Animal Models of Diabetic Complications Consortium (U01 DK076136)

Annual Report (2008)

"Angiogenic Signals in Diabetic Complications"

Duke University and Durham VA Medical Centers
University of Toronto

Principal Investigator
Thomas M. Coffman, M.D.

Co-Investigators
Brian H. Annex, M.D. (Duke)
Susan E. Quaggin (U of Toronto)

Address: Duke University Medical Center
Room 2018/MSRB2
106 Research Drive
Durham, NC 27710
Phone: 919-684-9788
E-mail: tcoffman@duke.edu

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 the past year, our work in this specific aim has focused in several areas. First, we have been interested in documenting the effects of diabetes on expression of and signaling by angiogenic signaling pathways in the models we are using for our studies. In previous studies, we and other AMDCC studies carried out studies suggesting that mice bearing the *Ins2*$^{+/C96Y}$ mutation (Akita mice) may have significant advantages as a platform for developing models of diabetic nephropathy (DN). Since genetic factors play a key role in susceptibility to DN in humans, we investigated the role of genetic background on kidney injury in Akita mice. To this end, we back crossed the *Ins2*^{C96Y} mutation onto the 129/SvEv and DBA/2 backgrounds and compared the extent of renal disease with the C57BL/6-Akita line. While male mice from all three lines developed marked and equivalent hyperglycemia, there were significant differences in the level of albuminuria with DBA/2>129/SvEv>C57BL/6. Renal and glomerular hypertrophy were seen in all of the lines, but significant increases in mesangial matrix were observed only in the 129 and C57BL/6 backgrounds. F1(DBA/2 x C57BL/6) animals had levels of albumin excretion similar to the more susceptible DBA/2 parental strain, suggesting dominant pattern of inheritance for albumin

susceptibility. However, the enhanced mesangial pathology in the F1 mice most closely resembled the C57BL/6 parental line, indicating genetic control of diabetic mesaignal expansion may be distinct from that of albuminuria. These findings, which are described in a submitted manuscript (Gurley et al), indicate that genetic background has a powerful influence on determining the severity of renal injury in Akita mice. Based on the observed segregation of these traits in the F1 animals, mapping of susceptibility loci could be possible through F1 inter-crosses or backcrosses with the individual parental lines.

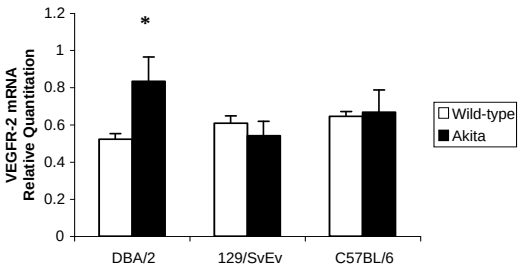

Figure 1. VEGFR-2 mRNA expression in renal cortex of wild-type and Akita mice.

To determine whether these strain differences in susceptibility to kidney injury in diabetes are associated with alterations in angiogenic signaling, we measured expression of VEGF and its major receptors in kidneys from the same animals in which the physiological assessments were carried out. Interestingly, we found that expression mRNAs for VEGF-A, VEGF-R1, soluble VEGF-R1, and VEGF-R2 were increased in the DBA/2-Akita mice, the strain with the most pronounced albuminuria (see Figure 1). Work is in progress to determine whether these alterations are reflected by changes in distal signaling including phosphorylation of AKT and eNOS.

Our project is considering two diabetic complications, nephropathy and peripheral artery disease. Our hypothesis suggests that key angiogenic signals may differ in these target tissues, explaining apparent differences in angiogenic activity. To explore this further, we have also carried out comparisons of components of the VEGF signaling pathway in renal cortex and skeletal muscle from the same animals, comparing a group of C57BL/6-Akita mice with C57BL/6 wild-type controls. In the kidney, diabetes was associated with a significant increase in VEGF-A protein, measured by ELISA from 22.7±4.9 to 38.8±2.6 pg/mg protein ($p<0.05$). By contrast there was no difference in the relatively low levels of VEGF protein in skeletal muscle of B6-Akita (3.4±0.3 pg/mg protein) and the wild-type controls (4.0±1.0 pg/ mg protein; p=NS). Coinciding with the higher VEGF levels in kidneys of diabetic animals, level of phosphorylated-AKT were increased by more than 80% in renal cortex of the Akita mice compared to wild-type controls ($p<0.05$). By contrast, levels of phospho-AKT were reduced by almost 70% in skeletal muscle of diabetic animals compared to controls. This marked discrepancy of responses to diabetes in key target tissues is likely to be critical in shaping the character of individual complications and will be further explored during the next year.

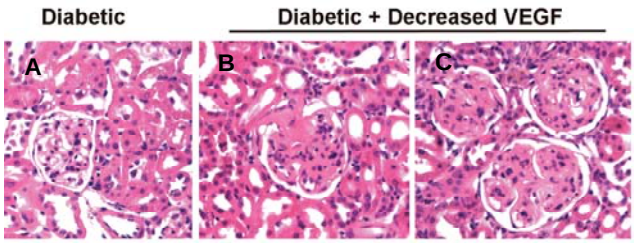

Figure 2. H & E stained sections from diabetic mice. (A) Normal VEGF production (B & C) Mice with induced knock-down of VEGF production in podocytes.

In parallel with these studies, we have carried out studies to directly test the contribution of local generation of VEGF in glomerular epithelial cells to the development of proteinuria and kidney pathology. To this end, we used a doxycycline inducible Cre-loxP gene targeting strategy to eliminate VEGF production in podocytes of adult mice. First, we induced diabetes in mice with streptozotocin using the AMDCC low-dose protocol. After 5 weeks, doxycycline was given to knockdown VEGF production in the glomeruli of diabetic mice. As shown in Figure 2, loss of VEGF production in the podocytes of diabetic mice resulted in profound glomerular injury within 3 to 5 weeks characterized by global sclerosis. Some of the mice died with accelerated renal failure. Taken together, these results

suggest that inhibition of VEGF enhances glomerular damage in diabetes and that enhanced VEGF production seen in the mouse strains with exaggerated proteinuria may be a compensatory, rebound phenomenon. Our future studies will continue to explore these issues.

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. In the previous year, we made a novel observation that alterations in sVEGFR-1 occur in peripheral skeletal muscle in the setting of diabetes mellitus and these may influence angiogenic signaling. A manuscript describing those findings has now been published, Hazarika et al. *Circulation Research* 2007 (see full citation under publications). These findings suggest that a soluble form of the VEGF Receptor-1 is down-regulated in the skeletal muscle of mice with a form of type 2 diabetes mellitus. We proposed that this down-regulation is a compensatory response to maintain critical down-stream signaling from the VEGF receptor including the ability to phosphorylate and activate Akt and endothelial nitric oxide synthase; which can be measured in tissues as the ratios of phosphorylated/total Akt (p-Akt/Akt) and eNOS (p-eNOS/eNOS). These data add to a small but growing body of literature which suggest that VEGFR-1 provides weaker signals than VEGFR-2; the major VEGF receptor involved in post-natal angiogenesis. Alternatively, VEGFR-1 may act as a scavenger receptor that frankly antagonizes VEGFR-2 signaling. In related work also supported in part by this grant and using the methods and approaches achieved during year 1 of this award, we found similar interactions between VEGF receptors 1 and 2 during development. That manuscript, Kappas et al. *J Cell Biol*, was recently accepted for publication (see full citation under publications).

In follow up to our findings concerning changes in VEGF and its receptor signaling in diabetes mellitus, we began to address two related questions. First, we considered whether the changes that we found in VEGF receptor ligand family within the skeletal muscle in a model of type 2 DM also occurred in type 1 diabetes. We therefore studied these parameters in mice with type I diabetes induced with alloxan, and compared them directly to animals with type 2 DM induced by high fat feeding. The mice with diet induced DM had lower fasting blood sugars (187±25 vs. 530±28 mg/dl), but the area under the curve following glucose administration was very similar between the two groups. We next tested whether the differences in downstream signaling seen in type 2 DM within the VEGF cascade are also seen in skeletal muscle in mice with type I DM, to the extent that pAkt/Akt and peNOS/eNOS reflect VEGF signaling. As shown

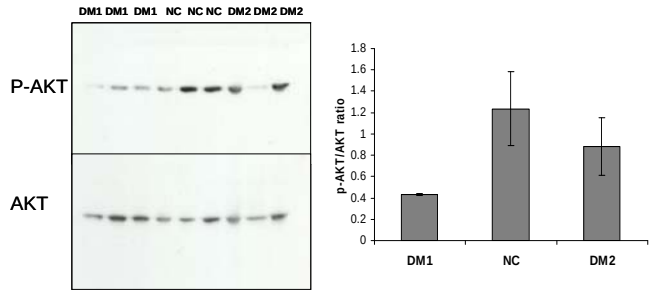

Figure 3. Measurements of phospho-AKT and total AKT in mouse skeletal muscle. Ratio of P-AKT/AKT is markedly reduced in the animals with type I diabetes (DM1).

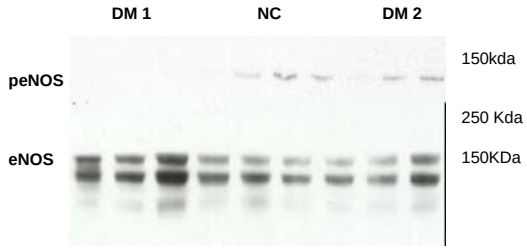

Figure 4. Measurements of phospho-eNOS and total eNOS in mouse skeletal muscle. Levels of phosphorylated eNOS were significantly reduced in DM1.

in Figures 3 and 4, VEGF signaling was significantly impaired in both groups of diabetic mice but if anything was more pronounced in the animals type 1 diabetes. As discussed above, we have also seen similar patterns in another model of type 1 DM, the Akita mouse.

Plans for the Upcoming Year

During the next year, we will continue our ongoing work assessing 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.*** These studies are in progress and should be completed over the next 12 months.

2. ***Identifying the mechanism of attenuated VEGFR2 signaling in skeletal muscle during diabetes.*** This is a complex question, but we have made significant progress and our data now suggest that VEGFR1 may antagonize VEGFR2 signaling in skeletal muscle. We have recently obtained VEGFR1 knockout mice from Dr. Vickie Bautch from UNC-Chapel Hill to allow direct testing of this question.

3. ***Development of a mouse line with a capacity for cell-specific, inducible expression of HIF-1 alpha.*** We have floxed HIF-1 alpha mice in hand. These will be crossed with our lines allowing podocyte-specific deletion, and we are attempting to obtain skeletal muscel Cre lines to facilitate similar studies in skeletal muscle.

4. <u>Collaboration:</u>

We have interacted with the group at Jackson Laboratories over the past year. The 129/SvEv(*Alb1-Ren2*)Tg line has been reposited at JAX. We are in the process of also transferring the 129/SvEv-*Ins2*^{Akita} line, but this has been impeded by breeding problems in our facility that now seem to be resolved.

5. <u>Address previous EAC comments:</u>

1. Coffman

a. The nephropathy work is progressing well (among the best) and the phenotype resulting from post-natal deletion of podocyte VEGF (and its alteration by hyperglycemia) is interesting.

b. *We like the work on peripheral vascular disease. This work might be rapidly translatable into human trials, given the availability of "drugs" to manipulate the VEGF axis already in human trials.*

c. *The HIF animals should be reposited with JAX by Spring 2008. Are the dox-inducible podocyte specific cres or the floxed VEGF mice available?*

Phenotypic characterization of the HIF animals is in progress at the Toronto site. When these are completed, the mice can be transferred to JAX. The dox-inducible podocyte specific Cres are available from Dr. Quaggin's lab. The VEGF-floxed animals were obtained from Genentech and can be obtained from them with permission.

d. *PAD is a good addition as it is a major diabetic complication. Dr. Annex and Dr. Abel should be communicating to compare/contrast/optimize models in order to derive the most consistent data possible. This can become another phenotyping area for the consortium. While it is of obvious academic interest to understand the underlying mechanisms of the enhanced PAD associated with diabetes, how this direction will be incorporated into the overall goals of the AMDCC should be clarified. When the tetHIF animals are available they will be an interesting model to test the role of HIF signaling. When do you think the tetHIF animals will be available?*

We agree that PAD models should be an important part of the AMDCC repertoire. As discussed above, we continue to look for a suitable skeletal muscle Cre line and have carried out some preliminary searches for appropriate promoter elements that could be used to generate such a line ourselves if necessary.

6. Publications:

1. Hazarika S, Dokun AO, Li Y, Popel AS, Kontos CD, Annex BH. Impaired angiogenesis after hindlimb ischemia in type 2 diabetes mellitus: differential regulation of vascular endothelial growth factor receptor 1 and soluble vascular endothelial growth factor receptor 1. *Circ Res* 2007;101:948-56.

2. Kappas NC, Zeng G, Chappell JC, Kearney JB, Hazarika S, Kallianos KG, Patterson C, Annex BH, Bautch VL. The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. *J Cell Biol* 2008 May 26. [Epub ahead of print].

3. Xie D, Hazarika S, Andrich AJ, Padgett ME, Kontos CD, Donatucci CF, Annex BH. High cholesterol feeding in C57/Blc6 mice alters expression within the VEGF receptor-ligand family in corporal tissue. *J Sex Med* 2008;5:1137-48.

4. Gurley SB, Snow KP, Hu A, Meyer TW, and Coffman TM. Influence of Genetic Background on Albuminuria and Kidney Injury in *Ins2*$^{+/C96Y}$ (Akita) Mice. Submitted.