNA concentrations in all RNA samples using real time PCR (PRISM 7500 Fast Thermocycler, ABI). cDNA reverse-transcribed from total RNA was amplified over 40 cycles.

Received September 17, 2007; accepted December 4, 2007.

Correspondence: Robin K. Ohls, M.D., Professor of Pediatrics, University of New Mexico Health Sciences Center, MSC10 5590, Albuquerque, New Mexico 87131-0001; e-mail: rohls@unm.edu

Supported by a grant from the Department of Pediatrics, University of New Mexico.

Abbreviations: Epo, erythropoietin, **HIF,** hypoxia inducible factor-1α, **ROP,** retinopathy of prematurity

In addition to Epo mRNA concentrations, HIF mRNA concentrations were measured in 23 retinal samples. In previous studies, the use of common "housekeeping" RNA, such as β-actin or GAPDH, did not remain constant under hypoxic conditions (13) or over the gestations evaluated in the second trimester. We tested HIF to determine whether, similar to other fetal tissues (11), retinal concentrations remain relatively unchanged throughout the gestations tested and could be used to normalize target mRNA data.

For quantitative PCR measurements, a standard curve was created by reverse transcribing serial dilutions of total RNA isolated from Heb3B cells, an immortalized cell line that produces Epo mRNA and protein constitutively. Undiluted Hep3B total RNA was assigned a value of "100" for both Epo and HIF assays (100 ng total RNA per 20 μL RT reaction).

Differences in mRNA concentrations and in Epo concentrations at each gestational age group (plotted as mean plus or minus SE) were analyzed using an unpaired t test and ANOVA analysis. The study was deemed not to constitute human subject research by the Institutional Review Board at the University of New Mexico, as no identifiable patient data were collected.

RESULTS

Epo mRNA concentrations were measurable in all fetal retinal samples tested. Epo mRNA concentrations increased with increasing gestational age (Fig. 1; $R = 0.65$, $p < 0.05$). Similar to previous fetal tissues tested, HIF mRNA concentrations were unchanged over the range of gestational ages tested (Fig. 2; $R = 0.23$, $p =$ NS). The quantity of HIF mRNA per total RNA measured in fetal retina was similar to that seen in RNA isolated from Hep3B cells (total RNA for all samples was 100 ng/20 μL reverse transcription reaction volume).

Both serum and vitreous Epo concentrations increased with increasing gestation (Fig. 3; $p < 0.05$, 12–14 wk *vs.* 18–20 wk and 21–24 wk). Vitreous Epo concentrations ranged from 1 to 12.5 mU/mL at 12–14 wk (median 7.5 mU/mL), 5–16.6 mU/mL at 15–17 wk (10.1 mU/mL), 1–29 mU/mL at 18–20 wk (14.1 mU/mL), and 15.7–34.1 mU/mL at 21–24 wk (26.8 mU/mL). Vitreous Epo concentrations were greater than serum Epo concentrations at each gestational age group tested ($p < 0.05$, 12–14 wk and 21–24 wk; $p < 0.01$, 15–17 wk and 18–20 wk).

DISCUSSION

The role of Epo in the developing eye has not been evaluated. Moreover, the role of Epo in the development of retinal vascular disease in preterm infants has not been determined. We found increasing concentrations of both Epo mRNA and protein with increasing gestation in the human fetal eye. Similar to other fetal tissues, we measured significant but unchanging HIF mRNA concentrations with increasing gestation. The finding of increasing Epo protein concentrations with increasing gestation, and importantly, Epo concentrations that exceeded those found in serum at each gestation tested suggests a role for Epo in human retinal development. In animal models, exposure to low levels of oxygen, deemed "hypoxic conditioning," results in up-regulation of Epo and its receptor (14). The increase in Epo concentration is thought to play a role in protecting against retinal damage, primarily by inhibiting gene expression for enzymes involved in apoptosis. In addition, neuroprotection of retinal photoreceptors can be induced by hypoxic preconditioning or by increasing systemic Epo concentrations (15).

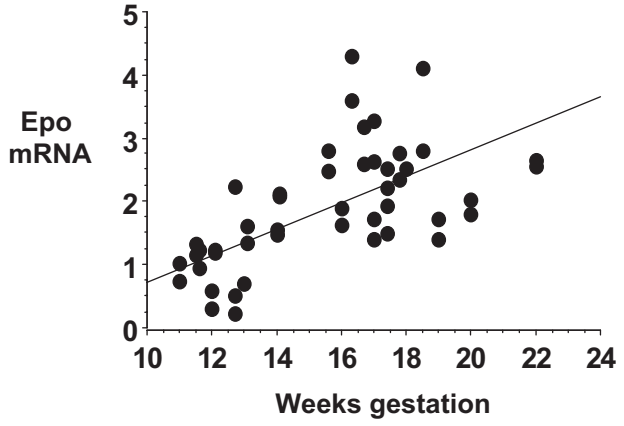

Figure 1. Retinal Epo mRNA concentrations in the developing human eye. Epo mRNA concentrations (45 samples measured) increase with increasing gestational age ($r = 0.65$, $p < 0.05$). Values are presented as retinal Epo mRNA concentration/Hep3B Epo mRNA concentrations per total RNA loaded.

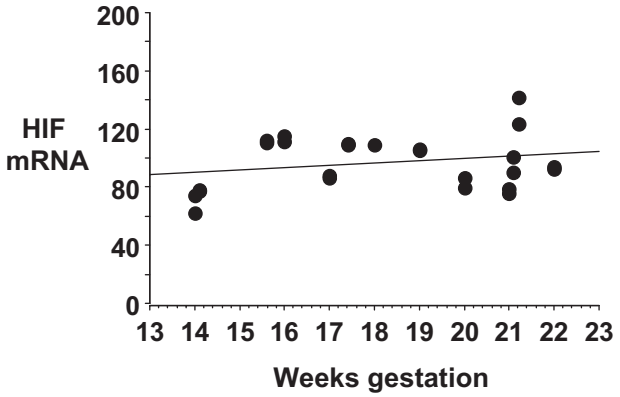

Figure 2. Retinal HIF-1α mRNA concentrations in the developing human eye. HIF mRNA concentrations (23 samples measured) were similar over increasing gestational ages ($r = 0.23$, $p =$ NS). Values are presented as retinal HIF mRNA concentration/Hep3B HIF mRNA concentrations per total RNA loaded.

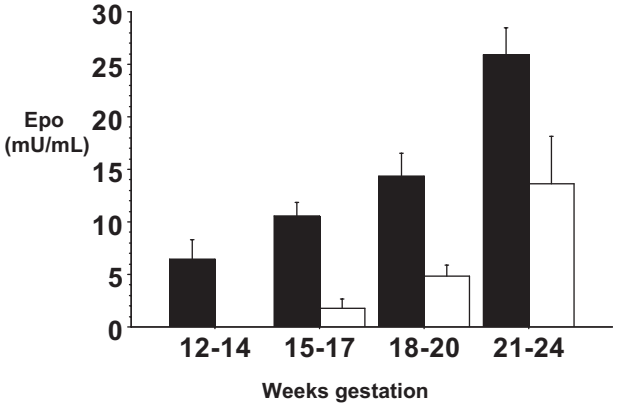

Figure 3. Serum and vitreous Epo concentrations. A total of 36 samples were measured for serum and vitreous Epo concentrations: 8 samples from 12–14 wk, 10 samples from 15–18 wk, 10 samples from 19–21 wk, and 8 samples from 22–24 wk. Values shown are mean ± SEM. Both serum (clear bars) and vitreous (solid bars) Epo concentrations increased with increasing gestation ($p < 0.05$, 12–14 wk *vs.* 18–20 wk and 21–24 wk). Vitreous Epo concentrations were greater than serum Epo concentrations at each gestational age group tested ($p < 0.05$, 12–14 wk and 21–24 wk; $p < 0.01$, 15–17 wk and 18–20 wk).

Watanabe and colleagues reported significant elevations in vascular endothelial growth factor (VEGF) and Epo in the vitreous of patients with proliferative diabetic retinopathy, although regression analysis showed that Epo was more strongly associated with active disease than VEGF (8). Those authors suggested that Epo is a potent ischemia-induced retinal angiogenic factor, because blockage of Epo is shown to inhibit retinal neovascularization and endothelial cell proliferation *in vivo*. Other authors have described Epo as an endogenous retinal survival factor (15–17).

Research has demonstrated that in addition to hematopoietic properties, Epo plays a developmental role in angiogenesis (18,19). Epo receptors are found on endothelial cells, neuronal cells, and accessory cells throughout the developing fetus. In response to Epo administration, angiogenic events such as endothelial cell proliferation, organization, and chemotaxis are demonstrated *in vitro* and *in vivo*. Epo is also shown to promote neurogenesis and has neuroprotective properties as well. Epo is found in the cerebral spinal fluid of both animals and humans, both neonatal and adult (20). Epo receptor expression has been demonstrated in most cerebral cell types, including endothelial cells, neurons, and astrocytes (20–22). Epo and Epo receptor expression is elevated during early development, but decreases up to 100-fold after birth (22,23). Epo receptor deficient mice have an increased rate of cerebral apoptosis, as well as areas of cerebral hypoplasia (24).

Similar to adult renal and fetal hepatic cells, neuronal cells have increased Epo gene expression in response to hypoxia (25). This pathway is mediated primarily by hypoxically regulated vascular growth factors (26). A variety of insults, including cerebral ischemia, seizures, and head injury result in increased growth factors. In response, the Epo/Epo receptor system is up-regulated, ultimately promoting cell viability (25–27). This neuroprotection is a result of repressing apoptosis and blocking nitric oxide-meditated toxicity (28,29). Animal studies have demonstrated dose-dependent effects, including reduced infarct volume, increased blood flow, and improvement in long-term outcome/relearning (14,30,31).

Although numerous randomized placebo-controlled studies have not reported significant differences in the incidence of ROP between Epo treated and placebo infants, retrospective reports have postulated that use of Epo may be associated with increased rates of ROP (32–35). Brown and colleagues reported an association between cumulative doses of Epo and risk for ROP (33); however, the authors were unable to separate the relationship among extreme prematurity, Epo doses, and ROP. A recent Cochrane review (36) speculated about a relationship between Epo and ROP, primarily due to the inclusion of a study, summarized in a letter to the editor, of early Epo and iron administered to preterm infants (32). Moreover, although numerous studies have reported an increased risk of ROP in relation to blood transfusions (37,38), no studies have reported Epo administration as a factor increasing the risk of ROP (2–4).

The relationship between exogenous Epo and ROP remains speculative, but should be monitored closely in future Epo studies. In addition, the relationship between endogenous Epo and ROP remains to be determined. It is possible that elevated vitreal Epo concentrations *in utero*, followed by decreased Epo concentrations after extremely preterm delivery create an abnormal cycle of vascular stimulus and suppression that eventually leads to ROP. We speculate that treatment of preterm infants with exogenous Epo may influence vitreal Epo concentrations; however, the timing of Epo treatment remains to be determined. We conclude that Epo is involved in human retinal vascular development and continue to evaluate the role of Epo in the developing human eye.

Acknowledgments. The authors thank Erik Leve and Mabel Padilla for technical assistance.

REFERENCES

1. Sola A, Chow L, Rogido M 2005 Retinopathy of prematurity and oxygen therapy: a changing relationship. An Pediatr (Barc) 62:48–61
2. Karna P, Muttineni J, Angell L, Karmaus W 2005 Retinopathy of prematurity and risk factors: a prospective cohort study. BMC Pediatr 5:18–25
3. Liu PM, Fang PC, Huang CB, Kou HK, Chung MY, Yang YH, Chung CH 2005 Risk factors of retinopathy of prematurity in premature infants weighing less than 1600 g. Am J Perinatol 22:115–120
4. Akkoyun I, Oto S, Yilmaz G, Gurakan B, Tarcan A, Anuk D, Akgun S, Akova YA 2006 Risk factors in the development of mild and severe retinopathy of prematurity. J AAPOS 10:449–453
5. Flynn JT, Bacalari E 2000 On "supplemental therapeutic oxygen for prethreshold retinopathy of prematurity (STOP-ROP), a randomized, controlled trial. I: Primary outcomes." J AAPOS 4:65–66
6. Chow LC, Wright KW, Sola A 2003 Can changes in clinical practice decrease the incidence of severe retinopathy of prematurity in very low birth weight infants? Pediatrics 111:339–345
7. Flynn JT, Chan-Ling T 2006 Retinopathy of prematurity: two distinct mechanisms that underlie zone 1 and zone 2 disease. Am J Ophthalmol 142:46–59
8. Watanabe D, Kiyoshi S, Matsui S, Kurimoto M, Kiryu J, Kita M, Suzuma I, Ohashi H, Ojima T, Murakami T, Kobayashi T, Masuda S, Nagao M, Yoshimura N, Takagi H 2005 Erythropoietin as a retinal angiogenic factor in proliferative diabetic retinopathy. N Engl J Med 353:782–792
9. Wang GL, Semenza GL 1993 General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci USA 90:4304–4308
10. Bunn HF, Poyton RO 1996 Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev 76:839–886
11. Ohls RK 2002 Erythropoietin and hypoxia inducible factor-1 expression in the mid-trimester human fetus. Acta Paediatr Suppl 91:27–30
12. Wen TC, Rogido M, Peng H, Genetta T, Moore J, Sola A 2006 Gender differences in long-term beneficial effects of erythropoietin given after neonatal stroke in postnatal day-7 rats. Neuroscience 139:803–811
13. Zhong H, Simons JW 1999 Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 259:523–526
14. Siren A-L, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, Keenan S, Gleiter C, Pasquali C, Capobianco A, Mennini T, Heumann R, Cerami A, Ehrenreich H, Ghezzi P 2001 Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci USA 98:4044–4049
15. Grimm C, Wenzel A, Acar N, Keller S, Seeliger M, Gassmann M 2006 Hypoxic preconditioning and erythropoietin protect retinal neurons from degeneration. Adv Exp Med Biol 588:119–131
16. Becerra SP, Amaral J 2002 Erythropoietin – an endogenous retinal survival factor. N Engl J Med 347:1968–1970
17. Fisher JW 2003 Erythropoietin: physiology and pharmacology update. Exp Biol Med (Maywood) 228:1–14
18. Carlini RG, Reyes AA, Rothstein M 1995 Recombinant human erythropoietin stimulates angiogenesis in vitro. Kidney Int 47:740–745
19. Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L, Dammacco F 1999 Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Blood 93:2627–2636
20. Juul SE, Anderson DK, Li Y, Christensen RD 1998 Erythropoietin and erythropoietin receptor in the developing human central nervous system. Pediatr Res 43:40–49
21. Shingo T, Sorokan ST, Shimazaki T, Weiss S 2001 Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. J Neurosci 21:9733–9743
22. Dame C, Bartmann P, Wolber E, Fahnenstich H, Hofmann D, Fandrey J 2000 Erythropoietin gene expression in different areas of the developing human central nervous system. Brain Res Dev Brain Res 125:69–74
23. Sola A, Wen TC, Hamrick SE, Ferriero DM 2005 Potential for protection and repair following injury to the developing brain: a role for erythropoietin? Pediatr Res 57:110R–117R

24. Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B, Lin CS, Nikodem VM, Hempstead B, Flanders KC, Costantini F, Noguchi CT 2002 Erythropoietin receptor signaling is required for normal brain development. Development 129:505–516
25. Sirén AL, Knerlich F, Poser W, Gleiter CH, Brück W, Ehrenreich H 2001 Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. Acta Neuropathol 101:271–276
26. Wenger RH 2000 Mammalian Oxygen sensing, signalling and gene regulation. J Exp Biol 203:1253–1263
27. Juul S 2002 Erythropoietin in the central nervous system, and its use to prevent hypoxic-ischemic brain damage. Acta Paediatr Suppl 91:36–42
28. Wen TC, Sadamoto Y, Tanaka J, Zhu PX, Nakata K, Ma YJ, Hata R, Sakanaka M 2002 Erythropoietin protects neurons against chemical hypoxia and cerebral ischemic injury by up-regulating Bcl-xL expression. J Neurosci Res 67:795–803
29. Erbayraktar S, Grasso G, Sfacteria A, Xie QW, Coleman T, Kreilgaard M, Torup L, Sager T, Erbayraktar Z, Gokmen N, Yilmaz O, Ghezzi P, Villa P, Fratelli M, Casagrande S, Leist M, Helboe L, Gerwein J, Christensen S, Geist MA, Pedersen LO, Cerami-Hand C, Wuerth JP, Cerami A, Brines M 2003 Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. Proc Natl Acad Sci USA 100:6741–6746
30. Grasso G, Buemi M, Alafaci C, Sfacteria A, Passalacqua M, Sturiale A, Calapai G, De Vico G, Piedimonte G, Salpietro FM, Tomasello F 2002 Beneficial effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage. Proc Natl Acad Sci USA 99:5627–5631
31. Sola A, Rogido M, Lee B, Genetta T, Wen TC 2005 Erythropoietin after focal cerebral ischemia activates the Jak-Stat signaling pathway and improves brain injury in postnatal day-7 rats. Pediatr Res 57:481–487
32. Romagnoli C, Zecca E, Gallini F, Girlando P, Zuppa AA 2000 Do recombinant human erythropoietin and iron supplementation increase the risk of retinopathy of prematurity? Letter to the Editor. Eur J Pediatr 159:627–628
33. Brown MS, Baron AE, France EK, Hamman RF 2006 Association between higher cumulative doses of recombinant erythropoietin and risk of retinopathy of prematurity. J AAPOS 10:143–149
34. Shah NC, Kim MR, Jadav P, Cohen LM, Jean Baptiste D, Weedon J 2005 Effect of recombinant human erythropoietin (rhEPO) on the development of retinopathy of prematurity. Pediatr Res 57:546
35. Manzoni P, Maestri A, Gomirato G, Takagi H, Watanabe D, Matsui S 2005 Erythropoietin as a retinal angiogenic factor. N Engl J Med 353:2190–2191
36. Ohlsson A, Aher SM 2006 Early erythropoietin for preventing red blood cell transfusion in preterm and/or low birth weight infants. Cochrane Database Syst Rev 3:CD004863
37. Hirano K, Morinobu T, Kim H, Hiroi M, Ban R, Ogawa S, Ogihara H, Tamai H, Ogihara T 2001 Blood transfusion increases radical promoting non-transferrin bound iron in preterm infants. Arch Dis Child Fetal Neonatal Ed 84:F188–F193
38. Dani C, Reali MF, Bertini G, Martelli E, Pezzati M, Rubaltelli FF 2001 The role of blood transfusions and iron intake on retinopathy of prematurity. Early Hum Dev 62:57–63