Ontogeny of VEGF, IGF-I, and GH in Neonatal Rat Serum, Vitreous Fluid, and Retina from Birth to Weaning

Houchang D. Modanlou,1 Zabra Gharraee,1,2 Jamal Hasan,1,2 Joshua Waltzman,3 Stephen Nageotte,3 and Kay D. A. Beharry1,2

PURPOSE. Vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-I, and growth hormone (GH) are major regulators of physical growth, as well as normal and pathologic retinal development. Ocular tissues are protected by the blood–ocular barrier. This study was conducted to test the hypothesis that the ontogenetic profiles of VEGF, IGF-I, and GH in the rat serum, vitreous fluid, and retina are compartment specific, and that the vitreous is a reservoir for retinal growth factors.

METHODS. Sprague-Dawley rat pups were killed at birth (postnatal day [P]0) and at P7, P14, and P21. At death, serum, vitreous fluid, and retinal homogenates were analyzed for ontogeny of VEGF, IGF-I, and GH.

RESULTS. VEGF levels were 10 times higher in the vitreous than in serum at all stages of development. Vitreous and serum VEGF levels progressively declined, with lowest concentrations at P21. Retinal VEGF levels increased with the highest concentration at P21. IGF-I levels in the vitreous decreased from P7 through P21. IGF-I levels in serum and retina homogenates increased with advancing postnatal age. Although IGF-I levels were four times higher in the vitreous than in the retina at P0, equilibration was achieved at P21. GH levels in the vitreous were 10 times lower than serum levels, were decreased at P14 and P21, and remained unchanged from P0 through P21 in the retina.

CONCLUSIONS. VEGF and IGF-I act in concert to promote retinal development with the vitreous fluid as a reservoir. The ontogenetic profiles of VEGF, IGF-I and GH in the serum and ocular compartments are specific. These differences should be considered when therapies for ROP are proposed. (Invest Ophthalmol Vis Sci. 2006;47:738–744) DOI:10.1167/iovs.05-1046

Retinopathy of prematurity (ROP) is a significant long-term morbidity among surviving preterm infants with birth weight less than 1250 g.1 Currently, ROP remains a nonpreventable disorder associated with extreme prematurity.2 Although many associated co-morbidities and neonatal therapeutic interventions have been reported to be associated with the occurrence of severe ROP, extreme prematurity and exposure to relative hyperoxia are the main predisposing risk factors.3,4 A recent observational cohort study has shown that a strict use of oxygen and avoidance of hyperoxia can minimize the occurrence of severe ROP and need for opthalmic intervention.5

ROP is a retinal vascular disease. There are two phases in its pathogenesis. In extremely preterm infants with incomplete retinal vascular development at birth, the progression of normal retinal vascular development is arrested, with retinal exposure to hyperoxia that results in vasobilitation (phase I). With recovery, in metabolically active but hypoxic retina, neovascularization occurs, leading to retinal detachment in severe cases (phase II). Low serum insulin-like growth factor (IGF)-I present in very preterm infants and suppression of vascular endothelial growth factor (VEGF) by retinal hyperoxia appear to be present during phase I of ROP, whereas in phase II, upregulation of VEGF during recovery results in excessive growth of retinal vessels, or neovascularization.6

Recently, some studies have reported that high levels of serum growth hormone (GH) at 1 month of age7 and low serum IGF-I concentrations in preterm infants8 were associated with the development of ROP. Because of the existence of the blood–retinal barrier, we suspect that serum growth factors may not represent growth factors present in ocular compartments of vitreous fluid and retina during normal retinal development. We therefore hypothesized that there are compartment-specific differences in the ontogenetic pattern of growth factors such as GH, IGF-I, and VEGF in the serum, vitreous fluid, and retinal tissue. To test our hypothesis, we investigated the ontogeny of these growth factors in the serum, vitreous fluid, and the retina of developing neonatal rats.

MATERIALS AND METHODS

All experiments were approved by the Memorial Health Services Institutional Animal Care and Use Committee (Long Beach, CA). Animals were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were treated humanely, according to the guidelines outlined by the U.S. Department of Agriculture (USDA), and the Guide for the Care and Use of Laboratory Animals (National Research Council). Euthanasia was conducted according to the guidelines of the American Veterinary Medical Association.

Experimental Design

Certified infection-free, timed-pregnant Sprague-Dawley rats carrying fetuses (10–14) of known gestational age (19 days) were purchased from Charles River Laboratories (Wilmington, MA). The pregnant rats were managed according to the ARVO Statement for the Use of Animals (National Research Council). Animals were handled according to the guidelines for the care and use of laboratory animals (National Research Council). Euthanasia was conducted according to the guidelines of the American Veterinary Medical Association.

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Corresponding author: Houchang D. Modanlou, University of California Irvine Medical Center, 101 The City Drive South, Route 81, Building 56, Suite 600, Orange, CA 92868; modanlou@uci.edu.
were placed in USDA-approved nesting cages and remained undis-
turbed until delivery (22 days’ gestation). Rats from five to six litters were pooled to eliminate litter differences and to equalize the number of runs in each group, within 24 hours of delivery. After pooling, the newborn rats were weighed, measured for linear growth, and randomly assigned to a dam (15 pups/litter) where they remained undis-
turbed until postnatal day (P)7, P14, and P21. The remaining rats were killed by decapitation at term. The total number of rat pups per age group was 30. At P7, P14, and P21, the rats were weighed, measured for linear growth, and killed by decapitation. Blood was collected in sterile tubes (Eppendorf, Fremont, CA) and placed on ice before processing. Because of the small volume at term and P7, blood from the term pups (n = 30) was pooled for a total of six serum samples and blood from the P7 rat pups (n = 30) was pooled for a total of 15 serum samples. Blood from P14 and P21 rat pups was not pooled and indi-
vividually analyzed.

Immediately after death, both eyes from each pup were enucleated and placed in ice-cold phosphate-buffered saline (PBS; pH 7.4). Enu-
culation was performed with the use of iris forceps and scissors for separation of the eyes from the surrounding connective tissue, nerve, and muscles. The eyes were dried on sterile gauze, and the vitreous fluid was aspirated with a 0.5-mL insulin syringe and placed on ice in sterile tubes (Eppendorf). For sufficient volume, vitreous fluid was pooled for a total of 6 samples for the term, P7, and P14 groups and 10 samples for the P21 group. After removal of the vitreous fluid, the corneas were removed and the eyecups were placed in ice-cold PBS (pH 7.4). The retinas were excised under a dissecting microscope and placed in a sterile tube (Eppendorf) containing ice-cold PBS on ice, before homogenization. Retinas from both eyes of five term and P7 pups was pooled for a total of 6 samples, and retinas from both eyes of three pups at P14 and P21 was pooled for a total of 10 samples. Retinas were homogenized in 0.5 mL ice-cold PBS on ice (Polytron homoge-
nizer; Brinkman Instruments, Westbury, NY). The samples were cen-
trifuged at 3000 rpm at 4°C, and a portion of the supernatant was removed for determination of total cellular protein levels. The remain-
ing supernatant was further centrifuged at 10,000 rpm for 20 minutes at 4°C, filtered, and frozen at −80°C until assayed. Retinal homoge-
nates were pooled for a total of 6 samples for the term and P7 groups and 10 samples for the P14 and P21 groups.

Assay for VEGF

VEGF levels in serum, undiluted vitreous fluid and retinal homogenates were assayed using commercially available sandwich immunoassay kits (R&D Systems, Minneapolis, MN). The assay predominantly binds the monomeric VEGF165, but also detects the VEGF121, isoform. The assay recognizes the 164-amino acid splice variant of mouse VEGF and has a 98% affinity to the rat sequence. The assay uses a monoclonal anti-
VEGF detection antibody conjugated to horseradish peroxidase and color development with tetramethylbenzidine/hydrogen peroxide (TMB solution). All assays were performed according to the manufac-
turer’s protocol. Samples were assayed in duplicate. VEGF levels in the sample were determined from a linear standard curve ranging from 0 to 2000 pg/mL. The coefficient of variation from inter- and intra-assay precision assessment was less than 10%. VEGF levels in the retinal homogenates were standardized using total cellular protein levels.

Assay of GH

GH levels in serum, undiluted vitreous fluid, and retinal homogenates were determined using active ultrasensitive GH enzyme immunoassay kits (Diagnostic Systems Laboratories, Webster, TX). The sensitivities of the GH assay was 0.66 pg/mL, and the intra- and interassay coeffi-
cient of variations were less than 10%. Samples were assayed in dupli-
cate. GH levels in the retinal homogenates were standardized using total cellular protein levels.

Assay of IGF-I

IGF-I levels in serum, undiluted vitreous fluid, and retinal homogenates were determined using a commercially available, nonextraction, en-
zyme immunoassay (EIA) kit (Diagnostic Systems Laboratories). This kit provides a highly sensitive antibody method that allows detection of extremely low levels of immunoreactive free IGF-I. The assay measures the true free IGF-I fraction plus the fraction readily dissociated from IGF binding proteins (IGFBPs) which together form the biologically active pool. Samples, standards, and control specimens were incubated with a free IGF-I antibody in microtitration wells. After incubation and washing, the wells were treated with an anti-free IGF-I detection antibody labeled with horseradish peroxidase. After it was washed, the plate was developed and the absorbance measured at 450 nm. The absorbance is measured is directly proportional to the free IGF-I levels. The sensitivity of the assay was 0.015 ng/mL and intra- and interassay coefficients of variations were <10%. Samples were assayed in dupli-
cate. IGF-I levels in the retinal homogenates were standardized using total cellular protein levels.

Total Cellular Protein Assay

On the day of the assay, retinal samples were homogenized and centrifuged as previously described, and a portion of the supernatant was removed for total cellular protein determinations. Total cellular protein was determined by the dye-binding protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The standard curve was linear from 0.05 to 1.45 mg/mL of protein.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine differ-
ences among the age groups for normally distributed data, and the Kruskal-Wallis test was used for non-normally distributed data. Post hoc analysis was performed using the Student-Newman-Keuls test for significance. Linear regression analysis was used to determine relation-
ships between mean variables. Significance was set at P < 0.05, and data are reported as the mean ± SEM, where applicable. All analyses were two-tailed and performed on computer (Prism; GraphPad Soft-
ware Inc., San Diego, CA).

RESULTS

Pooling and randomization resulted in an equivalent number of runs in each group at term (mean body weight: 6.5 ± 0.13 g; mean linear growth: 6.14 ± 0.07 cm). There were no signifi-
cant differences in mean maternal weights or litter sizes before pooling.

Ontogeny of VEGF

The ontogenic patterns of VEGF in the serum, vitreous fluid, and retina are presented in Figure 1. In the rat serum, VEGF levels (in picograms per milliliter) were high at birth (234.8 ± 26.9) and progressively declined at P7 (141.6 ± 8.0, P < 0.01 vs. P0), P14 (56.1 ± 16.7, P < 0.001 vs. P0), and P21 (20.8 ± 6.4, P < 0.001 vs. P0). A similar ontogenic pattern is noted in the vitreous fluid, although the levels were 10 times higher than that measured in the serum. Rat vitreous fluid VEGF levels (pg/mL) at term and P7 were comparable, but declined at P14 (1535.8 ± 204, P < 0.01 vs. P0) and P21 (895.7 ± 117.6, P < 0.001 vs. P0). A quite different ontogenic pattern was noted for retinal VEGF levels, which increased with advancing postnatal age. Despite the low levels of retinal VEGF (in picograms per milligram protein) measured at birth (177.2 ± 19.6) compared with that in the vitreous, retinal, and vitreous fluid VEGF levels approached equilibrium at P21 (461.0 ± 66.8, P < 0.05).

Ontogeny of IGF-I

Rat serum IGF-I levels (in nanograms per milliliter) increased with advancing postnatal age and was significantly higher at P14 (880.8 ± 40.7, P < 0.05) and P21 (912.9 ± 47.5, P < 0.05) compared with P0 (595.5 ± 46.8). In contrast, a progressive decline in rat vitreous fluid IGF-I levels (in nanograms per
Ontogeny of VEGF in the rat serum, vitreous fluid, and retina at birth (P0), P7, P14, and weaning from the dam (P21). VEGF levels declined in the serum and vitreous fluid, but tended to increase in the retina with advancing postnatal age. Blood from the term rat pups \((n = 30)\) was pooled for a total of 6 serum samples and from the P7 rat pups for 15 samples. Blood from the P14 and P21 rat pups \((n = 30 \text{ each})\) was not pooled. Vitreous fluid from the P0, P7, and P14 rat pups was pooled for 6 samples each and from the P21 rat pups for 10 samples. Retinas from both eyes of the P0 and P7 rats were pooled for 6 samples each and from the P14 and P21 rat pups for 10 samples each. Data are presented as the mean ± SEM.

**FIGURE 1.** Ontogeny of VEGF in the rat serum, vitreous fluid, and retina at birth (P0), P7, P14, and weaning from the dam (P21). VEGF levels declined in the serum and vitreous fluid, but tended to increase in the retina with advancing postnatal age. Blood from the term rat pups \((n = 30)\) was pooled for a total of 6 serum samples and from the P7 rat pups for 15 samples. Blood from the P14 and P21 rat pups \((n = 30 \text{ each})\) was not pooled. Vitreous fluid from the P0, P7, and P14 rat pups was pooled for 6 samples each and from the P21 rat pups for 10 samples. Retinas from both eyes of the P0 and P7 rats were pooled for 6 samples each and from the P14 and P21 rat pups for 10 samples each. Data are presented as the mean ± SEM.

Graphs showing mean VEGF levels for serum, vitreous fluid, and retina at different postnatal ages (P0, P7, P14, P21). The figures illustrate the decline in VEGF levels in the serum and vitreous fluid and the increase in the retina with increasing age. The statistical significance is indicated by asterisks: "p<0.01" and "p<0.001 vs Term".
Figure 2. Ontogeny of IGF-I in the rat serum, vitreous fluid, and retina at birth (P0), P7, P14, and weaning from the dam (P21). Serum IGF-I levels increased with advancing postnatal age, whereas vitreous fluid IGF-I declined. Retinal IGF-I levels tended to increase with postnatal age. Blood pooling in the study groups was as described in Figure 1. Data are presented as the mean ± SEM.

Figure 3. Ontogeny of GH in the rat serum, vitreous fluid, and retina at birth (P0), P7, P14, and weaning from the dam (P21). Serum and vitreous fluid GH levels decreased with advancing postnatal age, whereas retinal GH levels remained unchanged. Blood pooling in the study groups was as described in Figure 1. Data are presented as the mean ± SEM.
ciencies, regardless of whether they were treated with GH, had a significantly lower number of vascular branching points than the reference group. The effects of GH are mediated in large part through IGF-I. IGF-I is critical for normal retinal vascular development in mice and in humans. Patients with defects in the IGF-I or IGF-I receptor gene were found to have a reduced number of retinal vascular branching points. IGF-I and its receptor are expressed constitutively by human retinal endothelial cells, as well as by retinal pigment epithelial cells, but the fetal source of IGF-I is mostly the placenta and perhaps ingested amniotic fluid. Preterm birth is associated with a rapid decrease in serum IGF-I levels as maternal sources of IGF-I are lost. This is particularly true at postmenstrual ages corresponding to the third trimester, because IGF-I levels in the fetus rise rapidly during the third trimester of pregnancy, in conjunction with the development of fetal tissues. Both GH and IGFs of placental and maternal tissues play an important role in fetal growth and retinal vascular development. High GH and low IGF-I values in serum have been noted in preterm infants. Indeed, newborns show a state of GH resistance, characterized by GH hypersecretion and a low IGF-I level.

VEGF, an endothelial-cell-specific angiogenic and vasopermeable factor, is essential in retinal vascular development in normal and pathologic neovascularization. It is produced by retinal pericytes, retinal endothelial cells, and retinal pigment epithelial cells. Its expression is oxygen-regulated. Both retinal pigment epithelium and retinal glial cells, including Müller cells, release VEGF in response to hypoxic conditions. Human and animal studies have shown that oxygen is involved in retinal neovascularization. High levels of supplemental oxygen suppress the expression of VEGF in the immature retina of the preterm infant during phase I of ROP. IGF-I is necessary for activation of VEGF independent of oxygen. Hellstrom et al. showed that low IGF-I present in extremely low birth weight infants further diminishes VEGF activation resulting in arrested blood vessel formation in stage I of ROP. IGF-I has been shown to act synergistically with VEGF to increase angiogenesis. IGF-I and VEGF are complementary for endothelial cell function through the mitogen-activated protein kinase and Akt signal-transduction pathway, as minimal IGF-I is required for VEGF signaling. As our study showed, retinal IGF-I increased with advancing age similar to retinal VEGF protein. This finding provides further evidence that IGF-I is necessary for VEGF effects on retinal vascular maturation.

As we suspected, the ontogeny of these three growth factors in the developing newborn rat pups showed a compartment-specific pattern. Random serum GH levels showed a steady and significant decrease from birth to 21 days of postnatal life. A similar trend for GH was also noted in the vitreous fluid, whereas there was no change in the GH levels in the retinal tissue during the same period. There was a steady increase in serum IGF-I levels from birth to 21 days of age with statistically significant values by 14 and 21 days of postnatal life. A reverse trend in IGF-I levels was noted in the vitreous fluid. Although not statistically significant, IGF-I levels in the retinal tissue increased gradually with advancing age. Serum and vitreous fluid VEGF levels showed a significant decrease from birth to 21 days of postnatal life, whereas its levels in the retinal tissue increased gradually to reach significance by 21 days of postnatal life. These findings imply that the vitreous compartment is a reservoir for retinal IGF-I and VEGF during the postnatal retinal vascular development in newborn rat pups. Furthermore, significantly elevated levels of VEGF from P14 to P21 may reflect a more rapid vessel growth and maturation resulting in increased density of retinal vessels during this period. These findings support Arnold et al. who have suggested that the aqueous and vitreous humor fluids are
likely to play important roles in IGF transport and reservoir functions and are potential sites of synthesis of IGF-binding proteins within the various ocular tissues.

When the serum, vitreous fluid, and retinal tissue GH, IGF-I, and VEGF are correlated, the findings reveal that there was no correlation between random serum, vitreous fluid and retinal GH levels. Serum IGF-I did not correlate with retinal IGF-I, nor was there a correlation between vitreous fluid and retinal IGF-I level (data not shown). However, there was a negative correlation between serum and vitreous fluid IGF-I levels. Similar analysis showed a positive correlation only between serum and vitreous fluid VEGF levels. The latter finding is different from the findings of Burgos et al.46 in diabetic retinopathy showing no relationship between serum and vitreous fluid VEGF levels. The reason for this difference is most likely due to the fact that our study involved serial measurements during early development of normal rats.

In summary, in our study GH, IGF-I, and VEGF in the systemic circulation, vitreous fluid, and retina exhibited compartment-specific differences. Decreasing vitreous fluid IGF-I and VEGF during the course of postnatal retinal development in rats may imply that the vitreous fluid is a reservoir for IGF-I and VEGF. As previously reported, high random serum GH levels at birth may reflect low GH receptors. Although its levels were low, GH was detected in the vitreal fluid and retina. However, its role in normal retinal vascular development is uncertain at present. We suggest that compartmental differences in growth factors should be considered in conditions associated with retinal neovascularization, such as ROP.

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References


