328 Mechanisms in retinal angiogenesis and retinopathy
Tuesday, May 06, 2014 11:00 AM–12:45 PM
S 310E-H  Paper Session
Program #/Board #  Range:  3014–3020
Organizing Section:  Retinal Cell Biology

Program Number:  3014
Presentation Time:  11:00 AM–11:15 AM
Changes in retinal vessel caliber with flicker light stimulation in eyes with diabetic retinopathy
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Purpose: Changes in retinal vessel calibre in response to flickering light are believed to be mediated by nitric oxide release from the retinal microvascular endothelium. This study investigated the responses of retinal vessels to flickering light in diabetic patients with various grades of diabetic retinopathy (DR).

Methods: This cross-sectional observational study evaluated adult subjects with diabetes mellitus. The Dynamic Vessel Analyser (DVA) was used to measure retinal vascular responses to diffuse illuminance flicker. DR was graded from retinal photography. Each eye was assigned a retinopathy severity score according to the modified Airlie House classification system, and categorized as minimal nonproliferative diabetic retinopathy (NPDR), mild NPDR, moderate NPDR, severe NPDR, or proliferative retinopathy. Eyes were also classified as having any DR (minimal NPDR or worse), moderate DR (moderate NPDR or worse), or vision-threatening DR (severe NPDR or worse, or clinically significant macular edema) according to the Eye Diseases Prevalence Research Group definitions.

Results: There were 279 subjects in total, with a mean age of 59.9±9.2 years. The majority were male (73%) and the mean HbA1c level and mean duration of diabetes were 7.7±1.4% and 13.9±10.4 years respectively. After adjustments for age, sex, smoking, duration of diabetes, HbA1c, hypertension and hyperlipidemia, retinal arteriolar and venular dilation responses to flicker stimulation decreased continuously with increasing severity of diabetic retinopathy, (p = 0.008 and <0.001 respectively). Subjects with reduced arteriolar dilation responses were more likely to have any DR [odds ratio (OR) 1.20 (95% confidence interval 1.01 – 1.45) per standard deviation (SD) decrease, p=0.045]. Subjects with reduced venular dilation responses were more likely to have any DR [OR 1.27 (1.04 – 1.53) per SD decrease, p=0.02], moderate DR [OR 1.27 (1.06 – 1.49) per SD decrease, p = 0.007] and vision-threatening DR [OR 1.51 (1.14 – 1.50) per SD decrease, p = 0.002].

Conclusions: Retinal arteriolar and venular dilation responses to flickering light are diminished in subjects with DR, and decrease progressively with more severe stages of DR. Our findings suggest that the severity of DR is correlated with measurable differences in retinal microvascular endothelial function, supporting a role for the latter in the pathogenesis of DR.

Commercial Relationships: Laurence S. Lim, None; Peng Guan Ong, None; E Shyong Tai, None; Gemmy C. Cheung, None; Wallace S. Foulds, None; Tien Y. Wong, None
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Program Number:  3015
Presentation Time:  11:15 AM–11:30 AM
Amacrine cell-derived VEGF is required for development and maintenance of the retinal vasculature in mice

Purpose: The retinal vasculature of many organisms including humans and mice consists of three distinct plexus layers. While it is clear that the inner retinal vascular layer develops over a pre-existing astrocytic network and that development of vascular and neuronal networks are co-dependent, it is unclear how the outer retinal vascular networks form. As retinal neurons populate the retina and mature, oxygen demands change and activation of the oxygen sensing VHL/HIF-α/VEGF pathway in maturing neurons may be a strong driving force for development and maintenance of the outer plexus layers. In this study, we examined the contribution of amacrine and horizontal cells due to their close proximity to the intermediate and outer retinal vascular layers.

Methods: Transgenic mice expressing Cre recombinase specifically in amacrine and horizontal cells (Ptf1a-Cre mice) were mated with floxed VHL, HIF-1α, HIF-2α and/or VEGF mice to generate conditional knockouts. Amacrine and horizontal cells were genetically ablated using Ptf1a-Cre and forced expression of diphtheria toxin (DT) receptors.

Results: We show that amacrine and horizontal cell processes tightly associate with intermediate and outer plexus retinal capillaries. Pseudo-hypoxia in Ptf1a-Cre; VHL mutants induces formation of a dense intermediate plexus compared to controls, while a dramatically attenuated intermediate plexus is observed in Ptf1a-Cre; VEGF and Ptf1a-Cre; HIF-1α mutants. Co-deletion of HIF-1α, but not HIF-2α, rescued the vascular phenotypes of Ptf1a-Cre; VHL KO mice. Amacrine and horizontal cell ablation by DT injection also suppressed the formation of the intermediate plexus and DT injection after the retinal vasculature had developed resulted in attenuation of the vasculature. In all of these genetic manipulations the deep plexus was less affected.

Conclusions: Dysregulated VEGF release from amacrine and horizontal cells results in formation of a very dense intermediate vascular plexus, while elimination of VEGF (or of amacrine and horizontal cells themselves) prevents its formation. These data demonstrate a novel function of amacrine cells, directing formation of the intermediate plexus layer. Horizontal cells, on the other hand, are likely strictly dependent on the vasculature, but do not determine its formation or maintenance.

Commercial Relationships: Yoshihiko Usui, None; Toshihide Kurihara, None; Peter D. Westenskow, None; Edith Aguilar, None; Liliana P. Paris, None; Stacey K. Moreno, None; Carli M. Wittgrove, None; Daniel Feitelberg, None; Martin Friedlander, None
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Program Number:  3016
Presentation Time:  11:30 AM–11:45 AM
Ataxia telangiectasia mutated (ATM) dysregulation precipitates in diabetic retinopathy
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Purpose: We show that ATM dysregulation precipitates retinal vascular and neuronal changes in diabetes. Recent studies have demonstrated that ATM is a key regulator of the VEGF pathways, suggesting that ATM dysregulation could cause VEGF-mediated changes in the retinal vasculature. This work extends these findings to diabetic retinopathy.

Methods: Diabetic retinopathy is commonly associated with retinal vessel abnormalities. Diabetes also increases the risk of developing retinal neovascularization. In this study, we attempted to determine if ATM dysregulation could induce these changes in the retinal vasculature.

Results: We found that ATM dysregulation could induce these changes in the retinal vasculature. ATM dysregulation increased the risk of developing retinal vessel abnormalities. ATM dysregulation also increased the risk of developing retinal neovascularization.

Conclusions: Our findings suggest that ATM dysregulation could be a key factor in the development of diabetic retinopathy.

Commercial Relationships: Ashay D. Bhatwadekar, None; Maria Korah, None; Sergio Caballero, None; Justin Baas, None; Maria Grant, None
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**Purpose:** Diabetic retinopathy (DR) is a vasodegenerative condition with apoptosis of endothelial cells and pericytes resulting in widespread areas of ischemia. Contrary to the belief that duration of diabetes is one of the strongest predictors for development of DR, we identified a unique cohort of patients who in spite of long-standing (>40 yrs) poorly controlled diabetes, remained free of DR. We reasoned that hematopoietic stem cells (HSCs) and vascular progenitors were unique in this group of patients. Microarray analysis of HSCs from this ‘protected cohort’ revealed upregulation of tumor suppressor protein ataxia telangiectasia mutated (ATM). We hypothesized that the loss of ATM in bone marrow HSCs could result in inadequate retinal repair and accelerated development of DR.

**Methods:** We developed gender mismatched mouse chimeras in which hematopoietic tissue of wild type mice was replaced with that from ATM−/− mice, WT. ATM−/− chimeras. These chimeras were sacrificed 6 months post induction of diabetes with streptozotocin and tissues were harvested for further analysis. Long-term repopulating (LTR) and short-term repopulating (STR)-HSCs were evaluated as lin-Sca1+ c-kit+CD34− and lin-Sca1+ c-kit+CD34+ cells respectively using flow cytometry. Retinas were processed for trypsin digestion to evaluate the degree of DR and femurs were embedded to quantify the numbers of LTR and STR-HSCs.

**Results:** We observed a 50% decrease (p<0.05) in the number of LTR-HSCs in diabetic mice. This decrease in LTR-HSCs was further accelerated in diabetic WT.ATM−/− chimeras (p<0.05). The diabetic WT.ATM−/− also showed a tendency of myeloid bias and a 2-fold increase (p<0.05) in STR-HSCs was observed compared to non-diabetic WT.ATM−/− chimeras. Cell cycle analysis further revealed a profound decrease (p<0.05) in quiescent (G0 phase) LTR-HSCs in control and diabetic WT.ATM−/− chimeras. Also, a substantial portion of STR-HSCs was in either G1 or G2 phase, suggesting a complete decline of quiescent HSCs in WT.ATM−/− chimeras. Quantification of retinas in diabetic WT.ATM−/− chimeras showed an accelerated increase in the number of acellular capillaries.

**Conclusions:** In conclusion, our study suggests the critical role of ATM in protecting bone marrow HSCs from diabetic stress and highlights the importance of maintaining long-term repopulating ability of HSCs to participate in retinal vascular repair in DR.

**Commercial Relationships:** Ashay D. Bhatwadekar, None; Maria Korah, None; Sergio Caballero, None; Justin Baas, None; Maria Grant, None

**Support:** Thomas H Maren Junior Investigator Award

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**Program Number:** 3017
**Presentation Time:** 11:45 AM–12:00 PM

The role of 53bp1 and of the endothelial DNA repair cascade in the vasoprolifearative retinopathies

**Purpose:** Hypoxia is central in the vasoprolifearative retinopathies (DR, ROP) as it is the driving force for the endothelial cell (EC) proliferation. On the other hand hypoxia and reoxygenation lead to stalled replication forks and activation the DNA repair machinery leading to replication arrest and apoptosis. The DNA is repaired either by homologous recombination (HR) or non homologous end-joining (NHEJ). During the S Phase of the cell cycle, HR is the main mode of DNA repair. p53 binding protein 1 (53bp1) is a DNA repair factor that promotes NHEJ and competes with BRCA1 (that promotes HR) for the DNA repair mode. Given our previous finding that H2Ax and endothelial DNA repair are critical for hypoxia-driven angiogenesis in retinopathy (Economopoulou et al., Nat Med 2009), we here explored the role of 53bp1 in ROP.

**Methods:** We subjected ECs to hypoxia/reoxygenation (H/Reox) conditions and studied the phosphorylation of 53bp1 (p-53bp1) by IF and WB. In vivo we subjected 53bp1−/− and +/- mice to the ROP model. Their retinas were analysed for neovascularisation, EC proliferation and apoptosis and WB quantification of the DNA repair factors BRCA1 and Rad51.

**Results:** The exposure of ECs to H/Reox conditions resulted in an increase in p-53bp1. Furthermore, H/Reox upregulated other DNA repair factors, mostly involved in HR, like RAD51. The retinas of 53bp1−/− ROP mice showed significantly higher neovascularisation compared to their wt littermates. This was due to higher EC proliferation and lower apoptosis in the retinas of 53bp1−/− mice. Furthermore BRCA1 and Rad51 were significantly upregulated in the retinas of 53bp1−/− ROP mice compared to 53bp1+/- suggesting a higher rate of HR in the 53bp1−/− retinas.

**Conclusions:** Our study shows a novel role of 53bp1 in vasoprolifearative retinopathies. The absence of 53bp1 results in increased neovascularisation due to enhanced EC proliferation and decreased apoptosis in the retinas of ROP mice. We propose that the increase in BRCA1 and Rad51 in the retinas of 53bp1−/− mice lead to a more efficient EC proliferation under H/Reox conditions in the ischemic retina. Intriguingly, our results show an opposite role of 53bp1 to the function of histone H2AX in the ROP. Overall, our study strengthens the notion that the DNA Repair cascade is important in vasoprolifearative retinopathies and identifies the variable effects of different DNA repair proteins in this context.

**Commercial Relationships:** Matina Economopoulou, None; Ria Zengler, None; Lutz E. Pillunat, None; Andre Nussenzeitge, None; Triantafyllos Chavakis, None

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**Program Number:** 3018
**Presentation Time:** 12:00 PM–12:15 PM

**Laminin β2 and γ3 chains regulate microglial activation and the downstream effects of microglia on retinal vascular development**

**Purpose:** Saptarshi Biswas1, 2, Julianne Chu1, 2, Galina Bachay1, 2, Dale D. Hunter1, 2, William J. Brunken1, 2. Ophthalmology, SUNY Downstate Medical Center, Brooklyn, NY; 2SUNY Eye Institute, Brooklyn, NY.

**Program Number:** 3017
**Presentation Time:** 11:45 AM–12:00 PM

The role of 53bp1 and of the endothelial DNA repair cascade in the vasoprolifearative retinopathies

**Purpose:** Laminin β2 and γ3 chains regulate microglial activation and the downstream effects of microglia on retinal vascular development

**Commercial Relationships:** Ashay D. Bhatwadekar, None; Maria Korah, None; Sergio Caballero, None; Justin Baas, None; Maria Grant, None

**Support:** Thomas H Maren Junior Investigator Award

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**Laminin β2 and γ3 chains regulate microglial activation and the downstream effects of microglia on retinal vascular development**

Saptarshi Biswas1, 2, Julianne Chu1, 2, Galina Bachay1, 2, Dale D. Hunter1, 2, William J. Brunken1, 2. Ophthalmology, SUNY Downstate Medical Center, Brooklyn, NY; 2SUNY Eye Institute, Brooklyn, NY.

**Purpose:** Microglia play important roles in vascular plexus formation both by mediating vascular anastomosis and secreting pro- or anti-angiogenic cytokines. Here, we investigated the role of laminin β2 and γ3 chains in recruiting retinal microglia to the developing vascular plexus, subsequent activation of microglia, and the effect of microglial activation states on retinal vascular development.

**Methods:** Global and activated microglia density in different regions of the retina, their association with vascular branch points, and mitotic and apoptotic endothelial cell quantification were analyzed using immunohistochemistry and 3D reconstruction.

**Results:** Previously, we showed that there is an increase in the number of microglia associated with the vascular plexus in the laminin γ3−/- retina. Here, we show that the laminin γ3−/- retina has increased microglia density in the ganglion cell layer (GCL) both centrally and peripherally compared to wild type retina. Moreover, consistent with this in the laminin γ3−/- retina there is an increase in both the vascular branch points and microglia associated with them at the nascent plexus. In the laminin β2−/- retina, the normally orderly arrangement of microglia in the GCL is disrupted, and microglia aggregate around the persistent hyaloid vessels and malformed retinal vasculature. In the laminin γ3−/- retina, more activated microglia are present around the vascular plexus than in the wild type. The increased number of vascular branch points associated microglia in
the laminin γ3-/- retina suggests more anastomotic events, leading to a denser plexus. In contrast, in the laminin β2-/- retina, the number of activated microglia remains the same. The developing vascular plexus of the laminin γ3-/- retina also has increased endothelial cell proliferation compared to the wild type, whereas the number of apoptotic endothelial cells remains unchanged in the laminin γ3-/- retina compared to the wild type.

**Conclusions:** Our results suggest that laminins containing the β2 and γ3 chains differentially regulate distribution of microglia in the retina as well as the recruitment of microglia to the developing vascular plexus with β2 laminins having pro-angiogenic effects and γ3 laminins having anti-angiogenic effects.

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**Program Number:** 3019
**Presentation Time:** 12:15 PM–12:30 PM

**Targeting of calcium/calmodulin-dependent protein kinase II delta and gamma isoforms inhibits growth factor-induced retinal angiogenesis in vitro**

Sadaf Ashraf, Hannah McCauley, Alan W. Stitt, Graham J. McGeown, Tim M. Curtis. Centre for Experimental Medicine, Queens University Belfast, Belfast, United Kingdom.

**Purpose:** Previous studies from our group have shown that calcium/calmodulin-dependent protein kinase II (CaMKII) plays a critical role in VEGF-induced retinal angiogenic signalling. In the present study, we have extended this work to examine the wider contribution of CAMKII signalling to growth factor (GF)-induced retinal angiogenesis in vitro and the specific involvement of the γ and δ isoforms of CAMKII in this process.

**Methods:** Human retinal microvascular endothelial cells (hRMECs) were cultured and stimulated over a 24h period with a range of GFS (vascular endothelial growth factor [VEGF], fibroblast growth factor [FGF], insulin-like growth factor [IGF], hepatocyte growth factor [HGF], placental growth factor [PIGF] and platelet derived growth factor [PDGF]) at 50ng/ml. Total and phosphorylated protein levels of CaMKII were detected using western blotting. The effects of CaMKII inhibition using 10μM KN93 (CaMKII inhibitor) and its inactive analogue KN92 on GF-induced sprouting angiogenesis in vitro were evaluated. Small interference RNA (siRNA; 50nM) mediated knockdown of CaMKIIγ and δ isoforms was used to investigate the relevance of these isoforms in GF-induced retinal endothelial cell migration, tube formation and sprouting angiogenesis.

**Results:** Exposing hRMECs to VEGF, FGF, HGF, IGF and PIGF triggered a time-dependent increase in total and phospho-CAMKII protein levels. In contrast, PDGF had no effect on CAMKII phosphorylation or total CAMKII levels. All GFS with the exception of PIGF and PDGF stimulated sprout formation compared with controls. VEGF, FGF, HGF and IGF also stimulated hRMEC migration and tube formation. KN93 reduced GF-induced sprout formation to control levels, whereas KN92 (inactive analogue) had no effect. siRNA knockdown of CaMKIIβ isoform significantly reduced GF-induced sprouting angiogenesis, migration and tube formation to control levels, whereas siRNA targeting of CaMKIIγ had only a partial effect.

**Conclusions:** These results suggest that both CaMKIIδ and γ isoforms are involved in mediating GF-induced angiogenic activity. CaMKII is thus an important regulator of GF-induced retinal angiogenesis and treatments targeting the γ and δ isoforms of this protein have the potential to reduce abnormal angiogenesis in ocular diseases.

**Commercial Relationships:** Sadaf Ashraf,None;Hannah McCauley,None;Alan W. Stitt,None;Graham J. McGeown,None;Tim M. Curtis,None

**Support:** British Heart Foundation R2828CVS

**Program Number:** 3020
**Presentation Time:** 12:30 PM–12:45 PM

**Deletion of Thioredoxin Interacting Protein (TXNIP) Augments Hyperoxia-induced Vaso-obliteration in Ischemic Retinopathy**

Azza B. El-Remessy, None; Mohammed A. Abdelsaid, None; Adviey Ergul, None; Suraporn Matragoon, None

**Purpose:** We have recently shown that thioredoxin interacting protein (TXNIP) is required for VEGF-mediated VEGFR2 receptor activation and angiogenic signal. Retinas from TXNIP knockout mice (TKO) exhibited higher cellular antioxidant defense compared to wild type (WT). The current study was undertaken to examine the impact of TXNIP deletion on hyperoxia-induced vaso-obliteration in ischemic retinopathy model.

**Methods:** TKO and WT pups were subjected to oxygen-induced retinopathy model. Retinal central capillary dropout was measured at p12. Retinal redox and nitrative state were assessed by reduced-glutathione (GSH), thioredoxin reductase activity and nitrotyrosine formation. Western blot and QF-PCR were used to assess VEGF, VEGFR-2, Akt, iNOS and eNOS, thioredoxin expression, ASK-1 activation and downstream cleaved caspase-3 and PARP in retinal lysates.

**Results:** Retinas from TKO mice exposed to hyperoxia showed significant increases (1.5-fold) in vaso-obliteration as indicated by central capillary drop out area compared to WT. Retinas from TKO showed minimal nitrotyrosine levels (10% of WT) with no change in eNOS or iNOS mRNA expression. There was no change in levels of VEGF or activation of VEGFR2 and its downstream Akt in retinas from TKO and WT. In comparison to WT, retinas from TKO showed significantly higher level of GSH and thioredoxin reductase activity in normoxia but comparable levels under hyperoxia. Exposure of TKO to hyperoxia significantly decreased the anti-apoptotic thioredoxin protein (~50%) level compared with WT. This effect was associated with a significant increase in activation of the apoptotic ASK-1, PARP and caspase-3 pathway.

**Conclusions:** Our results showed that despite comparable VEGF level and signal in TKO, exposure to hyperoxia significantly decreased Trx expression compared to WT. This effect resulted in liberation and activation of the apoptotic ASK-1 signal. These findings suggest that TXNIP is required for endothelial cell survival and homeostasis especially under stress conditions including hyperoxia.

**Commercial Relationships:** Azza B. El-Remessy,None;Mohammed A. Abdelsaid,None;Adviey Ergul,None;Suraporn Matragoon,None

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