VEGF Mediated STAT3 Activation Contributes to Retinal Avascularity in a Rat Model of ROP

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Purpose
To investigate 1) whether upregulated vascular endothelial growth factor (VEGF) following repeated fluctuations in oxygen in the 50/10 oxygen-induced retinopathy (50/10 OIR) rat model of retinopathy of prematurity (ROP) contributes to avascular retina through activation of signal transducer and activator of transcription-3 (STAT3), and 2) the molecular mechanisms involved.

Introduction
ROP is one of the earliest and most prevalent causes of childhood visual impairment and blindness in the US and worldwide. The pathogenesis of ROP is associated with intravitreal neovascularization (INNV), new vessels that grow into the vitreous at the junctions of vascularized retina and “avascular” retina, and these can lead to retinal detachment and blindness. The avascular retina has been believed to be the source of angiogenic factors that cause INNV.

Several growth factors have been implicated in the pathogenesis of ROP, such as vascular endothelial growth factor (VEGF). In the rat 50/10 OIR model, increased VEGF signaling induced by repeated fluctuations in oxygen led to both avascular and hypoxic retina2 that contributed to the later development of INNV. Activation of NADPH oxidase following rescue in supplemental oxygen contributed to INNV via the JAK/STAT pathway3. The JAK/STAT pathway can be activated by certain cytokines and factors that bind membrane receptors to mediate cell proliferation, differentiation, migration, and apoptosis. Erythropoietin (EPO) is one factor that can activate JAK/STAT and was reported as a molecular target causing INNV. However, if delivered during growth factor-reduced, hypoxia-induced capillary constriction, exogenous EPO promoted physiologic retinal vascularization and actually reduced subsequent INNV. Greater understanding may lead to a strategy to promote physiologic vascularization and inhibit abnormal angiogenesis.

Methods
1. 50/10 Oxygen-Induced Fluctuations Rat Model (50/10 OIR model): As reported1 pups and their mothers were placed into the incubator, which cycled oxygen between 50% and 10% every 24 hour for 14 days. Litter numbers were between 12 and 14 pups for each experiment to assure consistency in outcomes.
2. Animals treated with AG490, EPO and VEGF antibody. Rat pups in the ROP Model were treated with 10 mg/kg AG490 (LC Laboratories), an inhibitor of JAK/STAT signaling, via intraperitoneal (IP) injections administered daily from postnatal day (p) 3 to p13. Separate litters received IP injections of EPO (41.6 µg/kg) or PBS control at p2, p4 and p6. Anti-VEGF antibody was given to rat pups by intraperitoneal injection at p12. Pups were removed from cycling for less than 30 minutes for treatments. Retinas from one eye of each pup were processed for protein and western blotting, and from the other eye for lectin-stained flat mounts.
3. Cell culture: Rat Müller cells (kindly provided by Dr. Varthy) were maintained in DMEM-high glucose, 4500 mg/L Sigma) with 10% FBS.
4. Protein Extraction and Western Blotting: Previously frozen retinal samples were homogenized in modified radio immune precipitation assay (RIPA) buffer with protease cocktail inhibitor and orthovanadate. Fifty µg of total protein for each sample was separated by NuPAGE® 4-12% Bis-Tris Gels, transferred to a PVDF membrane, and incubated with primary antibodies overnight at 4°C. All membranes were reprobed with β-actin to ensure equal protein loading.

Results

Figure 1. Retinal STAT3 activity increases in 50/10 OIR Model; inhibition of STAT3 activity reduces avascular retina. (A) phosphorylation of STAT3 in retinas from pups in Room Air or 50/10 OIR model. (B) phosphorylation of STAT3 was inhibited in retinas from pups with AG490 injection; (C) avascular retinal area was decreased in pups with AG490 injection (retinal flat mount). All data are means S.E.M. (*p<0.05, **p<0.001 and ***p<0.0001 vs. corresponding control).

Figure 2. Inhibition of STAT3 increases EPO production (A) without affecting VEGF expression and caspase 3 activity (B) in retina from pups in 50/10 OIR model with AG490. All data are shown as means S.E.M. *p<0.05, vs. PBS.

Figure 3. Inhibition of VEGF activity decreases p-STAT3 (A) and causes an increase in EPO expression (B) in retina from pups in 50/10 OIR model with anti-VEGF. All data are shown as means S.E.M. *p<0.05, ***p<0.001 vs. Vab Non; **p<0.01 vs. Vab Non; #p<0.05, ##p<0.001 vs IgG Inj (n=6).

Figure 4. Retinal p-STAT3 increased in Muller cells in 50/10 OIR model; VEGF activates STAT3 and inhibits EPO gene expression via JAK/STAT signaling in rat Muller cells (rMC). (A) p-STAT3 colocalized with Muller cell marker glutamine synthetase in retinas in 50/10 OIR model by IHC (Blue represents Hoescht 33342, Red represents anti-GS, green represents anti-pSTAT3). (B) p-STAT3 at Tyr 705 and Ser 727 was measured in rMC incubated with 20 ng/ml of VEGF for durations: 0, 5, 15, 20, 30 and 60 mins; (C) EPO mRNA was measured in rMC treated with AG490, anti-VEGF antibody (Vab) or control IgG by real time PCR. **p<0.05, ***p<0.0001 vs Con; #p<0.01 vs Vab; ##p<0.001 vs IgG+AG.

Figure 5. Exogenous EPO administered by IP injection reduces avascular area of retina in 50/10 OIR model. The avascular/total area of retina in rats treated with IP EPO or PBS from the 50/10 OIR model was analyzed. All data are shown as means S.E.M. *p<0.05, vs. PBS.

Conclusions
1. VEGF-mediated STAT3 signaling contributes to retinal avascularity in the rat 50/10 OIR model of ROP.
2. Increased VEGF by repeated fluctuations in oxygen inhibits angiogenic factor EPO in retina through JAK/STAT3 signaling.
3. Exogenous EPO reduces retinal avascularity in the 50/10 OIR model.

References

Acknowledgements
Supported by Research to Prevent Blindness

R01 5R01EY015130 MEH. R01EY017011MEH and MOD 6-FY08-590 MEH (PI: MEH).

Financial Disclosures: None