Animal Models of Diabetic Complications Consortium
(U01 DK076136-01)

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"Angiogenic Signals in Diabetic Complications"

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Table of Contents

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Hypothesis

In humans with diabetes, abnormal angiogenesis, defined as growth and proliferation of blood vessels from existing vascular structures, contributes to the development of end-organ damage. In this regard, "excessive" angiogenesis and increased activity of the vascular endothelial growth factor (VEGF) signaling pathway have been associated with diabetic complications such as retinopathy, and perhaps nephropathy. In contrast, inadequate angiogenesis with a reduced capacity to promote collateral blood vessel growth results in more severe manifestations of coronary and peripheral vascular disease in diabetes. However, the mechanisms responsible for the loss of control of angiogenesis in diabetes and how this dysregulation modulates tissue pathology are not clear. *We have hypothesized that abnormal signaling in VEGF-associated pathways is a critical factor in the pathogenesis of diabetic complications including nephropathy and peripheral artery occlusive disease (PAOD). Furthermore, we posited that distinct properties of key cellular targets in individual tissues determine the effects of diabetes on the local angiogenesis response, shaping the resulting pathology. We suggest for nephropathy the critical target cell is the podocyte and in PAOD it is skeletal muscle.*

Accordingly, to develop better models of diabetic nephropathy and PAOD, we will generate mouse lines with inducible alterations of angiogenic signaling pathways targeted to podocytes and skeletal muscle. Because both enhanced and diminished angiogenesis responses have independently been associated with diabetic complications, we will use models with up- or down-regulated angiogenic signaling. Some of these models have been generated and are ready to use; we propose others to be generated as a part of the consortium activities. The long-term goals of our studies are: (1) To understand how alterations in angiogenic factors contribute to the development of diabetic complications and (2) To develop mouse models of diabetic nephropathy and PAOD that more faithfully reproduce the respective human conditions.

Recent Progress and Major Accomplishments

SPECIFIC AIM I. To define the role of altered angiogenic signaling in podocytes on the development of albuminuria and nephropathy in diabetes. During this first year of funding, we have made significant progress toward achieving the objectives of this aim. First, we have generated founder lines over-expressing a stabilized version of the Hif2alpha protein under control of a tetO response element (**Figure 1**). These mice are currently being bred to various rtTA transgenic driver lines to determine the effect of upregulation of this protein in specific cell lineages including podocytes, or pericytes of the retina and kidney. We have also generated the transgene construct for a stabilized version of the Hif1 alpha protein under control of a tetO response element (**Figure 1**) as was proposed in the original application. This construct will be sent to JAX laboratories for injection and generation of founder mouse lines.

In addition, we have recently begun an analysis of separate line of HIF1alpha and HIF2alpha over-expression transgenic mice. In these lines, HIF expression is non-inducible and is triggered by podocyte-specific Cre driver lines. In the presence of Cre, a floxed STOP codon

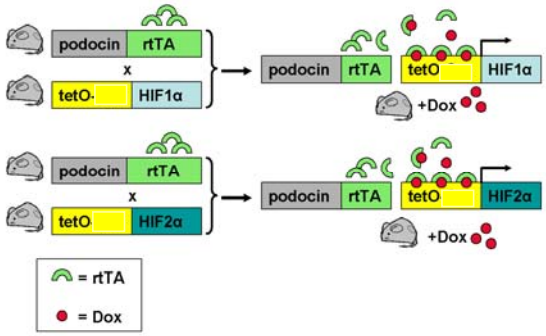

Figure 1: Strategy for inducible expression of stabilized HIF alpha subunits specifically in the podocyte. In the presence of doxycycline, HIFs are expressed only in podocytes. To generate increased expression of stabilized HIFs in podocytes. Inducible expression in other cell lineages can be achieved by crossing with different rtTA lines.

is excised, leading to transcription and translation of the HIF proteins under regulation of the endogenous Rosa locus with expression limited to podocytes. At 6 months of age, neither HIF1- or HIF2-alpha over-expressor lines exhibit an overt phenotype or signs of glomerular disease. Urine protein excretion is <1 gm/L by dipstick in both lines. To attempt to accentuate a renal phenotype, these mice were inter-crossed to generate double transgenics with over-expression of both HIF1 and HIF2 alpha proteins in the podocytes. At 3 months of age, we find mild protienuria by dipstick, but they are otherwise well. Over the course of the next year, we will administer STZ to induce diabetes and will follow for signs of worsening proteinuria and glomerular disease.

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Finally, we have generated quadruple transgenic mice with the following genotype: VEGFflox/flox/pod-rtTA/tetO-Cre. This combination of transgenes allows for inducible deletion of VEGF specifically in podocytes when mice are given doxycycline. We first documented robust and efficient deletion of the VEGF-A gene from adult podocytes. After doxycycline administration, we find that these mice develop glomerular disease over the course of 1 to 3 months. In a cohort of these mice, STZ was administered to induce diabetes before VEGF excision was induced. The STZ-treated animals developed an explosive and substantially accelerated course of renal disease apparent renal failure occurring within 4 weeks after doxycycline was administered. We are currently generating additional animals with this genotype to confirm this finding and to characterize the nature of their glomerular disease in detail with molecular, genome wide gene expression and physiologic analaysis. Biochemical studies are also underway to analyze potential crosstalk between hyperglycemic injury and reduced VEGF signaling in podocytes. We are optimistic that this may prove to be a useful animal model of diabetic nephropathy.

SPECIFIC AIM II. To define the role of altered angiogenic signaling in skeletal muscle in a model of peripheral artery occlusive disease. Deficient angiogenesis following ischemia may contribute to worse outcomes of peripheral arterial disease in patients with diabetes mellitus. Vascular endothelial growth factor and its receptors promote angiogenesis in PAD and, therefore, impaired activity of VEGF in diabetes might be one mechanism explaining the exaggerated severity of PAD in diabetic patients. A soluble form of the VEGF receptor Flt-1 (VEGFR1) is a naturally occurring inhibitor of VEGF. We hypothesized that in diabetes excess production of soluble Flt-1 might account for impaired angiogenesis and tested this hypothesis in diabetic mice. Following surgical induction of hind-limb ischemia, perfusion recovery was significantly attenuated in mice with from diet-induced, type II, diabetes (DM, n = 10) compared

to normal chow- fed control mice (NC, n =10). Hind limb skeletal muscle and age-matched DM and NC mice was collected, at baseline and 3 days after hindlimb ischemia, and analyzed for expression of VEGF (n = 10/group), full length and soluble VEGF receptors and downstream VEGF signaling (n = 20/group), using ELISA, RT-PCR and western blots (WB). At baseline, DM mice had increased VEGF (NC vs. DM, 26.6±2.6 vs. 53.5±8.8 pg/mg protein, $p<0.05$); decreased soluble and membrane bound VEGFR-1 (NC vs. DM; sVEGFR1; 1.44±0.30 vs. 0.85±0.08; VEGFR1 1.03±0.10 vs. 0.72±0.10; WB-densitometry, $p<0.05$); decreased VEGF signaling (NC vs. DM; p-AKT/AKT 0.76±0.2 vs. 0.38±0.1; p-eNOS/eNOS 0.36±0.06 vs. 0.25±0.04, WB-densitometry; $p< 0.05$); and no change in VEGF R2. Following ischemia, both DM and NC had comparable increases in VEGF A. Although sVEGFR1 and VEGFR1 expression increased in both groups, the fold-increase from baseline was greater in DM. Taken together, these findings suggest that in DM there are higher levels of VEGF, lower VEGFR1 and sVEGFR1 with lower receptor/signaling. Following ischemia, VEGF A increases in both NC and DM but sVEGFR1 and VEGFR1 increases disproportionately in DM, which may limit VEGF ligand binding to VEGFR2 and extent of the adaptive angiogenic response. A manuscript describing these studies is currently under review at *Circulation Research*.

Plans for the Upcoming Year

During the next year, we will continue our ongoing work on transgenic mouse generation, focusing in particular in generation of the line with inducible HIF-1alpha expression along with developing and/or identifying a driver transgene triggering expression specifically in skeletal muscle. We will also be completing our assessment of the consequences of inducible elimination of VEGF expression from podocytes in adult mice on the course of STZ-induced diabetes. We will also carry out appropriate crosses so that similar studies can be done on the Akita background. Finally, we will continue our ongoing work characterizing the activity of VEGF-associated signaling pathways in our target tissues of interest, skeletal muscle and the glomerulus, to allow direct comparisons of the extent of angiogenic signal activation in these tissues and to understand how altered VEGF signaling may contribute to diabetic complications in these tissues.

Preliminary Milestones for 2009 and Beyond

1. ***Complete phenotypic characterization of diabetic mice with time- and cell-specific targeting of the VEGF gene in glomerular podocytes.*** Our preliminary data suggest that elimination of VEGF production by podocytes dramatically accelerates the course of diabetes. If these data are confirmed, this would suggest that impaired generation of VEGF by glomerular epithelial cells may be a critical factor in the pathogenesis of diabetic nephropathy. Subsequent studies might focus on downstream targets mediating this effect and exploration of mechanisms of VEGF inhibition in diabetes.

2. ***Identifying the mechanism of attenuated VEGFR2 signaling in skeletal muscle during diabetes.*** Our preliminary studies described above clearly demonstrate impaired VEGF2R signaling in skeletal muscle in diabetic mice, despite elevated levels of VEGF ligand. This dynamic may be a major contributor to the abnormal adaptive angiogenic

response to ischemia in this setting. Further characterization of this mechanism should be enlightening from the standpoint of pathogenesis and perhaps in identification of novel therapeutic targets.

3. ***Development of a mouse line with a capacity for cell-specific, inducible expression of HIF-1 alpha.*** This transgenic mouse line will allow us to explore the role of angiogenic signaling pathways upstream of VEGF in diabetic complications.

4. Collaboration:

We have had significant interactions with the group at Jackson Laboratories over the past year. We are in the process of transferring two lines to the JAX repository: The 129/SvEv-*Ins2*^{Akita} line and the 129/SvEv(*Alb1-Ren2*)Tg. In addition, we anticipate that work will begin soon on our inducible-HIF-1alpha transgenic.

5. Address previous EAC comments:

NOT APPLICABLE THIS YEAR

6. Publications:

1. Gurley SB, Clare SE, Snow KP, Hu A, Meyer TW, Coffman TM. Impact of genetic background on nephropathy in diabetic mice. *Am J Physiol* 2006; 290:F214-22.

2. Gurley SB, Coffman TM. The renin-angiotensin system in diabetic nephropathy. *Seminars in Nephrology* 2007; 27: 144-152.

3. Hazarika S, Dokun AO, Li Y, Popel AS, Kontos CD, Annex BH. Impaired Angiogenesis Following Hind-Limb Ischemia in Type 2 Diabetes Mellitus: Differential Regulation of VEGFR1, R2, and Soluble VEGFR-1. *Submitted*.