Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: Direct correlation with clinical retinopathy of prematurity

Ann Hellstrom*†, Carole Perruzzi‡, Meihua Ju§, Eva Engström†, Anna-Lena Hård*, Jun-Li Liu¶, Kerstin Albertsson-Wikland†, Björn Carlsson‖, Aimon Niklasson†, Lena Sjödell*, Derek LeRoith¶, Donald R. Senger‡, and Lois E. H. Smith§**

*Department of Clinical Neuroscience, Section of Ophthalmology, and †International Pediatric Growth Research Center, Department of Pediatrics, The Queen Silvia Children's Hospital, 41685 Göteborg, Sweden; ‡Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA 02215; §Department of Ophthalmology, Children's Hospital, Harvard Medical School, Boston, MA 02115; ¶Endocrine Branch, National Institutes of Health, Bethesda, MD 20892; and ‖Research Centre for Endocrinology and Metabolism, Department of Internal Medicine, University of Göteborg, 41345 Göteborg, Sweden

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Retinopathy of prematurity is a blinding disease, initiated by lack of retinal vascular growth after premature birth. We show that lack of insulin-like growth factor I (IGF-I) in knockout mice prevents normal retinal vascular growth, despite the presence of vascular endothelial growth factor, important to vessel development. *In vitro*, low levels of IGF-I prevent vascular endothelial growth factor-induced activation of protein kinase B (Akt), a kinase critical for endothelial cell survival. Our results from studies in premature infants suggest that if the IGF-I level is sufficient after birth, normal vessel development occurs and retinopathy of prematurity does not develop. When IGF-I is persistently low, vessels cease to grow, maturing avascular retina becomes hypoxic and vascular endothelial growth factor accumulates in the vitreous. As IGF-I increases to a critical level, retinal neovascularization is triggered. These data indicate that serum IGF-I levels in premature infants can predict which infants will develop retinopathy of prematurity and further suggests that early restoration of IGF-I in premature infants to normal levels could prevent this disease.

Retinopathy of prematurity (ROP) is a major cause of blindness in children in the developed world (1). Current treatment results in destruction of retina and is only partially effective at reducing blindness.

ROP occurs in two phases. When infants are born prematurely, the retina is incompletely vascularized. In infants who develop ROP, growth of vessels slows or ceases at birth (2, 3), leaving maturing but avascular and, therefore, hypoxic peripheral retina. This is the first phase of ROP. The extent of nonperfusion of the retina in the initial phase of ROP appears to determine the subsequent degree of neovascularization, the late destructive stage of ROP, with the attendant risk of retinal detachment and blindness (4). If it were possible to allow blood vessels to grow normally in all premature infants, as they do *in utero*, the second damaging neovascular phase of ROP would not occur.

When ROP first was described in 1942, the etiology was unknown. However, the liberal use of high supplemental oxygen in premature infants soon was associated with the disease and hyperoxia was shown to induce ROP-like retinopathy in neonatal animals with incompletely vascularized retinas. This suggested that an oxygen-regulated factor was involved. Expression of vascular endothelial growth factor (VEGF) is oxygen-regulated and was found to be important for both phases of ROP (5–9). VEGF is necessary for normal vascular development (7–10). High supplemental oxygen affects the first phase of vascular growth in ROP animal models through suppression of VEGF expression (7). However, with current careful use of moderate oxygen supplementation, the oxygen level in patients is not a significant risk factor for ROP (11–13), yet, the disease persists, suggesting that other factors also are involved.

Because the greatest risk factor for development of ROP is low birth weight (11) (and gestational age), we hypothesized that insulin-like growth factor I (IGF-I) was critical to ROP. IGF-I is an important somatic growth factor that is correlated with birth weight (14, 15) and gestational age (16, 17). In particular, IGF-I is not maintained at *in utero* levels after premature birth (16) perhaps because of loss of IGF-I sources from placenta and from amniotic fluid that is ingested by the fetus. IGF-I levels in amniotic fluid increase rapidly from the second to the third trimester, the time period when the preterm babies who are at risk of developing ROP are born (18). Furthermore, many premature infants are undernourished, which further lowers IGF-I. We hypothesized that IGF-I is critical in normal retinal vascular development and that a lack of IGF-I in the early neonatal period is associated with lack of vascular growth and with proliferative ROP.

Methods

Measurement of Vessel Growth in IGF-I Knockout Mice. These studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. IGF-I null mice (IGF-I$^{-/-}$) were generated through inbreeding mice carrying heterozygous IGF-I-flox$^{+/-}$ (L/−) on a mixed C57/129sv background (19). Born as dwarfs with severe developmental deficiency, only 40% of the few born survived postnatal life. Their littermates, L/L or L/−, were virtually identical and normal. Genotyping using PCR and Southern blot analysis on tail DNA samples were performed as reported previously (20). At postnatal day 5 (P5), five IGF-I$^{-/-}$ and six IGF-I$^{+/+}$ sibling mice were killed and eyes were isolated and then fresh-frozen in OCT compound and serially sectioned (8 μm). Thirty sections were made through the pupil and optic nerve, and blood vessels were stained with fluoresceinated Griffonia Bandereiraea Simplicifolia Isolectin B4 (Vector Laboratories). The length of vascularized retina was measured from the optic nerve, along the surface of the ganglion layer, to the edge of the vascular front and represented as a percentage of the total length of the retina, from the optic nerve to the ora serrata.

Abbreviations: IGF-I, insulin-like growth factor I; VEGF, vascular endothelial growth factor; ROP, retinopathy of prematurity; Akt, protein kinase B; P*n*, postnatal day *n*; qRT-PCR, quantitative real time–PCR.

**To whom reprint requests should be addressed at: Department of Ophthalmology, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115. E-mail: lois.smith@tch.harvard.edu.

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Retinal Flat Mount. Eyes from 5 IGF-I$^{-/-}$ and 5 IGF-I$^{+/+}$ littermate control mice were enucleated at P5 after intracardiac perfusion with fluorescein-dextran in 4% paraformaldehyde (21). Retinas were isolated, flat-mounted with glycerol-gelatin, and photographed with a fluorescence microscope. VEGF mRNA was visualized according to standard protocol (7).

Laser-Capture Microdissection. OCT-embedded eyes from five IGF-I$^{-/-}$ mice and six IGF-I$^{+/+}$ littermate controls were sectioned at 8 μm in a cryostat, mounted on uncoated glass slides, and stored immediately at −80°C. Slides containing frozen sections were fixed immediately in 70% ethanol for 30 sec, stained with hematoxylin (Meyers) and eosin, followed by 5-sec dehydration steps in 70%, 95%, and 100% ethanol and a final, 10-min dehydration step in xylene. Once air-dried, the anterior avascular third of retinal sections was microdissected, without retinal pigment epithelium contamination, with a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). Each population was estimated to be greater than 95% "homogeneous" as determined by microscopic visualization of the captured cells. Material from 40 sections from more than four mice was combined, RNA was isolated and converted to cDNA as described, and specific cDNA was quantified by using quantitative real time–PCR (qRT-PCR).

RNA/cDNA Isolation. Total RNA was isolated from pooled microdissected retina from IGF-I$^{-/-}$ and control IGF-I$^{+/+}$mice (22). All cDNA samples were aliquoted and stored at −80°C. The VEGF mRNA compared with cyclophilin was measured for IGF-I$^{-/-}$ and control IGF-I$^{+/+}$ retina.

Analysis of VEGF Expression. PCR primers targeting VEGF and two unchanging control genes (cyclophilin and 18S) were designed by using PRIMER EXPRESS software (Perkin–Elmer) and synthesized (Oligos Etc., Wilsonville, OR). Amplicons generated during the PCR were gel-purified and sequenced to confirm the selection of the desired sequence. Quantitative analysis of gene expression was generated by using an ABI Prism 7700 Sequence Detection System (TaqMan) and the SYBR Green master mix kit (Perkin–Elmer). The sequences are as follows: VEGF, forward 5′-GGAGATCCTTCGAGGAGCACTT-3′ and reverse 5′-GGCGATTTAGCAGCAGATATAAGAA-3′; cyclophilin, forward 5′-CAGACGCCACTGTCGCTTT-3′ and reverse 5′-TGTCTTTGGAACTTTGTCTGCAA-3′; 18S ribosomal RNA, forward 5′-CGGCTACCACATCCAAGGAA-3′ and reverse 5′-GCTGGAATTACCGCGGCT-3′.

Clinical Studies. On an Institutional Review Board-approved protocol, all children with a gestational age less than 32 weeks at birth and without any obvious abnormalities born at The Queen Silvia Children's Hospital, Göteborg, between December 15, 1999 and March 15, 2000 were invited to participate in the present study. With written consent, 0.5 ml of blood was collected weekly from birth to hospital discharge. Serum IGF-I was measured in duplicate by an IGF binding protein-blocked RIA, without extraction and in the presence of ≈250-fold excess IGF-II (23) (Mediagnost, Tübingen, Germany). The intraassay coefficient of variation (CV) were 8.1%, 4.4%, and 4.5% at concentrations of 55, 219, and 479 μg/liter, respectively, and the interassay CV were 10.4%, 7.7%, 5.3% at concentrations of 55, 219, and 479 μg/liter, respectively.

ROP Examinations. Dilated retinal examinations with indirect ophthalmoscopy were performed weekly or biweekly from the age of 5 to 6 weeks until the retina was fully vascularized or the condition was considered stable. Children with plus disease and/or stage 3 ROP had more frequent examinations. ROP changes were classified according to the International Classification of ROP.

Retinal Endothelial Cells and Analyses of Akt Phosphorylation. Experiments with bovine retinal endothelial cells (VEC Technologies, Rensselaer, NY) were performed four times with similar results. Moreover, similar results were obtained with separate bovine retinal endothelial cell populations isolated as described previously (24). For analyses of Akt phosphorylation, cells were grown in complete culture medium (MCDB-131 Complete; VEC Technologies) to confluence in 24-well plates coated with bovine collagen type 1 (50 μg/ml Vitrogen; Cohesion, Palo Alto, CA). At confluence, cells were shifted for several days to endothelial basal medium (EBM) (Clonetics, San Diego) containing 2% FBS to reduce baseline phosphorylation of Akt. On the day of assay, cells were shifted to serum-free EBM for 4 h to reduce baseline further and then stimulated with VEGF, IGF-I, or both (R&D Systems) as indicated. Cells were lysed in electrophoresis sample buffer and subjected to electrophoresis in 10% polyacrylamide gels followed by electroblotting, as described (24). Blots were stained with phospho-Akt antibody (Ser-473; PharMingen), secondary antibody, and chemiluminescent substrate, also as described (24). To visualize total Akt, replicate blots were prepared and stained with an antibody that binds both phosphorylated and nonphosphorylated Akt (H-136; Santa Cruz Biotechnology).

Results

IGF-I Is Critical for Normal Retinal Vascular Growth. To test whether IGF-I is critical for normal retinal vascular development and, therefore, critical to the development of ROP (3, 4), we examined retinal vessels in IGF-I$^{-/-}$ mice (which lack both circulating and local IGF-I) and their normal littermate controls (IGF-I$^{+/+}$). The systemic level of IGF-I (vs. local production) contributes most significantly to retinopathy (25).

Mice were perfused with FITC dextran at P5, eyes were enucleated, and retinas were examined in cross-section and flat mount. There was significantly retarded vascular growth in the eyes of the IGF-I$^{-/-}$ mice (Fig. 1*A*) compared with IGF-I$^{+/+}$ controls with normal IGF-I levels (Fig. 1*B*). At P5, the percent distance of the vessels from optic nerve to periphery was 58 ± 4.8% for IGF-I$^{-/-}$ retinas vs. 70.3 ± 5.8% for IGF-I$^{+/+}$ controls ($P < 0.001$), indicating that IGF-I is critical for normal vascular development and that low IGF-I in the neonatal period could cause retardation of vascular growth.

VEGF is an important factor in normal vessel development and is found anterior to the growing vascular front (7–9). Vessels grow toward the moving wave of VEGF, which is induced as nonvascularized retina matures anteriorly (physiological hypoxia) and then is suppressed posteriorly as vessels supply oxygen (Fig. 2*A*). Inhibition of VEGF can cause retardation of vascular growth (5, 6, 10). To test whether the effect of low IGF-I on inhibition of vascular growth was a result of the absence of VEGF, we laser-microdissected the area of retina anterior to blood vessels in P5 IGF-I$^{-/-}$ and control IGF-I$^{+/+}$ retinal cross-sections to detect VEGF mRNA by using qRT-PCR (Fig. 2*B*). Anterior to the vessels in both IGF-I$^{-/-}$ and IGF-I$^{+/+}$ control retinas, VEGF mRNA was present in comparable amounts relative to cyclophilin control as measured by qRT-PCR. Thus, low IGF-I does not inhibit vascular growth through suppression of VEGF (24, 26). IGF-I control is either downstream of VEGF or permissive to its action in vascular development. This data also support the hypothesis that VEGF, in the absence of IGF-I, cannot stimulate normal retinal vascular development.

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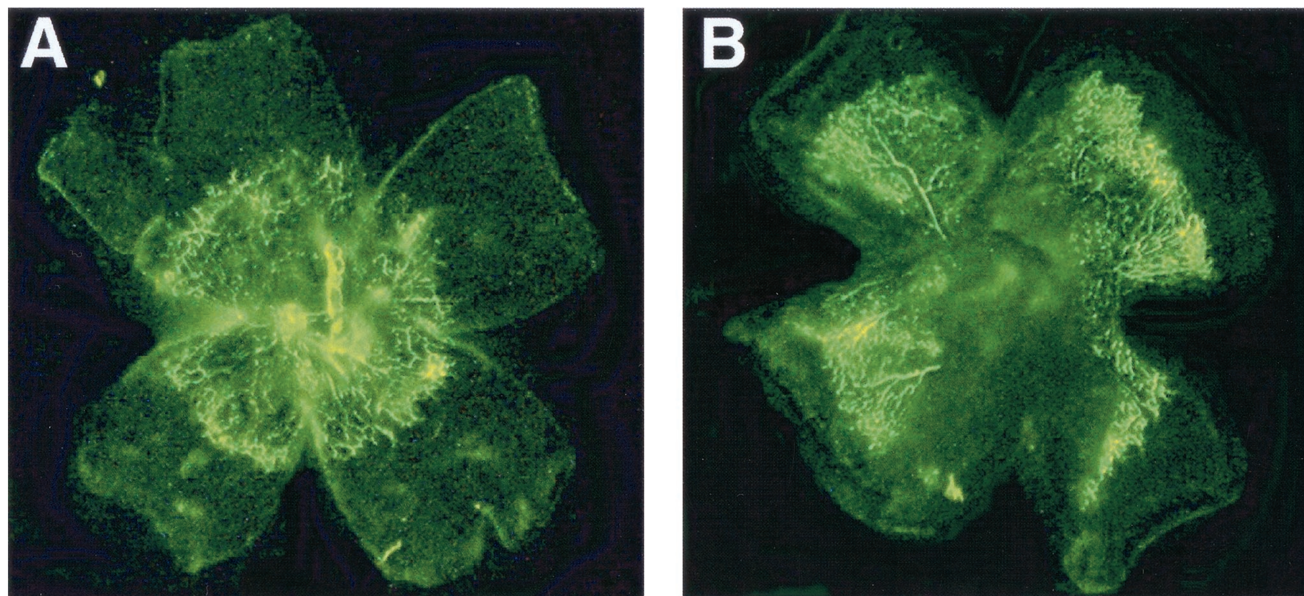

Fig. 1. Effect of IGF-I inhibition on vascular growth. Flat-mounted whole retina shows that, in IGF-I$^{-/-}$ mice (*A*), there is less progression of vascular development (bright area) compared with IGF-I$^{+/+}$ littermate controls (*B*).

Prolonged Low Level of IGF-I Is Associated with Both Suppression of Vascular Growth and Proliferative ROP. To test the hypothesis that a prolonged period of low IGF-I levels after birth was associated with lack of vascular growth followed by ROP in premature infants, we prospectively measured IGF-I plasma levels weekly after birth and coordinately examined retinas in all premature infants born at gestational ages 26–30 weeks at high risk for ROP (n = 31). ROP stages 0–4 were defined according to the International Classification (27), and, for our studies, ROP stages 2–5 were defined as ROP and ROP stages 0–1, as no ROP.

We first confirmed that lack of vascular growth is associated with proliferative ROP (3). The normal immature retina has a gradual transition from translucent vascularized retina into gray, nonvascularized retina without a distinct border between the two. In ROP, a sharp observable stationary border consisting of a line or ridge between vascularized and nonvascularized retina becomes apparent. In all patients with ROP (n = 10), there was a demarcation line anterior to which no vessels were seen. In all infants without ROP (n = 19), there was no ridge and no demarcation line, indicating more normal growth of the vascular front (data not shown).

The mean duration of time from birth to IGF-I reaching 30 ng/ml was 19 days (range, 1–79) in infants who developed no ROP (n = 19) compared with 58 days (range, 29–120) for those that developed ROP (n = 10) ($P \leq 0.0001$), confirming the hypothesis that prolonged low levels of IGF-I were associated with ROP. IGF-I might be lower *in utero* in younger fetuses and, therefore, related simply to gestational age. However, the mean IGF-I level at the same gestational age was consistently lower in infants who developed ROP than those who did not develop ROP with a difference at 34 weeks of 25 ng/ml for ROP (range 21–35) vs. 43 ng/ml for no ROP (range, 11–58) ($P \leq 0.002$).

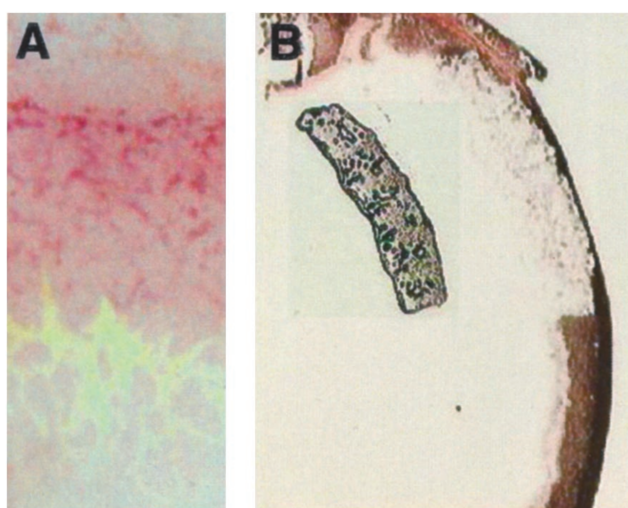

Fig. 2. Laser microdissection of retina anterior to growing vessels. (*A*) VEGF mRNA (pink) is visualized anterior to the growing vessels (green fluorescein) in flat-mounted retina. (*B*) The area containing VEGF (*Inset*) was removed by laser microdissection in both IGF-I$^{-/-}$ mice and control IGF-I$^{+/+}$ retinal cross-sections, and VEGF mRNA was analyzed by qRT-PCR relative to cyclophilin control.

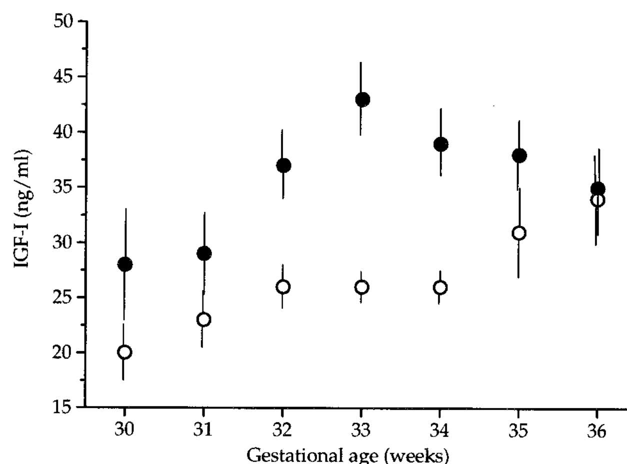

Fig. 3. Mean serum IGF-I at matched gestational ages in infants with and without ROP. The mean IGF-I level for infants with ROP (○) and without ROP (●) is shown vs. gestational age. (Bars = SEM.)

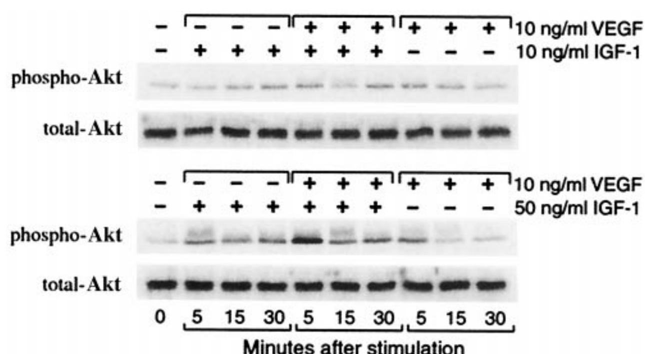

Fig. 4. After serum starvation to reduce baseline Akt phosphorylation, cells were stimulated with VEGF, IGF-I, or both for the times indicated. Replicate blots were prepared from total cell lysates and stained either with phospho-Akt (serine 473) antibody or antibody that recognizes Akt irrespective of phosphorylation status (total-Akt).

Maximum IGF-I during the gestational age of 30–35 weeks was significantly lower among children with ROP (38 ng/ml; range, 28–54 ng/ml) than children without ROP (52 ng/ml; range, 29–90 ng/ml) ($P < 0.04$). In all infants who developed ROP, the onset of the proliferative phase of ROP did not occur before IGF-I levels increased to >30 ng/ml. In summary, the development of ROP was strongly associated with a prolonged period of low IGF-I (<30 ng/ml) followed by rise to "threshold" (>30 ng/ml) at ≈34–35 weeks gestation, the mean onset of proliferative ROP in our cohort. Infants with early higher IGF-I levels had more normal vascular development and did not develop ROP (Fig. 3).

IGF-I Supports VEGF Activation of the Akt-Survival Pathway in Retinal Endothelial Cells. Late-stage ROP is characterized by initial cessation of vascular growth followed by a sudden proliferation of neovascularization at ≈34 weeks postconceptual age, whatever the chronological age of the infant. We postulated that low IGF-I prevented maximum VEGF-induced endothelial cell function because there was a supporting effect of IGF-I on VEGF-regulated retinal vascular endothelial cell survival and proliferation. We have shown previously that IGF-I is required for maximum VEGF stimulation of the MAPK pathway, which is important to cell proliferation (24).

Cell survival, which is also critical to both phases of ROP, is associated with activation of the Akt pathway, which can be accomplished in endothelial cells by stimulation with sufficient concentrations of VEGF (28–30) or IGF-I (31). However, the possibility that these two cytokines exert complementary effects toward Akt activation had not been explored. Therefore, we tested the effects of IGF-I on VEGF activation of Akt in retinal endothelial cells. We found that VEGF (10 ng/ml) and IGF-I (50 ng/ml) individually stimulated modest increases in Akt phosphorylation (2.5-fold), but that both together stimulated a 5-fold increase (Fig. 4). However, the complementary action of VEGF and IGF-I toward stimulation of Akt phosphorylation was not observed when IGF-I was reduced to 10 ng/ml. Thus, these data indicate that 50 ng/ml IGF-I, which approximates a more normal physiological circulating concentration in newborns, acts together with VEGF to activate Akt (as indicated by phosphorylation of serine 473) and, therefore, supports endothelial cell survival in retina. By contrast, when IGF-I is reduced to 10 ng/ml, comparable to the serum level present in premature infants likely to develop ROP, no such complementarity with VEGF is observed. Consequently, in such patients, lower than normal levels of IGF-I likely translate into reduced Akt activation and reduced endothelial cell survival, despite the presence of a constant level of VEGF.

Discussion

These studies demonstrate that IGF-I is necessary for vascular growth and rationalize the disease process of ROP, which begins with cessation of the growth of retinal vessels after premature birth. A key difference between vascular growth *in utero* and after birth is that IGF-I falls in premature infants after birth (16). Our findings suggest that if IGF-I increases quickly in premature infants after delivery, allowing normal vascular development, ROP does not occur.

VEGF has been shown to play a significant role in the development of blood vessels but is insufficient in the presence of low IGF-I levels to allow blood vessel growth (24, 26). VEGF is produced in the increasingly hypoxic avascular retina as metabolic demands increase with development and VEGF levels rise in the vitreous (32, 33). When IGF-I rises more quickly after

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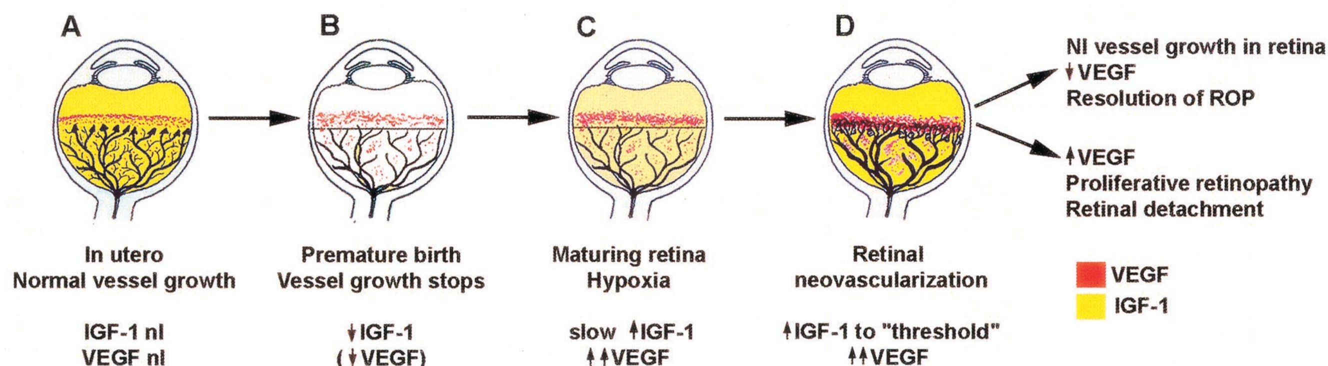

Fig. 5. Schematic representation of IGF-I/VEGF control of blood vessel development in ROP. (*A*) *In utero*, VEGF is found at the growing front of vessels. IGF-I is sufficient to allow vessel growth. (*B*) With premature birth, IGF-I is not maintained at *in utero* levels and vascular growth ceases, despite the presence of VEGF at the growing front of vessels. Both endothelial cell survival (Akt) and proliferation (mitogen-activated protein kinase) pathways are compromised. With low IGF-I and cessation of vessel growth, a demarcation line forms at the vascular front. High oxygen exposure (as occurs in animal models and in some premature infants) may also suppress VEGF, further contributing to inhibition of vessel growth. (*C*) As the premature infant matures, the developing but nonvascularized retina becomes hypoxic. VEGF increases in retina and vitreous. With maturation, the IGF-I level slowly increases. (*D*) When the IGF-I level reaches a threshold at ≈34 weeks gestation, with high VEGF levels in the vitreous, endothelial cell survival and proliferation driven by VEGF may proceed. Neovascularization ensues at the demarcation line, growing into the vitreous. If VEGF vitreal levels fall, normal retinal vessel growth can proceed. With normal vascular growth and blood flow, oxygen suppresses VEGF expression, so it will no longer be overproduced. If hypoxia (and elevated levels of VEGF) persists, further neovascularization and fibrosis leading to retinal detachment can occur.

birth as occurs in the non-ROP infants, VEGF does not accumulate because vascular growth can occur, which provides oxygen to the maturing retina and controls VEGF production (7, 8). When IGF-I is low for an extended period, vessels cease to grow, the maturing avascular retina becomes hypoxic, and VEGF accumulates in the vitreous. As IGF-I rises to a threshold level with high levels of VEGF present, a rapid growth of new blood vessels (retinal neovascularization) is triggered (Fig. 5). This rapid vascular growth likely is based on increased survival and proliferation of vascular endothelial cells because IGF-I and VEGF are complementary for endothelial cell function through the mitogen-activated protein kinase and Akt signal-transduction pathways. In particular, our data indicate that IGF-I (and perhaps other cytokines) is necessary at minimal levels to promote maximum function of VEGF.

This work has direct clinical implications for the diagnosis and treatment of ROP. These findings suggest that IGF-I levels can be used to predict which babies will develop ROP. The differences in patterns of IGF-I levels between patients that do and do not develop ROP suggest that increasing serum IGF-I early after birth may prevent this disease. After premature birth, potential sources of IGF-I are lost, including ingestion of amniotic fluid, which contains high levels of IGF-I. IGF-I may be increased to the levels found in infants without ROP through increased caloric intake (17), oral ingestion of IGF-I to mimic ingestion of amniotic fluid (34), or an i.v. supply to raise IGF-I to a more normal level. Because ROP is correlated with other developmental problems, increasing IGF-I levels to the level of infants without ROP also may improve neurological development (35) and somatic growth (34).

Both IGF-I and VEGF are also important in the second or neovascular phase of ROP (5–7, 24, 26). IGF-I is critical for retinal neovascularization (24). Thus, although we would predict that early intervention to increase IGF-I would allow normal vascular growth and prevent the development of the second, potentially destructive phase of ROP, late intervention after accumulation of VEGF might trigger or exacerbate retinal neovascularization.

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