Up-Regulation of VEGF by Retinoic Acid During Hyperoxia Prevents Retinal Neovascularization and Retinopathy

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PURPOSE. Retinopathy of prematurity (ROP) is directly associated with abnormal expression of retinal vascular endothelial growth factor (VEGF) in premature neonates. This study was to investigate whether the systemic administration of retinoic acid (RA) regulates retinal VEGF expression and prevents retinal neovascularization and retinopathy in the oxygen-induced retinopathy (OIR) mouse model.

METHODS. C57BL/6 mice were subjected to OIR by exposure to 75% oxygen from postnatal day (P) 7 to 12 of age. RA was intraperitoneally injected daily to pups from P6 to P9. Retinal whole mount staining and image analysis, immunostaining, Western blotting, quantitative RT-PCR, TUNEL assay, and electroretinography were performed to evaluate the effects of RA on VEGF expression, retinal neovascularization, and retinal neuron functions.

RESULTS. Systemic administration of RA in OIR mice promoted retinal VEGF mRNA and protein expression in phase I; the stabilized level of VEGF in phase I supported retinal vascular development and counteracted vaso-obliteration in OIR mice. Subsequently, the excessive generation of VEGF in phase II was attenuated; the retinal vascular leakage and apoptotic cells were significantly ameliorated. As a result, RA significantly prevented the development of hypoxia-induced retinal neovascularization and retinopathy in OIR mice and improved the functional recovery of retinal neurons downstream of photoreceptor cells as measured by focal electroretinography.

CONCLUSIONS. Systemic administration of RA regulates retinal VEGF expression and supports retinal vascular development in OIR mouse model. We propose that systemic administration of RA to extremely low birth weight, preterm infants during oxygen therapy could potentially be an effective therapeutic approach for the prevention of ROP.

Keywords: VEGF, OIR, ROP, retinal neovascularization, retinoic acid, hypoxia, IGF-1

Retinopathy of prematurity (ROP) continues to be a leading cause of childhood blindness globally and the condition typically affects premature, low birth weight infants exposed to oxygen therapy. Due to the advances in neonatal intensive care, many more extremely low birth weight infants survive. This success has not been entirely without consequence: in our modern era, ROP incidence has reached its third epidemic peak. In 2010, an estimated 14.9 million babies were born preterm; 50,000 newborn babies are blinded each year due to ROP.

ROP progresses in two distinct phases. Phase I involves the arrest of vascular growth and vaso-obliteration. Retinal vasculogenesis is normally completed by full-term gestation in humans, with the vascular beds formed in a wave of growth that moves from the optic disc to the retinal periphery. Thus, extremely preterm infants are born with an avascular peripheral retina. When a preterm infant is placed inside a higher concentration oxygen chamber, the pro-angiogenic vascular endothelial growth factor (VEGF), along with other cytokines, becomes acutely and significantly downregulated in the retina, which contributes to growth arrest of the normal retinal vasculature, and subsequently loss of vascular support to developing retinal neurons. As the infant matures, the avascular retina becomes increasingly hypoxic due to the increased metabolic demands of the maturing retina and a return to normal air supply (21% oxygen). This hypoxia induces a localized up-regulation of VEGF, leading to phase II of ROP: vasoproliferation and retinal neovascularization. Elevated concentrations of VEGF have been detected in the vitreous of patients with ROP. Vision loss is the ultimate consequence of aberrant neovascularization in phase II, which results from fibrosis and retinal detachment.

The degree of vaso-obliteration in phase I determines the severity of retinal hypoxia, which subsequently determines the production of VEGF in the retina in phase II. Thus, it is quite logical to propose that reducing the degree of vaso-obliteration in phase I will decrease the retinal ischemia, thereby lessening the generation of VEGF in phase II, resulting in a better visual acuity for ROP patients.

Significant efforts and achievements have been made targeting VEGF for therapy in phase II. For example, cryotherapy and laser photocoagulation are used to destroy the avascular retinal tissue to reduce the source of VEGF and anti-VEGF therapy is being tested to neutralize VEGF production. The later strategy is controversial at this time because VEGF is also essential for normal retinal development and...
homeostasis. In this report, we attempt an alternative approach to targeting VEGF in phase I. The objective was to promote VEGF expression in the hyperoxic phase I to counteract vaso-obliteration and support retinal vascular development. We hypothesized that reducing an acute drop of endogenous VEGF expression within the retina during phase I would decrease vascular ablation and subsequent hypoxia in phase II and related retinopathy.

The key issue therefore is to search for reagents or compounds that can promote generation of VEGF in retina in the hyperoxic phase in ROP. A study by Saito et al.9 demonstrated that retinoic acid (RA) up-regulates the VEGF gene in Y79 cells (retinoblastoma cells), and VEGF transcripts were increased by RA in a directly correlated time- and dosage-dependent manner. Similar effects from RA treatment were reported in bronchioalveolar carcinoma cells.10 In addition, RA is an active metabolite of vitamin A and regulates a wide range of biological processes including cell proliferation, differentiation, morphogenesis, eye development, and phototransduction.11–13 Considering that preterm infants are also at greater risk for vitamin A deficiency,14 and that vitamin A supplementation actually increases survival and improves respiratory functions of preterm infants,15 RA was a good candidate to test our hypothesis.

We tested systemic dosing with RA using the oxygen-induced retinopathy (OIR) murine model, a model that provides both a vascular ablation (phase I) and neovascular (phase II) response.16 We show that systemic administration of RA in OIR mice promoted VEGF expression and reduced the acute drop of VEGF in retinas in phase I. Consequently, the generation of retinal VEGF in phase II was attenuated. As a result, RA significantly prevented the development of hypoxia-induced retinal neovascularization and retinopathy in OIR mice.

**Materials and Methods**

**Mouse Model of OIR**

All animals in all experiments were treated in accordance with the guidelines of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and monitored by the Institutional Animal Care and Use Committee of the two institutes participating in this study. All mice were provided with food and water and kept under controlled room temperature and lighting conditions of 12-hour light–dark schedule.

The OIR mouse model was produced in mouse C57BL/6 pups by following the method described by Smith et al.16 Briefly, P7 pups (7-day-old mice) and their nursing dams were exposed to hyperoxic conditions (75% oxygen) for 5 days. On P12, the pups along with their dams were returned to room air (21% oxygen). RA (Sigma, St. Louis, MO, USA) was freshly prepared as 1.664 M in vehicle of dimethyl sulfoxide (0.1% v/v; Sigma) and corn oil. OIR mice of the same litter were selected randomly for different treatments: one group for RA injection, another for vehicle injection as O2 control. These littermates were treated with intraperitoneal injection once a day from P6 to P9 with RA concentration of 3.34 μmol/kg or vehicle only as control.17,18 Age-matched mice were maintained in room air in the same room where the O2 incubator was placed, and received the same vehicle intraperitoneally for the duration of each experiment on OIR mice as described above.

**Quantification of Retinal Whole Mount**

Mice were killed, and their eyes were enucleated for experiments on P10, P12, P17, and P28. Usually, one eyeball was used for whole mount analysis, and the other eyeball from the same pup was prepared and stored for qRT-PCR, Western blot, or other analysis. Whole mount preparation and imaging quantification were performed following the methods published by Connor et al.19 In brief, eyes were fixed for 1 hour in 4% paraformaldehyde, followed by retinal dissection. Retinas were stained overnight in Alexa Fluor 594-conjugated G. simplicifolia isoleciton B4 (1:100 dilution; Molecular Probes, Eugene, OR, USA). Retinal flat mounts were generated and images were acquired from each retina using Eclipse Nikon C1si laser confocal microscope (Nikon Instruments, Inc., Tokyo, Japan). Adobe Photoshop (Adobe Systems, San Jose, CA, USA) was used for quantitative analysis. Areas of vaso-obliteration and areas of neovascular tufts in pixels were compared to the total retinal area in pixels and given as percentages.

**Quantitative RT-PCR**

RNA was immediately isolated from retinas using an E.Z.N.A. MicroElute Total RNA kit (Omega BioTek, Norcross, GA, USA) according to the manufacturer's instruction. After quantification of the RNA concentrations, cDNA was synthesized by reverse transcription from 1.0 μg of RNA, using the PrimeScript RT reagent Kit With gDNA Eraser (Takara, Otsu, Shiga, Japan). Synthesized cDNA was divided into aliquots and stored at −80°C.

For real-time qPCR, SYBR Remix Ex Taq II (Tli RNaseH Plus) Kit (Takara) was used by following the manufacturer's instruction. The assays were run on an ABI 7300 system (Applied Biosystems, Foster City, CA, USA). Data were analyzed according to the manufacturer's protocol. Data were calculated by the 2−ΔΔCt method and relative gene expressions are presented as fold change of transcripts for target genes normalized to GAPDH.

We determined specificity of each primer with the BLAST module. Primers were synthesized by Invitrogen and the primer sequences (5′ to 3′) are as follows: VEGF (Exon 3): GCTACTGCGCGTCCGATGTAG (forward); CACTCCAGGGCTTCATCGTTA (reverse); VEGF (Exon 8): ACAAGGAGGAGA CATTGAGGTAG (forward); CAGGGCCAGGAATTGGTTG (reverse); IGF-1: TGGATGCTCTTCAGTTCGTGTG (forward); AGCTCGGGAAAGCAACACTCATG (reverse); GAPDH: TGTGCTCGTCGGTGAATC (forward); TTGCTGTTGAAGTCCGAG GAG (reverse).

**TUNEL Assay**

Serial 12-μm-thick frozen sections of eyeballs from each treatment were cut in transverse planes. Retinal apoptosis was detected using TUNEL assay kit following the instruction provided by Manufacturer (Koshide Biotechnology, Inc., Wuhan, Hubei, China). Retinal sections were counterstained with Hoechst 33342. For each section, TUNEL-positive green nuclei were counted under higher magnification per microscopic field per section for all the eyeballs.

**Immunoblotting**

The retinas were homogenized in soluble protein lysis buffer with protease inhibitor cocktail on ice (Sangon Biotech, Shanghai, China). The samples were vortexed briefly and shaken for 30 minutes at 4°C. Following a centrifugation at 16,000g for 20 minutes at 4°C, total protein in the supernatants was collected and quantified using a BCA protein assay kit (Solarbio, Beijing, China).

Equal amounts of denatured protein were loaded and separated on 12% SDS-PAGE (Sigma) and electrotransferred
onto 0.2-μm polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% nonfat milk in PBS, probed with primary goat anti-mouse VEGF polyclonal antibody (Santa Cruz, San Jose, CA, USA) and anti-GAPDH polyclonal antibody (R&D Systems, Minneapolis, MN, USA), respectively; following with corresponding goat anti-mouse IgG-HRP or donkey anti-Goat IgG-HRP (R&D Systems); and developed with a high-sensitivity chemiluminescence kit (Millipore) and visualized by an electrochemiluminescence detection system (FluorChem HD2; Alpha Innotech, San Leandro, CA, USA).

Permeability

To observe the permeability of the mouse retinal vessels, tracer experiments were performed as described previously with minor modification. Under deep anesthesia with pentobarbital sodium, the pup’s chest was opened, and a sterile 24-gauge needle on a syringe was inserted into the left ventricle. The syringe was pulled outward slightly to aspirate the blood, ensuring the needle was in the ventricle. Each pup was perfused with 300 μL of PBS containing the mixture of 100 μg/mL Hoechst 33342 (Mer, 616 D; Molecular Probes) and 20 mg/mL FITC-dextran (Mer, 2000 kD; Molecular Probes). Five minutes after perfusion, eyeballs were removed and fixed in 4% paraformaldehyde for 1 hour, and retinas were flat mounted and photographed with a Nikon C1si confocal microscope.

Focal-ERG

Dark-adapted mice were handled under dim red room lighting (variable 4–15 lx). After 2 hours of dark adaptation, pupils were dilated with a sequential application of tropicamide and phenylephrine eye drops. Short-term anesthesia of 30 minutes was induced by a single intraperitoneal injection of 50 mg/kg ketamine HCl and 7 mg/kg xylazine. Upon loss of the blink reflex, corneas were protected by Gentec lubricant solution (Novartis, East Hanover, NJ, USA).

Focal-ERG recordings were collected on OIR mice of age P10 after dark adaptation, using a Micron III Focal-ERG system (Phoenix Research Labs, Pleasanton, CA, USA). Aiming of the light stimulus was accomplished by viewing the retina with dim red illumination (LED), high camera gain, and video averaging (15 frames). A circular LED white-light stimulus of 10 disc-diameters was projected onto the central retina (30 ms duration) with a bright intensity setting corresponded to an energy delivery of 50,400 cd/s/m² of projected retina area. This illuminated region was 36% of the total retinal area, covering the region most affected in the mouse OIR model. Mice were maintained at 37°C on a regulated temperature pad. The lens mount of the focal-ERG provided the gold corneal electrode. Platinum cutaneous needle electrodes were used for the reference and ground, inserted into the head cap and hind flank skin. Triggering of the light stimulus and acquisition of ERG traces were accomplished with LabScribe-2 software using a Phoenix Research Labs ERG plug-in. Twenty stimulus traces were used to obtain an average ERG trace. Amplitudes of the b-wave were determined from averages of the left and right eyes for each mouse.

Histology

Mice were killed after ERG analysis. Each eyeball was fixed in 4% paraformaldehyde and then processed for standard paraffin-embedded sections. The retina sections were cut through the optic disc of each eye and stained with hematoxylin and eosin. Slides were digitized using an Olympus VS-120SL virtual microscopy system (Center Valley, PA, USA) and Digital Slide Box software (Leica Microsystems, Buffalo Grove, IL, USA).

Statistical Analysis

All statistical analyses were carried out using the SPSS 17.0 statistical software (SPSS 17.0, Chicago, IL, USA). For the comparison of normally distributed variables, we used the one-way ANOVA, the between two different groups used the LSD. P values < 0.05 were considered to be statistically significant.

Results

RA Regulates VEGF Production in Biphases in OIR Mice

To determine whether RA can regulate VEGF expression in retina of newborn pups under hypoxic conditions, we analyzed VEGF mRNA and protein in OIR mice after systemic administration of RA as described in detail in Materials and Methods. The OIR mouse is a well-characterized VEGF-dependent model, in which suppression of retinal VEGF occurs when pups are exposed to high oxygen from postnatal day 7 to 12 (phase I); consequently, elevation of retinal VEGF occurs after returning the pups to room air (phase II). We measured retinal VEGF mRNA by quantitative RT-PCR (qRT-PCR) using two different primer pairs at age P10 (phase I) and P17 (phase II) after the treatments of OIR mice with or without RA. As expected, VEGF mRNA levels were significantly suppressed by more than 50% in the retinas of 75% O2 pups on P10 with vehicle (control) intraperitoneal injection (Figs. 1A, 1B; O2 versus Air, P < 0.01). In contrast, the retinal VEGF mRNA expression was significantly greater in their RA-injected littersmates (O2 RA), showing a significant increase in comparison to the O2 controls (Figs. 1A, 1B). After returning animals to room air, the avascular region of the retina became hypoxic, and retinal neovascularization reached its peak on P17 in the OIR mouse model (Smith et al., 1994). As expected, the retinal VEGF mRNA was dramatically elevated in O2 pups at age P17 (Figs. 1C, 1D; O2 versus Air, P < 0.01); however, the level of VEGF mRNA in RA-treated OIR pups (O2 RA) was significantly lower in comparison to that of untreated OIR pups (Figs. 1C, 1D; O2 RA versus O2, P < 0.05). Thus, in OIR mice, RA treatment increased retinal VEGF mRNA concentration in phase I, resulting in a reduction of VEGF transcripts in phase II.

Next, VEGF protein expression in the retinas of each treatment (Air, O2, and O2 RA) was analyzed by immunoblotting of whole retinal protein extracts. Consistent with the qRT-PCR data, a suppression of VEGF expression was apparent in O2 mice in phase I at age P10 (Fig. 2, top, P10). In contrast, the induction of VEGF protein was striking in RA-treated OIR pups (Fig. 2, O2 RA), expressing a comparable amount of VEGF protein to the room air controls. Immunoblotting with anti-GAPDH IgG confirmed nearly equal loading of retinal protein. Furthermore, at time points in phase II, such as P13 (data not shown) or P17 (Fig. 2, bottom, P17), the O2 pups exhibited a significantly increased amount of VEGF protein under the hypoxic status of the retinas in comparison to air controls. Retinal VEGF protein content was significantly reduced in RA-treated OIR mice compared to their nontreated littersmates at age P17 (Fig. 2, O2 versus O2 RA, P < 0.05). The histogram on the right summarizes the abundance of VEGF as measured by densitometry relative to GAPDH loading controls from three independent experiments at each time point. Shown are the fold changes versus the VEGF level of room air controls. VEGF164 was the key isof orm detected by this monoclonal anti-VEGF IgG, whereas VEGF188 and VEGF200 are slightly visible. To further confirm the pattern of VEGF expression shown by Western blot, we used immunofluorescence imaging on the retinal sections from P10 and P17. In the retina of room air pups of age P10, staining with monoclonal VEGF antibody was
FIGURE 1. RA regulates retinal VEGF mRNA expression in OIR mice. VEGF-A mRNA levels in the retinas of mice in room air, O2 control (vehicle intraperitoneally), or O2 with RA injection (O2 RA), respectively, were measured at age P10 (A, B) and age P17 (C, D). Relative gene expression was achieved by qRT-PCR with cycle numbers normalized to GAPDH as a loading control. Data were normalized to (Air) normal control means. Data are expressed as mean ± SD of four independent experiments. *P < 0.05.

FIGURE 2. Expression of retinal VEGF protein in OIR mice is regulated by RA. Total retinal lysates from age P10 and P17 mice, respectively, were prepared and an aliquot of same amount of protein lysate from each sample was resolved by SDS-PAGE under reducing conditions. The membrane was probed with anti-VEGF IgG, subsequently reprobed with anti-GAPDH IgG. The histogram on the right summarizes the abundance of VEGF as measured by densitometry relative to GAPDH loading controls from three independent experiments (mean ± SD). Shown are the fold changes versus the VEGF level of room air controls. *P < 0.05.
restricted to the ganglion cell layer (GCL), the borders of inner nuclear layer (INL) and outer nuclear layer (ONL), and to a few of the retinal capillaries (Fig. 3A). Negative control retinas (IgG serum) showed no staining in the corresponding areas (data not shown). There were extremely low signals of VEGF immunoreactivity in retinas of O2 mice at this age (Fig. 3B). However, the immunostaining was increased in the retinas of their littermates treated with RA (Fig. 3C, O2 RA, arrows). At P17, the hypoxic retinas (O2 pups) exhibited an increased immunostaining of VEGF (Fig. 3E), whereas the staining was reduced significantly in O2 RA retinas (Fig. 3F), comparable to that of the normal controls (Fig. 3D).

Taken together, the data provide strong evidence that systemic administration of RA has modified retinal VEGF mRNA and protein expression in both phases of the OIR model. Thus, RA appears to be an excellent candidate for us to further determine whether it can attenuate ROP progression in OIR model.

**RA Prevents Oxygen-Induced Retinal Vasoobliteration and Neovascularization**

To determine whether the RA-mediated modification of VEGF expression in OIR mice has any effects on retinal vasoobliteration and neovascularization, we performed retinal whole mount fluorescent staining with isolectin G4 from *Griffonia simplicifolia* to visualize the retinal vasculature (Fig. 4). At age P10, the central avascular area in the RA-
treated OIR pups (O2 RA) was consistently reduced in comparison to their O2 littermate controls, although the vaso-oblitration was not statistically significant at this age (P > 0.05) (data not shown). At age P12, the central avascular area in O2 RA pups showed a significant reduction in comparison to their O2 littermates (Figs. 4B, 4C, 4K, P < 0.05). In addition, the morphology of the vessels in Figures 4B and 4C showed a distinguished difference under higher magnifications (Figs. 4G, 4H). Capillaries in O2 RA mice (Fig. 4H) displayed an uniformly distributed network and relatively normal morphology in general as compared with O2 littermate controls (Fig. 4G). At P17, area of capillary ablation in the central retina in the O2 RA pups (Figs. 4E, 4J) was significantly reduced compared with that of O2 littermate controls (Figs. 4E, 4I, 4L, P < 0.01). Some of the RA-treated retinas had a fully revascularized central area as shown in Figure 4E. At P17, the retina had the maximum neovascular response in O2 pups as the retinal neovascularization (NV) tufts had become apparent (Fig. 4I, green), whereas the RA-treated O2 littermates showed significantly less area of retinal NV tufts (Fig. 4J, green; Fig. 4M) (P < 0.01).

Reduction of Apoptosis in RA-Treated OIR Mice

VEGF is a survival factor for retinal endothelial cells and neurons. The hyperoxia-induced vaso-oblitration in phase I of OIR is primarily driven through apoptosis of vascular endothelial cells.21 With up-regulated VEGF in phase I in O2 RA retinas, we would expect less apoptosis in the retina in comparison to O2 controls. TdT-dUTP terminal nick-end labeling method was used to compare the extent of apoptosis in developing retinas of P14 pups. Figure 5 illustrates the number of apoptotic nuclei in the retinal sections of the normal controls (Fig. 5A), untreated O2 (Fig. 5B), and RA-treated O2 pups (Fig. 5C). We observed 50% reduction in the number of apoptotic nuclei in the O2 RA retinas in comparison to O2 retinas (P < 0.05). These data are in good agreement with the observation that vaso-oblitration is minimized in O2 RA retinas shown in Figure 4.

Suppression of Retinal Vascular Leakage

VEGF also plays a critical role in vascular permeability, which unsurprisingly affords it another name, vascular permeability factor. Retinal hyperpermeability has been described in OIR mice.20 It has been shown previously that increased vascular leakage and a compromised microvascular tight junction are signs of pathological retinal neovascularization. We questioned whether administrating RA would ameliorate the retinal vascular leakage in OIR mice. The permeability of the retinal blood vessels was assessed using Hoechst as a tracer described previously.25 FITC-dextran (Mr, 2000 kDa) and Hoechst 33342 (Mr, 616 Da) were injected into the left ventricle of mice at P17. Dextran was detected in the retinal vascular lumen (green) as shown in Figure 6. The leakage of the vessels was detected by Hoechst 33342 dye as it stained the nuclei of retinal cells surrounding or adjacent to the leaking vessels (Fig. 6B, blue). The extensive area of nuclear staining of retinal cells indicates the hyperpermeability of the vessels. In contrast, the nuclear staining was mostly localized in the vicinity of vascular lumen (Fig. 6A) in the RA-treated OIR mice, an indication that RA ameliorates retinal vascular leakage.

Electroretinography (ERG)

In the mouse OIR model, the ablation of the superficial, middle, and deep vascular plexus causes hypoxia induced loss of bipolar cells. Photoreceptor cells are affected less because their inner segments are closer to the retinal pigment epithelium and the oxygen supply from the choroidal vasculature. The B-wave amplitude of the ERG trace is derived from the neural electrochemical activity downstream of activated photoreceptors, which are mostly bipolar and ganglion cells. By the end of the neovascular phase in OIR mice, age P21, any surviving bipolar and retinal ganglion cells can complete synaptic connections and establish functionality. If RA treatment improves overall survival of bipolar and ganglion cells, this could result in a meaningful improvement of retinal function. To evaluate if RA treatment can ultimately improve retinal function in vivo, the average B-wave amplitude for each mouse was determined by averaging both eyes after allowing for recovery by age P36. O2 RA mice had a 45% greater B-wave amplitude compared to their control O2 littermates: 77 ± 19 µV for RA-treated versus 54 ± 19 µV for O2 controls (P = 0.05, Figs. 7A, 7B). Histology results of these mice shown in Figure 7D are consistent with the ERG data. In the OIR model, regions of the retina that experience loss of microvasculature in Phase I experience death of neurons in GCL and INL (Fig. 7D, b). In contrast, at the same retinal region, RA-treated littermates showed a higher density of bipolar cell nuclei and ganglion cell nuclei (Fig. 7D, a), indicating that RA prevents the loss of retinal neurons, at least in part, by supporting retinal vasculature in GCL and INL of the retina.

RA Does Not Regulate Insulin-Like Growth Factor 1 (IGF-1) Expression in Retina

Thus far, we have mainly emphasized the importance of VEGF and how RA affects VEGF expression in the process of retinal vascular development and ROP progression. Another factor that is highly crucial when considering the impact of VEGF is the expression of IGF-1. In particular, IGF-1 is required for maximal VEGF-mediated activities in the retina.24 Thus, to ascertain whether IGF-1 mRNA is also regulated by the systemic administration of RA in mice, we examined the expression of IGF-1 mRNA in the retinas of four treatments on mice of ages of P10 and P12 (Fig. 8). IGF-1 gene expression in the neural retina was essentially the same between room air controls and O2 controls with or without treatment of RA at both of these time points (P > 0.05). The data suggest that neither RA nor hyperoxia substantially altered the level of IGF-1 mRNA in the retinas of neonatal mice. With a normal level of IGF-1 expression in these pups, the VEGF activities induced by RA treatment progressed unimpeded. This is different from preterm infants who have a deficient level of IGF-1. We elaborate this point further in the Discussion.

DISCUSSION

Prevention of retinal NV by increasing VEGF level in phase I of ROP has been proposed by previous investigators.29 However, how to promote retinal VEGF expression appropriately during oxygen therapy of preterm neonates is a key obstacle to application. Here, we have demonstrated the strategy of remodulating endogenous VEGF expression in the neural retina. Systemic administration of RA prior to and during oxygen exposure in OIR mice modulates endogenous VEGF production, improves the stability of the retinal microvasculature and better vascular regrowth, decreases the retinal apoptosis and vessel leakage, and allows recovery of the retinal neurons and their functions. RA plays an important role in regulating the growth, differentiation, and organogenesis of a wide variety of cell types and organs by regulating the expression of numerous factors.
FIGURE 4. Retinal whole-mounts analysis of vaso-obliteration (VO) and neovascularization (NV) in RA-treated OIR mice. Representative flat-mounted retinal images of age P12 and P17 mice are shown by fluorescent labeling of the microvasculature with G. Simplicifolia lectin-Alexa Flour 549. Mouse pups exposing to O2 displayed a central capillary dropout area (VO, yellow). Quantitation of VO at age P12 and P17 (10 pups each) shows a statistical significance in RA-treated pups (K, L). (G, H) are the higher magnifications of (B, C), respectively, at the corresponding middle spot of the retinas (B, C). At age P17, the retinal NV area (green) in OIR pups of RA-treated mice had a significant smaller area in comparison to OIR pups (M). n = 10 retinas of 10 mice of different litters. *P < 0.05; **P < 0.01. Scale bars: 25 μm (G, H), 500 μm (A–F, I, J).
genes through ligand-activated nuclear receptors. In fact, RA plays an essential role in the eye development: mice lacking activity of RA have extremely small eyes with gross morphologic defects in the choroid and sclera and retinal dysplasia. The roles of RA in angiogenesis and regulation of VEGF production in different organs or systems, however, have been divergently reported as an angiogenic inhibitor or stimulator. The molecular mechanisms underlying the cell type-specific role of RA in angiogenesis appear to be variable in a given system or environment.

We propose RA to be a potential candidate for intervention against ROP in patients for several reasons. First, RA could be systemically administered in preterm infants during phase I to possibly modulate VEGF expression in retina. Under normal retinal development, VEGF is distributed in a gradient and mediated and guided by neuroglia. Direct supply of VEGF to the eye of neonates (such as intravitreal injection) is not appropriate because VEGF distribution cannot be spatially controlled; VEGF floods the eye and promotes acute breakdown of the blood retinal barrier. Systemic application of RA is not only a more feasible route for giving a drug to neonates, but it also generates an endogenous supply of VEGF to retinas with the benefit that is cell mediated. From our results, RA enhanced retinal vascular development and survival of retinal cells.

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Secondly, systemic administration of RA might benefit both the retina and the lungs of preterm infants. Preterm infants regularly receive vitamin A treatment, and supplementation in
FIGURE 7. RA improves retinal function and neuron recovery in OIR mice. Focal-ERG analysis of dark-adapted RA-treated and nontreated OIR littermates was completed at age P36. (A) Examples of focal-ERG traces show improved B-wave amplitudes in the RA-treated pups. Photoreceptor-derived A-wave amplitudes were not different, reflecting the fact that most photoreceptor cells survive. (B) Average B-wave amplitudes from RA-treated littermates ($n = 4$) were 45% greater than their nontreated littermates ($n = 3$). B-wave values were averaged from the right and left eyes for each mouse. $P = 0.05$. (C) Fluorescein angiography illustrating a greater number of thick, bright, tortuous vessels in O2 controls compared to O2 RA mice. Micron-III images are from the same eyes as in (A) and were captured immediately after focal-ERG testing. (D) Example histology, prepared after the ERG analysis above, shows the same region of the retina section from (a) RA-treated and (b) untreated OIR littermates of age P36. Loss of bipolar cells in the central retinal region of OIR mice, adjacent to the optic disc is displayed ([b], arrow). A lower density of bipolar cell nuclei and ganglion cell nuclei are seen in (b) compared to the RA-treated retina (a). Note that the density of photoreceptor nuclei in the ONL is not different, consistent with above A-wave amplitudes (A) that were not significantly different. Scale bar: 100 μm.
Retinoic Acid Prevents OIR

Figure 8. RA treatment does not change retinal IGF-1 gene expression in OIR mice. IGF-1 mRNA levels in the retinas of pups were measured at ages P10 and P12, respectively, representing the four treatments: normal room air control with vehicle injection (Air), room air with RA injection (Air RA), 75% O₂ control with vehicle injection (O₂), and 75% O₂ plus RA injection (O₂ RA). Relative gene expression was measured by qRT-PCR with cycle numbers normalized to GAPDH gene expression. Values are shown relative to room air control values. Data are expressed as mean ± SD of four independent experiments. P > 0.05.

extremely low birth weight infants has been shown to reduce the rate of death or bronchopulmonary dysplasia (BPD) by 11%. RA, a product of vitamin A oxidation, is also important for normal lung development and maturation. RA can reduce hyperoxic lung injury in newborn mice. RA has been proposed to treat BPD in premature infants. BPD and ROP are two of the most devastating hyperoxia-induced disorders in premature neonates.

Thirdly, RA has been used in therapies in humans. For example, it is especially effective in therapy against acute promyelocytic leukemia. The pharmacology, pharmacodynamics, and toxicology of RA in humans are well understood. In clinical study, up to 48,000 IU or 50.4 μmol retinol of oral vitamin A has been delivered to newborn infants without any reported toxic effects. It has long been recognized that excess RA during embryonic development can be teratogenic. The RA dose used in this study (3.34 μmol/kg) is significantly lower than the dose that caused teratogenic changes in mice (233 μmol/kg). In addition, early gestational time is the sensitive period for excess RA induction of teratogenesis rather than after the birth. While the precise mechanisms of how RA up-regulates retinal VEGF mRNA in OIR mice remain to be elucidated in detail, it is known that increased activation of VEGF promoter by RA is mediated through SP1 in some cell lines and cancer cells. In those systems, elevated levels of SP1 lead to binding of SP1 to cognate sites in the VEGF promoter, increasing VEGF expression. It is possible that the same mechanism applies to the retinal cells for up-regulating VEGF mRNA in this model. In addition, reactive oxygen species in retinal cells in the neontates might also contribute to the modification of VEGF production by RA.

The murine VEGF gene is alternatively transcribed to yield the VEGF₁₂₀, VEGF₁₆₄, VEGF₁₈₈ isoforms. VEGF₁₆₄ is the potent isoform in retinal angiogenesis. Mice expressing only VEGF₁₆₄ showed normal vascular development in retina. We tested VEGF₁₆₄ for VEGF transcript by designing two different primer pairs at different exons (3 and 8, respectively), and consistent results were achieved (Fig. 1). Although VEGF₁₆₄ is not the only factor that affects ROP progression, it is the key one. The importance of this study is that it demonstrates that RA can modify VEGF expression in the retina of OIR mice and rescue their phenotypes.

IGF is important in fetal growth and development during all stages of pregnancy. The IGF-1 level rises significantly in the third trimester of pregnancy. Thus, premature birth is associated with a low level or loss of IGF-1 from the maternal source. It has been reported that VEGF action is highly dependent on IGF-1; VEGF cannot stimulate normal retinal vessel growth in the absence of IGF-1. IGF-1 controls maximum VEGF activation in the Akt endothelial cell survival pathway. Thus, we speculate that a low level of IGF-1 could explain why vitamin A supplementation showed a nonsignificant trend in ROP therapy. Unlike preterm human infants, the OIR mice were born at full term. Thus, they had a normal level of IGF-1 and other growth factors that ensured the full activities of VEGF in retinal cells.

In conclusion, we have shown that systemic administration of RA regulates retinal VEGF expression and supports retinal vascular development in an OIR mouse model. We propose that systemic administration of RA to extremely low birth weight, preterm infants during oxygen therapy could potentially be an effective therapeutic approach for the prevention of ROP.

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References


