Animal Models of Diabetic Complications Consortium
(U01 DK61018)

Annual Report
(2008)

"Generating Mouse Mutants With Diabetic Nephropathy"
Vanderbilt University School

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Part A:

Principal Investigator's Summary

Accumulating evidence implicates endothelial dysfunction in the pathogenesis of diabetic complications, particularly nephropathy, retinopathy, neuropathy, and macrovascular disease, and therefore, the Vanderbilt component of the AMDCC is focusing on the role of microvascular dysfunction in development of diabetic nephropathy. Specifically, we are attempting to investigate the role of ieNOS and prostacyclin synthase (PGIS), two endothelial genes encoding biochemically interrelated enzymes and in which polymorphisms associated with altered enzyme activity have been implicated in human diabetic nephropathy.

Both PGIS and eNOS activity are critical for the maintenance of normal endothelial function. COX2 appears to be the major source of urinary prostacyclin excretion in man, and prolonged COX2 inhibition is associated not only with reduction of PGIS but also with excess cardiovascular mortality from thrombotic events. This is consistent with a cardioprotective action of prostacyclin. Functionally significant polymorphisms in eNOS and PGIS have been identified in humans. ENOS and PGIS activity are not only topographically linked but also biochemically linked through oxidative stress, which not only uncouples eNOS, but also results in increased peroxynitrite levels, which directly reacts with and inactivates prostacyclin synthase. Both eNOS uncoupling and peroxynitrite-induced inactivation of prostacyclin synthase have been demonstrated to be direct consequences of hyperglycemia. It has been hypothesized that as a result of this, diabetics exhibit impaired endothelial dependent acetylcholine induced vasodilation and glomerular barrier function which is reflected as albuminuria. This may also be associated with the global cardiovascular disease associated with diabetic nephropathy.

Responsible Investigator: Raymond C. Harris, M.D.

1. Project Accomplishments:

Recent Progress and Major Accomplishments

I Proposed Goals of the Vanderbilt AMDCC

The goal of **Aim 1** is to determine the role of endothelial eNOS activity in the progression of diabetic nephropathy by generating floxed eNOS mice and studying them in the DN susceptible DBA2/J Akita mouse. During the past year, we completed the construction and assessment of the targeting construct, and the Vanderbilt transgenic core electroporated the construct into 29S6/SvEvTac ES cells. After confirmation by Southern blotting, four clones were transferred to Jackson Labs. Three of the four clones were injected into blastocyst on 4/2/08 and 4/3/08. As of 5/13/08, they reported that Clone 1C7 had 12 pups, with 6 chimeras (3 high males, 1 high female, 2 medium males), Clone GB3 had 12 pubs born with 7 chimeras (5 high males, 1 high female, 1 medium male) and Clone 6B2 had 23 pubs born, with 14 chimeras (13 high males, 1 high female). The high male chimerics will be mated to C57BL/6J to look for germ-line transmission. Once we have established germline transmission we will mate the proven germline chimeric mice to either 129S1 (stock# 002448) or to stock # 3946 - 129S4/SvJaeSor-*Gt(ROSA)26Sortm1(FLP1)Dym*/J (129-FLPe).

The goal of **Aim 2** is to determine the role of endothelial prostacyclin synthase in the progression of diabetic nephropathy nephropathy by generating floxed PGIS mice and studying them in the DN susceptible DBA2/J Akita. In this regard, we have completed the construct and have electroporated 129P3ES cells with the floxed PGIS targeting vector. The original screen of ES cells was negative. The construct was re-electroporated into ES cells and rescreened. Although we obtained a number of chimeric pups, we have yet to achieve germline transmission. A meeting with the Vanderbilt transgenic core leadership is scheduled for later this month to discuss strategy going forward.

Plans for the Upcoming Year: Our goal is to have the floxed mice available by late 2008. We then hope to send them to Jackson Labs for speed congeneic backcrossing to 129/sv and DBA2/J Akita backgrounds. In the meantime, we also will make the Tie-2-Cre mice available to Jackson Labs for backcrossing to DBA2/J background.

Preliminary Milestones for 2009 and Beyond: Our goal is to be able to make the appropriate crosses to produce endothelial-specific deletion of either eNOS or PGI synthase during 2009 and determine the effects on development of diabetic nephropathy.

II Ongoing Studies of Murine Models of Diabetic Nephropathy

A) Characterization of the role of endothelial nitric oxide synthase deficiency in development of diabetic nephropathy.

In the previous funding cycle of the AMDCC, we found that in a model of type II diabetes (db/db mice), eNOS deficiency led to marked acceleration of diabetic nephropathy. In further studies, we have now found that although lipid abnormalities induced by either ApoE deficiency or LDL receptor deficiency did not by themselves accelerate diabetic nephropathy, in the presence of endothelial dysfunction seen with eNOS deficiency, diabetic nephropathy was significantly accelerated. We have also begun to examine potential markers and signaling pathways mediating podocyte injury in this model of diabetic nephropathy and of interest, we have found that in the eNOS-/- db/db model of diabetic nephropathy, there is increased podocyte expression of the prorenin receptor, as determined by in situ hybridization and immunofluorescence.

We have also undertook a proteomic approach to investigate potential underlying mechanisms mediating the accelerated nephropathy seen in the eNOS knockout diabetic mice. We isolated glomeruli from mice with a sieving/magnetic bead approach and utilized MALDI-TOF MS to analyze differential glomerular protein expression. We found a number of proteins that were differentially expressed and are studying potential underlying pathophysiologic mechanisms. One protein of interest is peroxiredoxin 6, a mitochondrial thioredoxin that serves both as an antioxidant and a phospholipase A2 inhibitor. We have confirmed by both RT-PCR and immunoblotting that peroxiredoxin 6 is decreased in the glomeruli from diabetic kidneys of mice with eNOS deficiency. We have obtained the peroxiredoxin knockout mice from AB Fisher at the University of Pennsylvania and are awaiting their release from quarantine so that we can begin to study whether peroxiredoxin 6 deficiency leads to acceleration of diabetic nephropathy.

B) The Role of Superoxide Dismutase-1 is Development of Diabetic Nephropathy

Growing evidence has implicated superoxide overproduction as a common pathogenic pathway in diabetic nephropathy (DN). However, the precise role of antioxidant enzyme in this disease is still incompletely understood. We have reported that renal expression of superoxide dismutase-1 (SOD1/CuZn-SOD), a cytosolic SOD isoenzyme, is prominently down-regulated in KK-strain *Ins2Akita* diabetic mouse which exhibits progressive DN but not in DN-resistant C57BL/6-strain *Ins2Akita* (C57BL/6-Akita) mouse (JASN 18: 61A). To determine the importance of SOD1 down-regulation in DN, we here generated SOD1-deficient C57BL/6-Akita mouse and examined their renal phenotype up to the age of 20 weeks. Renal superoxide production measured by water-soluble tetrazolium salt formazan assay was significantly increased in SOD1 deficient (SOD1-/-) C57BL/6-Akita males compared to wild-type (SOD1+/+) C57BL/6-Akita males. Further, SOD1-/- C57BL/6-Akita mice exhibited significantly increased albuminuria and lower glomerular filtration rate, although differences were not observed in blood glucose, HbA1c, body weight, and kidney weight between SOD1-/- and SOD1+/+ C57BL/6-Akita mice. Finally,

histological examination revealed an increase in mesangial matrix expansion in SOD1-/- C57BL/6-Akita mice at the age of 20 weeks. Significant renal phenotypes were not observed in SOD1-/- C57BL/6 (non-diabetic control) mouse. In conclusion, the present study demonstrates an important role for SOD1 isoenzyme in preventing renal injury under chronic hyperglycemic condition.

2. Collaboration:

With Jax: As indicated above, when our floxed mice are available, we have made arrangements with Jackson Labs to undertake the appropriate backcrosses onto the strains of interest.

With the MMPCs: We will continue to utilize the Phenotyping facilities at the Vanderbilt MMPC for functional characterization of the mice generated in this project.

3. Responses to Previous EAC Comments

Specific EAC Comments to Vanderbilt AMDCC

1) This group focuses on the "endothelial dysfunction" phenotype characteristic of human microvascular disease in diabetes. They have generated targeting vectors and mouse lines necessary to continue their studies. Very productive.

Your BKS-eNOS mice should be reposited with JAX by Spring 2008. These mice should also be phenotyped for CV (hypertension, endothelium-dependent vasodilation, atherosclerosis and cardiac function) and neuropathy. Interesting comparison to Dr. Smithies eNOS mice.

We are in the process of completing the transfer. We are planning to have Vanderbilt MMPC characterize the cardiovascular phenotype and have already had discussions with the director, David Wasserman.

2) Conditional eNOS blasts should be shipped to JAX for injection ASAP.

See above

3) Longer term diabetes studies should be carried out with the BKS-eNOS-/+ mice.

We are presently carrying out the longer term studies- we have a group of mice that we will follow for up to one year.

In addition, in the general EAC comments, Comment #7 **was: "*Is the value of outbred mouse lines being fully utilized for the study of diabetic complications?*** *Such lines may help define the mechanisms by which human ancestral differences affects phenotypes. Alternatively, because the genetic variation of outbred strains is relatively small (compared to the human population), using a range of inbred strains (such as the phenome panel) might provide a useful starting point."*

In this regard, we will submit a pilot project that will characterize the CD-1 outbred mouse line, which appears to be susceptible to develop diabetic nephropathy.

4. Publications:

Kanetsuna, Y, Takahashi, K, Nagata, M, Gannon, MA, Breyer, MD, Harris, RC and Takahashi, T. eNOS Deficiency Confers Susceptibility to Diabetic Nephropathy in Nephropathy Resistant Inbred Mice. Am. J. Pathology 170:1473-84, 2007

Breyer MD, Tchekneva E, Qi Z, Takahashi T, Fogo AB, Zhao HJ and Harris RC. Genetics of diabetic nephropathy: lessons from mice. Seminars Nephrol. 27:237-47, 2007.

Breyer, MD, Tchekneva, E, Qi, Z, Takahashi, T, Fogo, AB and Harris, RC. Examining diabetic nephropathy through the lens of mouse genetics. Current Diabetes Reports 7:459-66, 2007.

Breyer, MD, Qi, Z, Tchekneva, E and Harris, RC. Insights into the genetics of diabetic nephropathy through the study of mice. Current Opinion Nephrology and Hypertension 17:82-86, 2008