Animal Models of Diabetic Complications Consortium (U01 DK076136)

Annual Report (2010)

"Angiogenic Signals in Diabetic Complications"

Duke University and Durham VA Medical Centers, University of Toronto, and University of Virginia

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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.

Progress Toward Stated Milestones:

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 covered several areas. First, we have continued our previous work focused on understanding strain-specific genetic modifiers that affect susceptibility to diabetic kidney disease, and their relationship with expression of and signaling by angiogenic signaling pathways. In previous studies, we and other AMDCC investigators have 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). We have now completed and published studies showing that there are strong, strain-specific modifiers of kidney injury in Akita mice (Gurley et al). For example, there were significant differences in the level of albuminuria in Akita mice on the different genetic backgrounds: DBA/2>129/SvEv>C57BL/6. Although renal and glomerular hypertrophy were seen in all of the lines, significant increases in mesangial matrix were observed only in the 129 and C57BL/6 backgrounds. F_1(DBA/2 x C57BL/6) animals had levels of albumin excretion similar to the more susceptible DBA/2 parental strain, suggesting a dominant pattern of

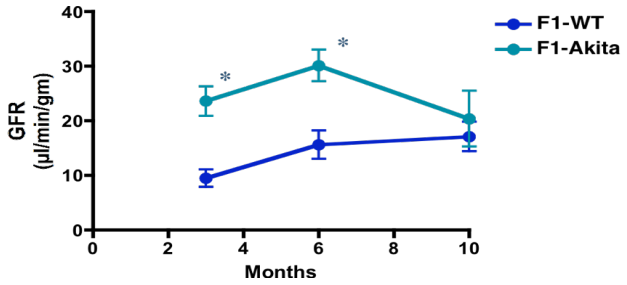


Figure 1: GFR in F_1(DBA/2 x C57BL/6) mice measured by FITC-inulin. (*p<0.01 vs. F1-Wild-type)

inheritance for albumin susceptibility. Furthermore, comparing age-matched wild-type and Akita mice on each background, the presence of the *Ins2*^{C96Y} mutation and diabetes was associated with higher GFR (Gurley et al). The extent of the increase in GFR in the C57BL/6- and 129/SvEv-Akita mice was ≈35-40% and approached statistical significance (p=0.052 and 0.07, respectively). The proportional increase in GFR was much greater in the F1(C57BL/6 x DBA/2)-Akita animals (≈150%, p=0.011); this is also the group with the highest levels of albuminuria. Thus, genetic background has a powerful influence on determining the severity of renal injury in Akita mice.

Because the F1 mice had among the highest propensity for injury, we have now examined GFR changes over time, from 3- to 10-months of age, compared to non-diabetic F1(C57BL/6 x DBA/2) mice. As shown in Figure 1, significant hyperfiltration was observed in the Akita group at 3 and 7 months, but by 10 months GFRs decreased in the Akita group such that this difference was abolished. We will continue to follow this group to see if there is any further fall in GFR, which would fulfill one of the important AMDCC criteria defining a useful nephropathy model. One problem with this model is that the 10-month old F_1 mice are beginning to appear frail and may not be suitable for longer-term analysis. Accordingly, we have explored in detail changes in GFR in another model consisting of a consitutively expressed renin transgene combined with the Akita mutation on the 129 background.

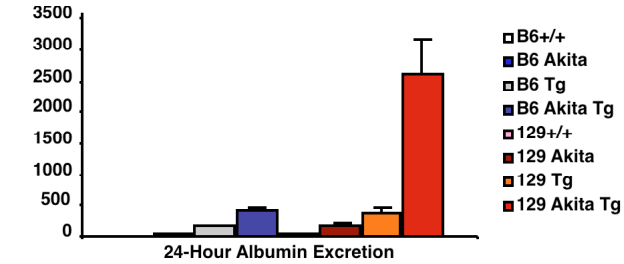


Figure 2. 24-hour urine albumin excretion. Albuminuria is dramatically enhanced in 129/SvEv-*ReninTg-Akita* mice (Red bar).

As a potential approach for enhancing the extent of diabetic kidney injury, we reasoned that chronic activation of the renin-angiotensin system (RAS) might be useful, in view of the generalized efficacy of RAS antagonists to ameliorate the progression of diabetic nephropathy in humans. We therefore took advantage of a transgenic mouse line (*ReninTg*) developed in the Smithies' lab in which renin is expressed constitutively under the control of the albumin promoter (*PNAS* 99:8288, 2002). We generated inbred transgenic mice on both the

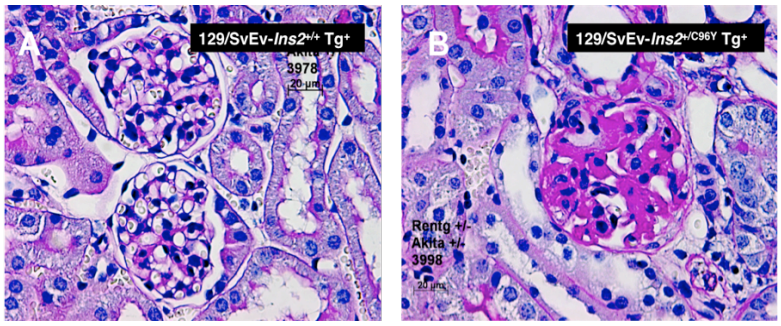


Figure 3. Photomicrographs of mouse kidney sections stained with PAS. Kidneys from 129/SvEv-*ReninTg- Akita* mice marked worsening of glomerular pathology (B) compared to non-diabetic 129/SvEv-*ReninTg* mice (A), or non-transgenic 129/SvEv mice (not shown).

C57BL/6 and 129/SvEv backgrounds, which we showed in our recent paper (Gurley et al) to have relative resistance and susceptibility, respectively, to diabetic kidney injury in the Akita model. We then crossed these *Renin-Tg* mice with the corresponding C57BL/6 or 129/SvEv-*Akita* mouse line in order to generate Akita mice bearing the renin transgene on each of the backgrounds. As shown in Figure 2, the 129/SvEv-*ReninTg-Akita* mice have marked augmentation of albuminuria compared to 129/SvEv-*ReninTg* or C57BL/6-*ReninTg-Akita* groups, indicating a powerful interaction between activation of the RAS and diabetes on a susceptible genetic background (129/SvEv) to dramatically enhance albuminuria. Glomerular pathology was also substantially increased in the 129/SvEv-*ReninTg-Akita* animals as shown in Figure 3. We also measured GFR in the various groups of 129/SvEv animals at 6-months of age, corresponding to the time that albuminuria was measured (Figure 2). As shown in Figure 4, significant hyperfiltration was present in both of the diabetic groups, and the level of GFR was virtually identical in both groups of Akita mice, whether or not the renin transgene was present.

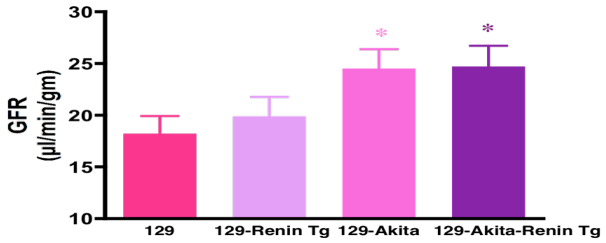


Figure 4. GFR in 6-month old 129/SvEv mice. There were significant increases in GFR (hyperfiltration) in diabetic Akita mice and this was not affected by the renin transgene.

Based on the observed segregation of these traits in the F1 animals and the powerful enhancement of proteinuria and pathological changes by the renin transgene, mapping of susceptibility loci should be possible through F1 inter-crosses or backcrosses with the individual parental lines, using the transgene as a genetic sensitizer. We are now carrying out additional preliminary studies to explore the feasibility of such an analysis.

In previous years of the grant, we carried out studies to examine the role of altered levels of VEGF in modulating kidney injury with diabetes, as described in last year's Annual Report. During the past year, we have examined the contributions of another important angiogenic factor, angiopoietin 1 (Ang 1), in diabetic kidney injury. These studies were carried out in Dr. Quaggin's laboratory. It had been previously suggested that Ang1 acting through its receptor Tie-2 played a role in developmental angiogenesis, as well as in stabilization of the mature vasculature. Previous studies had also suggested that: (1) Ang1 is produced by podocytes, (2) exogenous administration of Ang1 had renoprotective effects in *db/db* mice and (3) Ang1 may alleviate diabetic retinopathy. Genetic elimination of *Ang1* or *Tie-2* causes early embryonic lethality in mice. Thus, Dr. Quaggin's laboratory generated mice with a conditional *Ang1* allele by homologous recombination in ES cells. To document functionality of the Ang1 allele, whole body deletion was carried out using a ubiquitously expressed *Cre*-recombinase transgene. As expected, there was 100% lethality during embryogenesis, although the major abnormality found in E10.5 embryos was in the heart, not in the vasculature. To examine the role of Ang1 in the glomerulus, the *Ang1*-floxed mice were crossed with a

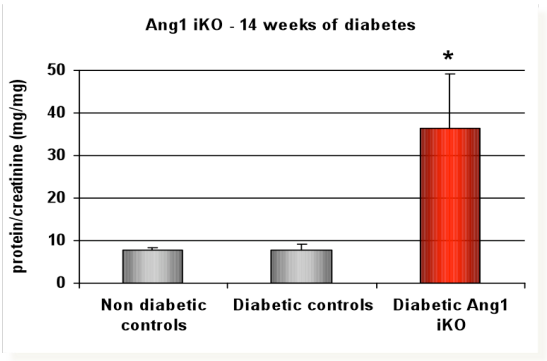


Figure 5. Urine protein excretion in mice. Levels of urinary protein excretion were significantly increased in STZ-treated mice lacking Ang1 in podoctyes (diabetic Ang1 iKOs) compared to wild type diabetic or non-diabetic controls. (*p<0.05 vs controls.

podocyte-specific *Cre* line to delete Ang1 specifically in podocytes, a major source of Ang1 in the kidney. In contrast to previous findings with VEGF, deletion of Ang1 specifically from podocytes does not cause an apparent phenotype, indicating that Ang1 is not required for maintenance of normal glomerular structure and function. In order to determine whether production of Ang1 by the podocyte plays any role in the development of glomerular injury in diabetes, mice lacking Ang1 specifically in podocytes were made diabetic using STZ, and proteinuria and glomerular pathology were assessed. As show in Figure 5, levels of proteinuria, measured after 14 weeks of diabetes, were significantly increased in diabetic mice lacking Ang1 in podocytes compared to diabetic controls, which had levels of protein excretion that were not different from non-diabetic controls. Similarly, the extent of glomerular pathological abnormalities was significantly worse in the animals with deletion of Ang1 from podocytes compared to diabetic controls with essentially normal glomerular structure (Figure 6). Thus, Ang1 plays a protective role in the glomerulus in diabetes, and deletion of Ang1 from podocytes markedly accelerates glomerular injury in diabetes.

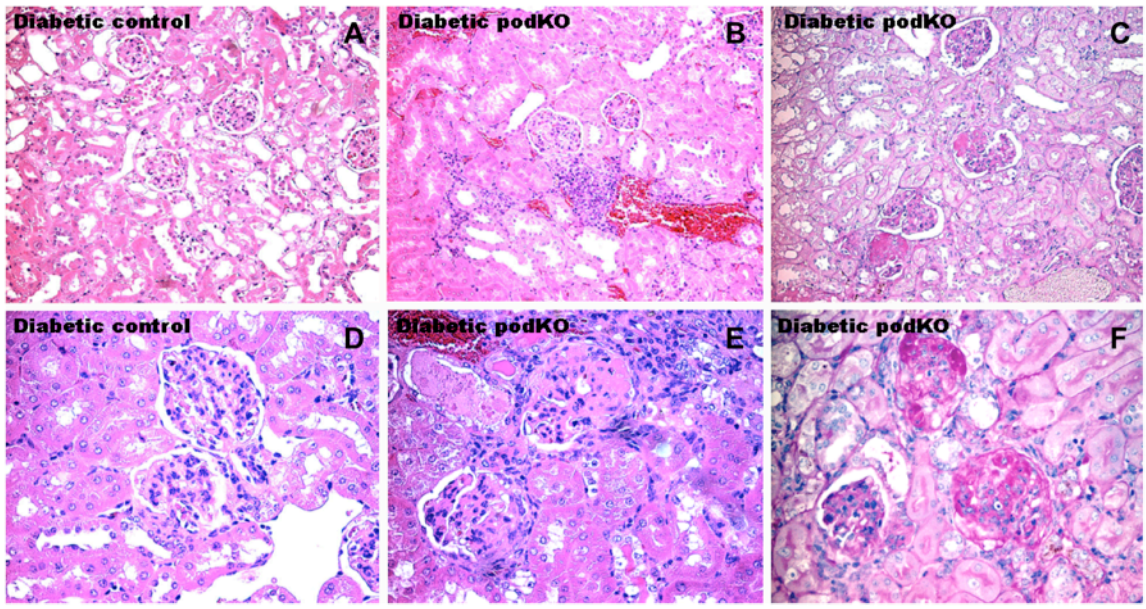


Figure 6. Glomerular histopathology in diabetic mice. Diabetes was induced in mice with STZ, kidneys were harvested after 14 weeks, and sections were fixed and stained for histopathological analysis. (A, D) Kidneys from diabetic controls showed minimal abnormalities. (B,C, E, F) Kidneys from diabetic mice lacking Ang1in podocytes (podKO) showed significant glomerular abnormalities including increased matrix deposition and sclerosis.

SPECIFIC AIM II. To define the role of altered angiogenic signaling in skeletal muscle in a model of peripheral artery occlusive disease.

In the prior progress reports, we described how as a part of the UO1 we described how alterations in the vascular endothelial growth factor receptor-ligand family were altered in mice fed a high fat diet. Briefly, we described 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 DM. We proposed that this down-regulation is a compensatory response to attempt to maintain critical down-stream signaling from the VEGF receptor which include the ability to phosphorylate (and thus activate) Akt and endothelial nitric oxide synthase; with measures being 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 is either of much weaker, or even a frank antagonist to VEGFR-2; the latter being the major VEGF receptor involved in post-natal angiogenesis. We had briefly described how alloxan treated mice had similar decreases in markers of VEGF receptor signaling (see Fig 3 and 4 in last years report, and one of our stated goals for this year was to examine and compare different models. In last years report (Fig. BHA1) we showed skeletal muscle changes in VEGF are likely to be different between type 1 and (high-fat feeding) type 2. We also showed (Fig. BHA2) that the reductions in pAkt/Akt and pe-NOS/eNOS (measures of down-stream VEGF signaling) that we observed in mice fed a high fat diet were present, and indeed may be more pronounced in type 1 models. Having established that there were similar changes in VEGF ligand in models of Type 1 and 2 DM, we next sought to determine whether there were similar alterations in the receptors. In fact, we showed similar effects on soluble VEGF receptor in the two models. However, as shown in Figure BHA3, below, the changes in the receptors (especially VEGFR2) were different at the protein (left panel) but not at the mRNA level. Since one of our goals is to identify an animal model where we can optimize glucose control to determine the consequences on PAD (ie, perfusion recovery and necrosis), we have begun to explore the Akita mouse line in order to evaluate changes caused by hyperglycemia and the extent to which such changes are ameliorated by normalization of blood glucose.

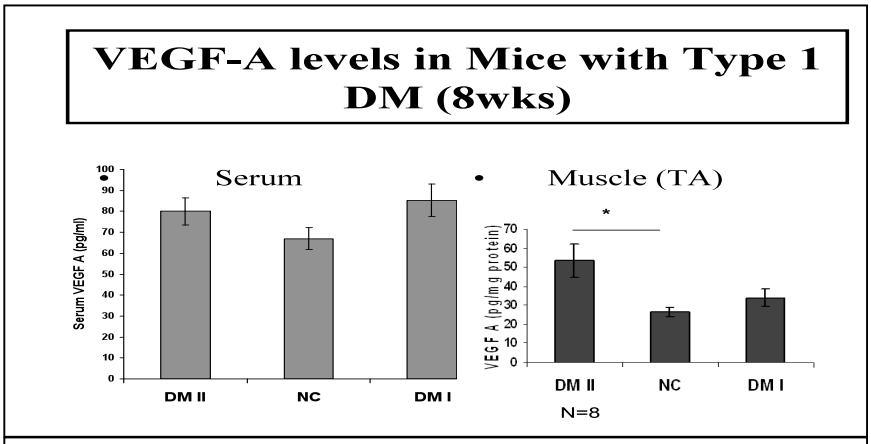
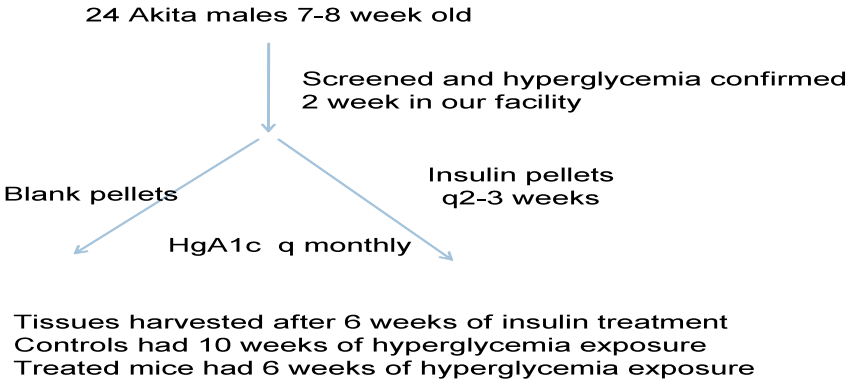
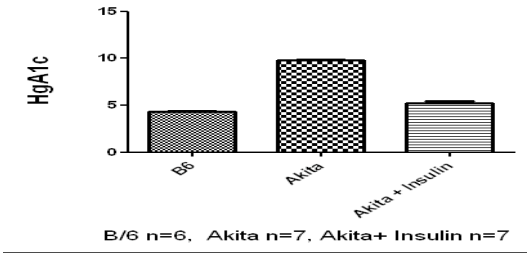


Figure 7. Mice with Type I DM have changes in levels of VEGF in skeletal muscle (right panel) that are very different from mice fed a high fat diet. Serum levels are not changed.

Experimental design

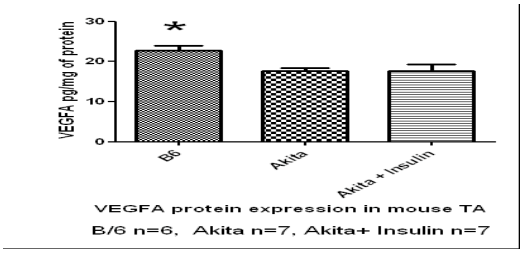


Comparison of HgA1c in C57BL/6, Akita and Akita treated with insulin



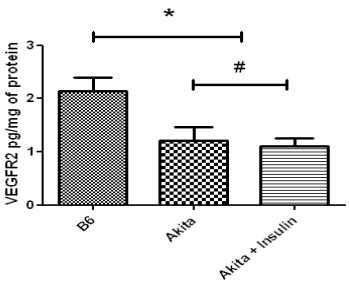
Despite controlling blood sugar and hemoglobin A1c in the mice, skeletal muscle expression of VEGF was different between Akita KO and wt mice but was NOT different based on whether the glucose was controlled.

Decreased VEGFA protein expression in TA of type I DM mice



We had previously shown that mice fed a high fat diet had reductions in sVEGFR1 but we did not find this in the Akita KO mice (data not shown) and though cGMP (a marker of NO generation) was not different between Akita and Wt mice, we found that Akita KO mice have reduced levels of VEGFR2

VEGFR2 protein expression is decreased in TA of type I DM mice



VEGFR2 protein expression in mouse TA

B/6 n=6, Akita n=7, Akita+ Insulin n=7

*=P<0.05, # = No sig diff

From a clinical perspective, patients with peripheral arterial disease are treated in an identical manner whether they have type 1 or type 2 DM but is also the same regardless of blood sugar. Thus, if confirmed these data have clear therapeutic implications. Thus our ongoing work on this for the next and following years will be:

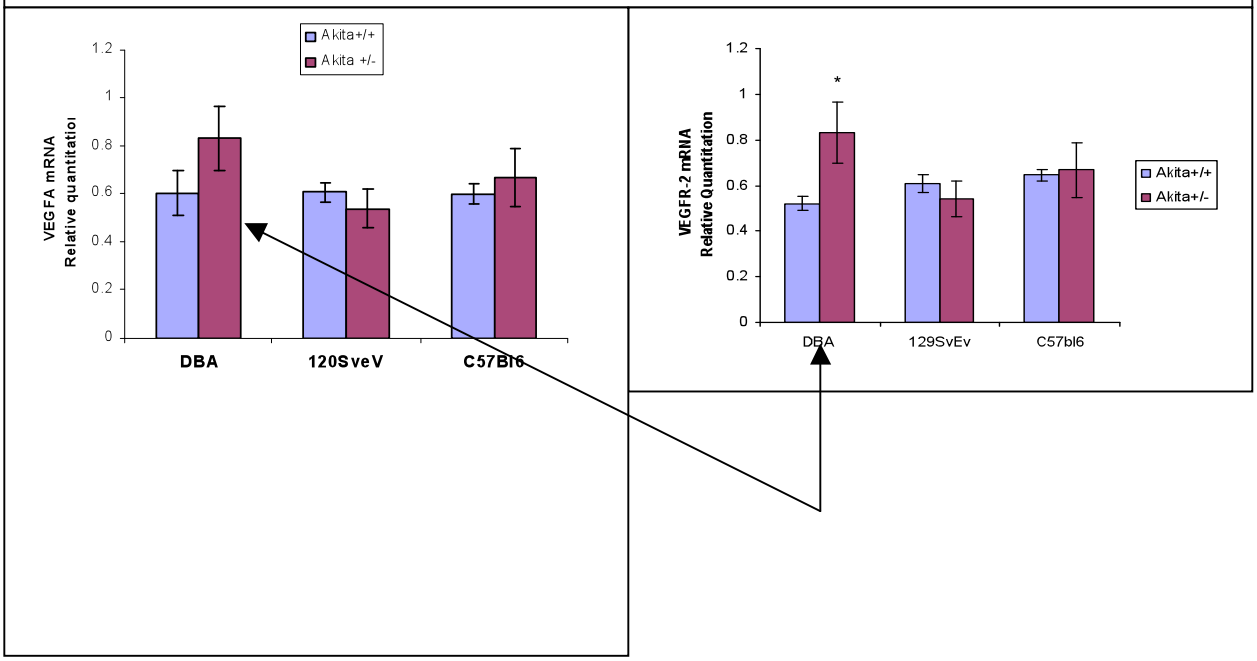
- Definitely establish the VEGF receptor ligand changes in muscle between the different models.
- Control for differences in the amount and extent of hyperglycemia.
- Examine the effects of glucose control.
- Examine apoptotic markers.
- Examine additional strains (i.e. Akita knock out mice).
- Compare and contrast muscle and kidney from same mice. (see below)

The hypothesis of the original application was that in humans with diabetes, abnormal angiogenesis contributes to the development of end-organ damage.
“Excess” angiogenesis = nephropathy
“Inadequate” angiogenesis = PAD
We hypothesize that abnormal signaling in VEGF-associated pathways is a critical factor in the pathogenesis of diabetic complications including nephropathy and peripheral artery disease (PAD).

Figures to follow will show:

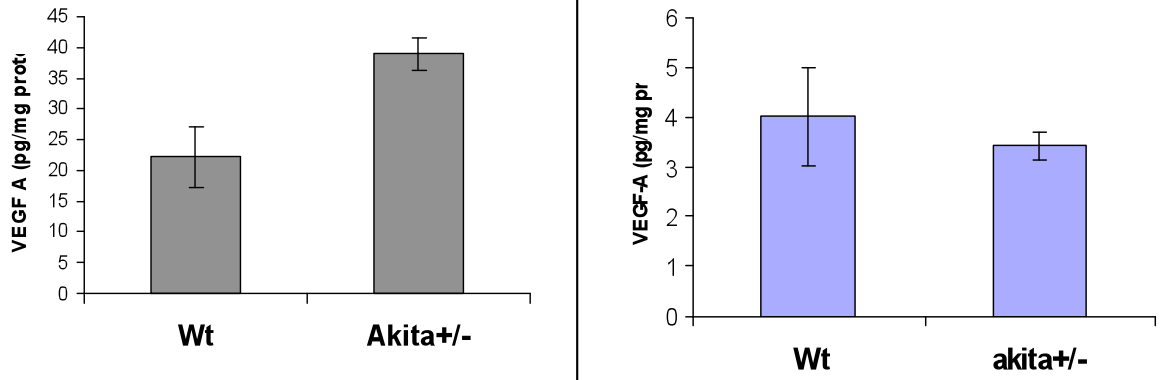
-Changes in VEGF that occur in muscle may not occur in kidney and may be directionally opposite and consistent with the central hypothesis. Specifically, Akita KO mice in the DBA strain background have the greatest amount of nephropathy. Nephropathy, we hypothesized may be related to the degree of excess VEGF signaling. In the figure below, kidneys from DBA mice have greater changes in VEGF and VEGFR2; both involved in ligand mediated receptor signaling.

VEGF mRNA (left) and VEGFR-2 mRNA (right) are both elevated in kidney of Akita KO vs. wild type mice in the DBA genetic background.



Finally, changes in the VEGF ligand are directionally opposite in muscle and kidney.

Kidney Cortex Increase VEGF **VEGF-A ELISA Assays** **Tibialis Anterior Muscle Decrease VEGF**



$p<0.05$ Akita +/- vs. Wt

We went on to show directionally opposite changes in pAkt/Akt (data not shown). We have also begun to utilize models with altered levels of VEGF receptor expression and are breeding these into appropriate strains.

Plans for the Upcoming Year

During the next year, we will continue our ongoing work assessing the consequences of inducible elimination of angiopoietin 1 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. Based on feedback from the EAC, we will also continue to explore the nature of naturally occurring genetic modifiers that affect the severity of diabetic kidney injury, including any associated metabolic alterations that accomplany these changes. 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 angiogenic signaling may contribute to diabetic complications in these tissues.

1. <u>Collaboration:</u>

Within the AMDCC, we have continued our ongoing collaborations with the two groups at UNC (Smithies and Maeda labs). In particular, we have been using the renin transgenic mice produced by the Smithies' lab to augment the severity of diabetic renal disease. In studies going forward, we hope to use this transgene as a genetic sensitizer to characterize strain specific genetic modifiers of kidney injury. In addition, we have interacted with the group at Jackson Laboratories over the past year to successfully transfer the 129/SvEvTac-*Ins2*^{Akita} line. The Jax group will carry out baseline phenotyping of the line. Finally, we have initiated a collaboration with Dr. Chris Newgard, a non-AMDCC investigator here at Duke. Dr. Newgard is an expert in the area of metabolomics and we plan to carry out an unbiased metabolomic screen of some of our models to look for metabolic signatures that are associated with enhanced renal disease.

2. Address EAC comments:

Coffman

"Excellent work and productive. We like the candidate genes being studies and mTOR mouse project should be quite interesting."

We appreciate these positive comments from the EAC.

"The GFR data is intriguing and points to the emerging need for measuring not only the usual and relatively easily assessed rodent risk factors (glycemia, weight gain/food intake, kidney weight and blood pressure) but also additional ones such as GFR."

We are in complete agreement with the EAC that GFR is an important outcome measure, and have added this to most of our ongoing studies. We have found that the protocol for measuring FITC-inulin clearances developed previously by the AMDCC is straightforward and reliable.

"The skeletal muscle studies are also interesting but seem somewhat off target from the major foci of AMDCC."

The rationale for the skeletal muscle studies is related to studies proposed by our co-investigator Dr. Annex focusing on peripheral artery disease (PAD) as a key diabetic complication. A key determinant of the outcome and severity of PAD is the extent of angiogenesis in skeletal muscle. Insofar as PAD is considered an important diabetic complication, we suggest that these studies are relevant to the major objective of the AMDCC.

"The VEGF story is also interesting but so much information seems to keep arguing for an "optimal" level that it will be difficult to define this especially if the optimum differs in normal versus diabetes."

We agree that the role of angiogenic factors such as VEGF in diabetic nephropathy is complex, but it is our hope that the genetic models we are using will provide a useful approach for understanding the consequences of altered levels for these factors.

3. Publications:

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