The role of VEGF and IGF-1 in a hypercarbic oxygen-induced retinopathy rat model of ROP

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Purpose: We have previously described a severe form of oxygen-induced retinopathy (OIR) in the neonatal rat, analogous to human retinopathy of prematurity (ROP), where carbon dioxide is added to the inspired environment (hypercarbic OIR). We studied the time course of emergence and resolution of neovascularization (NV) in normocarbic OIR and hypercarbic OIR and the associated changes in VEGF and IGF-1 mRNA levels in hypercarbic OIR.

Methods: 550 newborn Sprague-Dawley rats were raised in 22 expanded litters of 25. Beginning at day 1 of life, rats were exposed to 7 daily cycles of hyperoxia (80% O₂, 20.5 h) and hypoxia (10% O₂, 0.5 h) with a gradual return to 80% O₂ over 3 h. Inspired CO₂ was maintained at 0.2% for 200 rats (normocarbic OIR) and 10% for 100 rats (hypercarbic OIR). Rats were sacrificed after a subsequent 5 day room air recovery period. An additional 250 rats raised in room air served as age matched controls. Retinae from left eyes were dissected and flatmounts were ADPase-stained. The presence and severity of NV was scored in a masked manner. Right eyes in hypercarbic OIR litters and room air controls were processed for analysis of VEGF and IGF-1 mRNA.

Results: In normocarbic OIR, NV started to emerge before room air recovery began at day 8. It was maximal at day 10 and resolved by day 20. In hypercarbic OIR, a similar pattern was seen, with emergence prior to day 8, peak at day 13 and resolution by day 20. In hypercarbic OIR, retinal VEGF mRNA was decreased at day 8 and increased at day 10 compared to room air controls, correlating with maximal NV. Retinal IGF-1 mRNA was not increased at any time in hypercarbic OIR compared to room air controls.

Conclusions: Neovascularization resulting from normocarbic OIR or hypercarbic OIR occurs before room air recovery. Retinal VEGF mRNA was downregulated and subsequently upregulated prior to maximal NV in hypercarbic OIR. Neovascularization in the hypercarbic OIR model does not appear to be associated with increased retinal IGF-1 mRNA.

Oxygen-induced retinopathy (OIR) in the neonatal rat provides a model for retinopathy of prematurity (ROP) and other diseases characterized by abnormal retinal angiogenesis. We have previously described a severe form of OIR in the neonatal rat, where carbon dioxide is added to the inspired environment [1]. We will refer to this model as hypercarbic oxygen-induced retinopathy (Hypercarbic OIR).

The neonatal rat OIR model has been used extensively by several laboratories [1-5]. The timing of the emergence and resolution of neovascularization (NV) has not been the focus of previous studies. In the first part of the present study, we characterized the time course of emergence and resolution of NV in a normocarbic OIR model [2] and a more severe hypercarbic OIR model [1].

Vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF-1) have been implicated in abnormal retinal angiogenesis. In both the mouse and rat OIR models, other laboratories have reported an increase in retinal VEGF mRNA and protein associated with the emergence of NV [6-8]. While VEGF’s role in mouse and rat OIR models has been well characterized, the role of IGF-1 is less well understood [9]. IGF-1 has been shown to act synergistically with VEGF to increase angiogenesis [10]. This increase in angiogenesis is in part due to the ability of IGF-1 receptor to increase VEGF’s activation of mitogen-activated protein kinase [11]. Knockouts or antagonists of IGF-1 receptor have been shown to be protective against neovascularization [11,12]. In premature infants, a low serum IGF-1 level in the early postnatal period with a subsequent increase, has been associated with severe ROP [9,13].

In the second part of our study, we evaluated the expression of VEGF and IGF-1 mRNA in our more severe hypercarbic OIR model. We hypothesized that the emergence of NV in hypercarbic OIR would be associated with increased expression of retinal VEGF and IGF-1 mRNA levels.

METHODS

All experiments were performed in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at our institution.

Animals: Pregnant Sprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN). Newborn pups from mothers delivering on the same day were mixed and randomly assigned within 24 h of birth to expanded litters of 25. One mother was assigned to each litter for the entire study.
Mothers received standard laboratory diet and water ad libitum. Light was cycled on a 12 h on, 12 h off schedule and the room temperature was maintained at approximately 21 °C. We have previously used such expanded litters for our neonatal rat studies [1,14-19] and have reported an increased incidence and severity of NV in large litters due to postnatal growth retardation [2,20].

**Normocarbic OIR model:** Neonatal rats were raised in 8 expanded litters of 25 each (n=200). Litters were housed in plastic chambers (18x21x18 inch, PlasLabs, Lansing, MI) in which oxygen and carbon dioxide levels were monitored and controlled by an OpMosphere (model A42X03, BioSpherix Ltd., Redfield, NY). As in previous OIR studies in our laboratory [1,2,18], on day 1 of life, litters received 7 daily cycles of oxygen (a gradual increase from 21% to 80% O₂ over 3 h, 80% O₂ for 20.5 h, and a rapid drop to 10% O₂ which was held for 0.5 h). We will refer to this model as “normocarbic OIR” to distinguish it from “hypercarbic OIR.”

Two litters were sacrificed at day 8 (while the animals were still in 80% oxygen), two on day 10 (after 2 days of room air recovery), two on day 13 (after 5 days of room air recovery) and two on day 20 (after 12 days of room air recovery). Time points were chosen from previous studies suggesting that NV was commonly seen at day 13 [1,2,14-17].

**Hypercarbic OIR model:** Neonatal rats were raised in four expanded litters of 25 each (n=100). These litters received 10% inspired CO₂ in addition to the cyclic hyperoxia and hypoxia described above for normocarbic OIR [1]. The rats were similarly recovered in room air. One litter of rats was sacrificed on day 8, day 10, day 13, and day 20, as described for the normocarbic OIR study.

**Room air controls:** An additional 250 rats in 10 expanded litters were raised in room air to serve as age matched controls for grading as well as mRNA expression. Litters were sacrificed as described for normocarbic OIR at day 8 (two litters), day 10 (two litters), day 13 (three litters) and day 20 (three litters).

**Retinal analysis of neovascularization:** At the end of each study, all rats were deeply anesthetized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (15 mg/kg). To evaluate vessel morphology, left eyes were removed and fixed with 10% neutral buffered formalin for 90 min at 4 °C. The cornea, lens, and vitreous were surgically removed and the retina was dissected and flatmounted. Retinae were processed for magnesium-activated adenosine diphosphatase (ADPase) staining as described by Lutty and McLeod [21]. ADPase-stained retinae were flatmounted on microscope slides in Aquamount (Lerner Laboratories, Pittsburgh, PA) with a coverslip. ADPase-stained retinae were graded by an independent examiner for NV in a masked manner using a standard method previously validated in our laboratory [18]. Room air control retinae (n=191) were included in the masked evaluation of NV to reduce the bias toward false positives.

Neovascularization was defined as clumps, sheets, or tufts of endothelial cells morphologically distinct from the normal vasculature, arising at the junction of the vascular and avascular retina [2,15-18]. Retinae were scored for the severity of NV by counting the number of clock hours containing NV by an independent masked observer (Figure 1) [18]. Cross sectional histology was not performed in this study due to the high correlation of our grading method to the number of cells above the inner limiting membrane of the retina [18].

**Total RNA isolation from individual retinae:** We have previously demonstrated that there is close correlation between the occurrence and severity of NV in paired right and left eyes [18]. Since left eyes were flatmounted and stained with ADPase, we could not use those retinae for analysis of mRNA.

Right eyes were removed from anesthetized rats and retinae were dissected in chilled PBS. Vitreous and ciliary bodies were removed and the retina was snap frozen in liquid nitrogen. Selected retinae with varying degrees of NV were homogenized in 0.8 mL TriPure™ isolation reagent (Roche Molecular Biochemicals, Indianapolis, IN) that included 200 µg of glycogen. Total RNA was treated with DNase I (10 units, Roche Molecular Biochemicals, Indianapolis, IN) for 30 min at 37 °C followed by heat inactivation (65 °C, 15 min). RNA was further purified by two acid phenol:chloroform extractions (5:1, pH 4.5, Ambion RNA Diagnostics, Austin, TX) followed by a chloroform:isoamyl alcohol extraction (24:1, Invitrogen, Carlsbad, CA). RNA was precipitated overnight at -20 °C.

From the hypercarbic OIR litters, all retinae from animals with NV (two from day 8, 12 from day 10, nine from day 13, and one from day 20) and a random sample of retinae from animals without NV (15 from day 8, three from day 10, four from day 13, and 10 from day 20) were processed for
mRNA analysis. Age matched room air control retinas were also processed for mRNA (six from day 8, six from day 10, 12 from day 13, and 18 from day 20).

**Northern blot analysis:** Total RNA (10 µg) from individual retinas in hypercarbic OIR and corresponding room air control litters was separated by electrophoresis on a 1.2% agarose, 2.2 M formaldehyde gel and transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) by rapid downward transfer (Turboblotter, Schleicher & Schuell). Each gel contained an aliquot of pooled RNA from 3 day room air control rats to normalize the hybridization signal between gels. The transferred RNA was probed with α-32 P-dATP and α-32 P-dCTP Radprime labeled (Invitrogen, Carlsbad, CA) cDNA probe of the 164 amino acid form of rat VEGF (nucleotides 1 to 577, GenBank accession number M32167; gift from P. Tofilon) [22] or IGF-1 (nucleotides 824 to 1158, GenBank accession number M15481) [23]. Membranes were washed for 20 min in 1X SSC, 0.1% SDS at 50 °C followed by a second wash for 20 min with 0.1X SSC, 0.1% SDS at 50 °C. Blots were exposed to a phosphorimager screen and expression was quantified using ImageQuant software (version 1.11; Molecular Dynamics [Amersham], Piscataway, NJ). For VEGF, the bands appearing at approximately 4.5 kb were quantified. Total RNA content was normalized between samples by probing blots with a labeled DNA probe for rat 36B4 (ribosomal protein P0; nucleotides 136 to 969; GenBank accession number X15096) [24]. A similar approach has been used by other groups studying VEGF [6].

VEGF mRNA expression levels were represented as a ratio of VEGF signal to 36B4 signal in arbitrary units and normalized relative to the 3-day control VEGF/36B4 ratio on the same blot. IGF-1 mRNA expression levels were represented in a similar manner, using 3-day controls across gels.

**Quantitative real time RT-PCR:** Single retinal mRNA expression for both VEGF and IGF-1 was also determined by quantitative real time reverse transcriptase PCR (qRT-PCR). First strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturers instructions from 1 µg total RNA primed with oligo dT. PCR reactions were set up using the FastStart master SYBR Green I reagents (Roche Molecular Biochemicals, Indianapolis, IN), 2 µl of cDNA and 10 pmol (VEGF and IGF-1) or 8.5 pmol (36B4) PCR primers. PCR was performed on a LightCycler (Roche Molecular Biochemicals, Indianapolis, IN) and quantified using RelQuant relative quantitation software (version 1.01; Roche Molecular Biochemicals, Indianapolis, IN). The following primers were used: VEGF 3' UTR 5'; 5'-CAG AAA CAC GAC AAA CCC ATC C-3'; VEGF 3' UTR 3'; 5'-TAA GCC ACT CAC ACA CAC AGC C-3'; IGF-1 5'; 5'-CTC TGG TTC ACC TTT ACC AC-3'; IGF-1 3'; 5'-TTT CCT CTA CCT CTA CTT C-3' and 36B4 5'; 5'-CCG TGT GAG TGT ACA CTA CC-3'; 36B4 3'; 5'-GTA GTC AGT CTC CAC AGA CAA AGC-3'. 36B4 was used as a reference gene and was amplified in a separate reaction at the same time as either VEGF or IGF-1. mRNA concentrations were reported as a ratio of VEGF or IGF-1 to 36B4 and compared relative to an analogous ratio of a 3 day control for VEGF, and 13 day control for IGF-1, included in the same run. This allowed samples to be compared across runs. Representative PCR products were sequenced to confirm specific amplification of desired products.

**Statistical analysis:** Comparisons of incidence between times were made with the Fisher exact tests. Comparisons of severity of NV and levels of VEGF mRNA and IGF-1 mRNA were made using Wilcoxon tests. Bonferroni corrected p values less than 0.05 were considered statistically significant.

# RESULTS

**Animal survival and retinai analyzed for neovascularization:** As in our previous studies using expanded litters, not all rats survived. Rates of survival for the normocarbic OIR study and hypercarbic OIR study are shown in Table 1. Data from rats surviving to the completion of each study were used for all analyses. Five (1%) of 388 ADPase-stained retinae were ungradable and were therefore excluded from analysis (3 from the normocarbic OIR study and 2 from the hypercarbic OIR study).

**Incidence and severity of neovascularization:** For the normocarbic OIR study, preretinal NV was observed in 4 (12%) retinae at day 8, 15 (52%) at day 10, 11 (31%) at day 13, and 0 (0%) at day 20 (Figure 2A). In the hypercarbic OIR study, preretinal NV was observed in 2 (12%) retinae at day 8, 12 (75%) at day 10, 9 (69%) at day 13 and 1 (8%) at day 20 (Figure 2B). Only 2 (1%) of 191 of the age matched room air control retinae were graded as positive. For the normocarbic OIR study, preretinal NV, when present, ranged from 1-3 clock hours on day 8, 1-7 clock hours on day 10, and 1-6 clock hours on day 13 (Figure 3A). In the hypercarbic OIR study, preretinal NV, when present, ranged from 1 clock hour on day 8, 1-5 clock hours on day 10, 1-7 clock hours on day 13 and 1 clock hour on day 20 (Figure 3B).

**Single retinal VEGF mRNA:** In room air controls, retinal VEGF mRNA expression increased over time from day 8 to day 20 by both Northern blot analysis (Figure 4 and Figure 5A) and qRT-PCR (Figure 5B). Comparing hypercarbic OIR and controls at each time, VEGF mRNA levels were decreased in hypercarbic OIR relative to controls at day 8 (p=0.0004 by Northern and qRT-PCR) and increased on day 10 (p=0.0005

### Table 1. Rat survival and incidence of neovascularization

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Total rats</th>
<th>Survival</th>
<th>Number with NV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normocarbic OIR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 days</td>
<td>50</td>
<td>33 (66%)</td>
<td>4 (12%)</td>
<td></td>
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<tr>
<td>10 days</td>
<td>50</td>
<td>32 (64%)</td>
<td>15 (52%)</td>
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<tr>
<td>13 days</td>
<td>50</td>
<td>37 (74%)</td>
<td>11 (31%)</td>
<td></td>
</tr>
<tr>
<td>20 days</td>
<td>50</td>
<td>35 (70%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Hypercarbic OIR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 days</td>
<td>25</td>
<td>18 (72%)</td>
<td>2 (12%)</td>
<td></td>
</tr>
<tr>
<td>10 days</td>
<td>25</td>
<td>16 (64%)</td>
<td>12 (75%)</td>
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<tr>
<td>13 days</td>
<td>25</td>
<td>14 (56%)</td>
<td>9 (69%)</td>
<td></td>
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<tr>
<td>20 days</td>
<td>25</td>
<td>13 (52%)</td>
<td>1 (8%)</td>
<td></td>
</tr>
<tr>
<td>Room air controls</td>
<td>8 days</td>
<td>50</td>
<td>40 (80%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>10 days</td>
<td>50</td>
<td>40 (80%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>13 days</td>
<td>75</td>
<td>54 (72%)</td>
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<tr>
<td>20 days</td>
<td>75</td>
<td>57 (76%)</td>
<td>0 (0%)</td>
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</tbody>
</table>

Rat survival and incidence of neovascularization in normocarbic OIR, hypercarbic OIR, and room air controls.
by Northern and \( p=0.006 \) by qRT-PCR), which corresponds to the times just prior to maximal neovascularization. In addition, qRT-PCR indicated a significant increase in VEGF mRNA in hypercarbic OIR retinae at day 13 \( (p=0.0001) \). VEGF mRNA levels had returned to near normal control levels by day 20 as measured by both Northern analysis and qRT-PCR.

**Single retinal IGF-1 mRNA:** In room air controls, retinal IGF-1 mRNA expression increased over time from day 8 to day 13 in age matched room air controls as measured by both Northern blot analysis (Figure 4 and Figure 6A) and real time RT-PCR (Figure 6B). IGF-1 mRNA levels plateaued (Northern) or slightly decreased (qRT-PCR, \( p=0.0001 \)) between days 13 and 20.

Comparing hypercarbic OIR and age matched room air controls at each time, there was some discrepancy between Northern blot analysis and qRT-PCR. By Northern blot analysis, IGF-1 mRNA levels were decreased in hypercarbic OIR at days 8 \( (p=0.002) \), 10 \( (p=0.007) \) and 13 \( (p=0.05) \) but returned to normal by day 20 (Figure 6A). By qRT-PCR, IGF-1 mRNA levels were decreased in hypercarbic OIR only at day 8 \( (p=0.02) \) and had returned to normal by day 10, remaining unchanged from control values at days 13 and 20. At no time was retinal IGF-1 mRNA increased above control levels by either Northern blot analysis or qRT-PCR.

**Correlations between mRNA and neovascularization:** Single retinal analysis allows evaluation of NV and retinal mRNA at an individual animal level, specifically in animals with and without NV. On day 13, animals with NV had higher retinal VEGF mRNA by Northern blot (Figure 7A). On day 13, retinal VEGF mRNA levels demonstrated a weak but positive correlation with severity of NV in the contralateral eye \( (R=0.63, p=0.03, \text{Northern}, \text{Figure } 8A; R=0.60, p=0.04, \text{qRT-PCR, Figure } 8B) \). No significant correlations were found at any time between retinal IGF-1 mRNA and severity of NV by Northern blot analysis or qRT-PCR.

**DISCUSSION**

In the present study, we found that in both normocarbic OIR and hypercarbic OIR, NV emerges prior to room air recovery and is maximal after 2-5 days of room air recovery. Neovascularization almost completely resolved after 12 days of room air recovery (by day 20 of life). We studied the role of
retinal VEGF mRNA and IGF-1 mRNA in hypercarbic OIR and found that maximal NV was associated with increased retinal VEGF mRNA, but retinal IGF-1 was not increased at any time compared to room air controls.

The emergence of NV prior to room air recovery in OIR models is a phenomenon that has not previously been well characterized. In previous studies, investigators have typically studied NV after a period of room air recovery [1,3-5]. Our finding of NV on the last day of cyclic oxygen is analogous to clinical ROP where stage 3 ROP can occur while an infant is still on oxygen. Although hyperoxia may still be the triggering insult, the cycles of hypoxia or normoxia may be sufficient to allow temporary “recovery” from hyperoxic insult and initiation of preretinal NV. Our findings of preretinal NV prior to room air recovery provides support for using the neonatal rat as a clinically meaningful model for stage 3 ROP.

The spontaneous resolution of NV in this model for OIR differs from what has been previously observed [25], but few details of the time course have previously been reported. This resolution does not necessarily limit the usefulness of this model in studying NV, but, in experimental treatment trials, the timing of treatment delivery and the timing of the retinal evaluation must account for this time course. Our data are useful to those using this model to investigate agents that might reduce NV. In addition, analysis of growth factors and other molecular mediators during the resolution phase may be useful in elucidating new avenues of research directed at anti-angiogenesis.

In the hypercarbic OIR model used in the present study, 10% CO₂ was added to the inspired gaseous environment. We previously found an increased incidence and severity of NV in hypercarbic conditions [1]. Although hypercarbia has been implicated as a risk factor for ROP [15,26-29], some clinical studies report neither blood CO₂ tension nor duration of hypercarbia or hypocarbia during the first 2 weeks of life to be associated with development or severity of ROP [30]. Such clinical studies have the disadvantage of trying to simultaneously assess the role of multiple risk factors. Animal studies such as ours have the advantage of studying a single variable, such as hypercarbia, while keeping other variables constant. We have previously speculated that hypercarbia acts via acidosis [15,16], although we cannot rule out the potential effect of carboxy radicals or the role of local vasodilation and increased local delivery of oxygen.

Figure 4. Representative Northern blots for VEGF and IGF-1. Representative phosphorimages of Northern blots shown for VEGF (top), IGF-1 (middle), and 36B4 (bottom). Expression was quantified using ImageQuant software. Total RNA content was normalized between samples to 36B4. VEGF or IGF-1 mRNA expression levels were represented as a ratio of VEGF or IGF-1 signal to 36B4 signal in arbitrary units and normalized relative to the 3-day control VEGF/36B4 or IGF-1/36B4 ratio on the same blot (note first lane).

Figure 5. Retinal VEGF mRNA expression in hypercarbic OIR. Retinal VEGF mRNA in hypercarbic OIR measured by Northern blot analysis (A) and qRT-PCR (B). Retinal VEGF mRNA levels were reduced on day 8 (while in high oxygen) and increased on day 10 (following 2 days of room air recovery) compared to age matched room air controls by both methods. qRT-PCR continued to show a significant increase of retinal VEGF mRNA levels at 13 days. VEGF mRNA had returned to normal levels by day 20. P values are for comparisons of OIR and room air controls at each time. Non-significant comparisons are labelled “NS” (p>0.05).
The reduction of retinal VEGF mRNA on the last day of cyclic oxygen is similar to the findings of Pierce et al. in the mouse model of OIR at the end of a period of constant oxygen exposure [31]. Our finding of the emergence of NV at this time may at first be difficult to reconcile with a depressed level of VEGF. We speculate that VEGF may transiently increase during the daily hypoxic periods of the cyclic oxygen regime (10% O2 for 30 min per day). Further studies would be needed to confirm or refute this hypothesis. Even if this hypothesis is correct, the periods of VEGF upregulation may be only brief and difficult to quantitate. An alternative hypothesis is that non-VEGF pathways may mediate this early NV.

Regarding retinal IGF-1 mRNA, at no time did we find increased retinal IGF-1 mRNA in our hypercarbic OIR model, compared to age matched control retinae. Other investigators [32] have reported a decrease in retinal IGF-1 mRNA under conditions of hypoxia but not hyperoxia. Based on our results, we conclude that NV in OIR is not associated directly by increased retinal IGF-1 mRNA. It is possible that the decrease of IGF-1 mRNA within the retina reflects the effects of overall growth retardation in rat pups exposed to hypercarbic cyclic oxygen, and further studies are warranted to examine other organs in these growth-retarded rat pups.

While we found no increase in retinal IGF-1 mRNA during NV, we did not study serum levels of IGF-1 protein, nor did we measure the levels of IGF-1 protein within the retina. Retinal NV has been associated with a relative increase in vitreous IGF-1 protein in the analogous condition of diabetic retinopathy [33]. Although IGF-1 has been shown to increase the activity of VEGF, studies of prematures with ROP [9,13] have suggested that decreased serum levels of IGF-1 protein early in postnatal developments are associated with later NV in ROP. One mechanism of reduced IGF-1 causing NV might be its effect on normal retinal vascular development. Reduced serum IGF-1 may slow retinal vascular development, resulting in a larger peripheral avascular area, which in turn may lead to increased VEGF. Further work on the time course of serum and retinal IGF-1 protein levels and their relationship to NV in this model is warranted. Our study would have been strengthened by measuring protein levels of IGF-1 in the serum and retina. Due to the differences in retinal processing for mRNA and protein, all experiments would have needed to be duplicated. Further elaboration of retinal protein levels in this model is warranted and remains the subject of further study.

In addition to a direct effect on normal and abnormal angiogenesis, IGF-1 may act synergistically with VEGF in promoting NV. VEGF protein expression has been shown to increase in retinal pigment epithelium (RPE) cells in vitro upon the addition of IGF-1 in models of hypoxia-induced VEGF expression [34]. Treatment of tumor cell lines with IGF-1 resulted in increased VEGF protein expression by increasing the half-life of the mRNA [35]. This synergistic role of VEGF and IGF-1 in vitro has been confirmed by other investigators [10]. A similar synergy between VEGF and IGF-1 may exist in the hypercarbic OIR model and in ROP, though we were unable to demonstrate an increase in absolute IGF-1 mRNA expression within the retina.

We used single retinae for individual analysis of retinal mRNA levels, which allowed us to look at relationships in individual animals between severity of NV in one eye and retinal growth factor mRNA levels in the contralateral eye.

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Figure 6. Retinal IGF-1 mRNA expression in hypercarbic OIR. Retinal IGF-1 mRNA in hypercarbic OIR measured by Northern blot analysis (A) and qRT-PCR (B). Retinal IGF-1 mRNA was decreased by Northern blot analysis at days 8, 10, and 13 but returned to normal by day 20. Retinal IGF-1 mRNA was decreased by qRT-PCR at day 8, returned to normal by day 10, and remained unchanged from control values at days 13 and 20. P values are for comparisons of OIR and room air controls at each time. Non-significant comparisons are labelled “NS” (p>0.05).

Figure 7. Retinal VEGF mRNA expression in retinae with and without neovascularization. Median expression of retinal VEGF mRNA in eyes of rats with and without neovascularization by Northern blot analysis (A) and qRT-PCR (B). Retinal VEGF mRNA was higher (p=0.02) on day 13 by Northern blot analysis in eyes of rats with neovascularization in the contralateral eye than without neovascularization. There were no other times demonstrating a statistically significant difference between rats with and without neovascularization (NV).
Previous studies of OIR in rodent models have largely relied on pooled mRNA samples. Among hyperoxic OIR rats, although retinal VEGF mRNA levels were on average higher than room air controls, the only difference we found was between animals with and without NV was a increase in retinal VEGF mRNA at day 13. Although some comparisons are limited by low statistical power, our data suggests there are other factors besides VEGF and IGF-1 that may play a role in the development of NV. It is possible that such factors may influence post-transcriptional expression and/or activity of these two growth factors or may be independent.

When we compared levels of VEGF and IGF-1 mRNA by Northern blot versus qRT-PCR we found slight discrepancies. These differences may be attributable to the more sensitive nature of qRT-PCR, especially for lower abundance messages. Although differences in absolute values and resultant significance were observed, overall trends in mRNA levels were similar between the two methods.

In summary, we have characterized the time course of NV in two different rat models of oxygen-induced retinopathy, normoxic OIR and hyperoxic OIR. These data are useful when designing experiments to investigate the relationship of various angiogenic or anti-angiogenic factors to the presence and/or severity of NV in rat models of ROP. In addition, we have temporally correlated the emergence and resolution of NV with VEGF and IGF-1 mRNA expression in the hyperoxic OIR model. Retinal VEGF mRNA was downregulated and subsequently upregulated prior to maximal NV in hyperoxic OIR. Neovascularization in the hyperoxic OIR model does not appear to be mediated directly by increased retinal IGF-1 mRNA.

Figure 8. Correlation of retinal VEGF mRNA to neovascularization severity. Correlation of retinal VEGF mRNA to neovascularization severity (clock hours) in contralateral eyes of rats in hyperoxic OIR litters on day 13 by Northern blot analysis (A) and qRT-PCR (B). Positive correlations (Spearman) were seen between retinal VEGF mRNA levels and severity of neovascularization by both techniques. NV refers to neovascularization.

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