Animal Models of Diabetic Complications Consortium
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"Generating Mouse Mutants With Diabetic Nephropathy"
Vanderbilt University School
Principal Investigator

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Part A:
Principal Investigator's Summary

There is abundant evidence that endothelial dysfunction is involved the pathogenesis of microvascular diabetic complications, particularly nephropathy, retinopathy and neuropathy. The goal of the Vanderbilt component of the AMDCC is to investigate the role of microvascular endothelial dysfunction in the development of diabetic nephropathy. Specifically, we are attempting to investigate the role of eNOS and prostacyclin synthase (PGIS), two endothelial genes encoding biochemically interrelated enzymes that produce substances important for vasodilation and maintenance of the integrity of the endothelium. 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. 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. In prior years, we had completed the construction and assessment of the targeting construct, and the Vanderbilt transgenic core electroporated the construct into 29S6/SvEvTac ES cells. 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 previous 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. Because we did not obtain successful targeting from this first round of injections, we reinjected our clone 1A6 and clone 6A9 on 11/09. From Clone 1A6, 10 of 15 pups were chimeric and from Clone 6A9, 8 of 12 pups were chimeric. Six highly chimeric mice were identified from each clone. From breeding with C57Bl6J females with the chimeras from clone 1A6, 3 of the chimeric males were proven to have germline transmission (1A6#1, 1A6#3 and 1A6#4). From Clone 6A9, 2 chimeric males have proven germline transmission (6A9#7 and 6A9#11). We have crossed these floxed mice with 129S4/SvJaeSor-*Gt(ROSA)26Sortm1(FLP1)Dym*/J (129-FLPe), which successfully removed the *Neo* cassette. We are now in the process of crossing these mice with a Sox2-Cre, which selectively targets the endothelium, to determine if we are able to delete eNOS successfully and selectively from the endothelium.

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. We have therefore performed an additional injection and are waiting to determine if we obtain chimeric pups that have achieved germline transmission.

Plans for the Upcoming Year: Our goal is to confirm successful deletion of the eNOS gene within the next 6-8 weeks. We then hope to send them to Jackson Labs for speed congenic backcrossing to 129/sv and DBA2/J Akita backgrounds. In the meantime, we also will also make Sox2Cre mice available to Jackson Labs for backcrossing to DBA2/J and 129/sv backgrounds so that we will be able to selectively knock out eNOS in the endothelium. We are also hopeful that we will have PGIS synthase floxed mice available for backcrossing sometime in 2010.

Preliminary Milestones for 2010 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 2010 or early 2011 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 order to investigate the role of altered eNOS activity in the development of diabetic nephropathy, we have studied *db/db* (BKS) mice, at 17, 26, 36 and 52 weeks. Moderate mesangial expansion was found at 26 weeks, with progressive increases with aging. GFR increased from 276±38 ul/min/mouse at 17 wks to 354±29 at 26 wks and 408±35 at 36 wks, followed by a decline to 221±71 at 52 wks (BKS control: 289±19). Neither immunohistochemistry nor immunoblotting indicated any significant alteration of glomerular eNOS monomer expression, but eNOS dimerization progressively decreased beginning at 26 wks old (dimer/monomer ratio: 0.28±0.02 at 14 wks; 0.23±0.03 at 26 wks; 0.18±0.02 at 36 wks and 0.13±0.04 at 52 wks), indicating eNOS uncoupling. In addition, there was decreased phosphorylation of eNOS at Ser 1177, an essential step in eNOS activation, beginning at 26 wks, without significant change of phosphorylation of Thr 495, a marker of enzyme inhibition. Immunofluorescent localization confirmed increased COX-2 expression in glomerular mesangium, endothelium and Bowman's capsule by 26 wk. In endothelium, the increased COX-2 partially co-localized with eNOS, suggesting the possibility of a compensatory role for COX-2 in the face of defective eNOS activity. In *db/db* (BKS) mice with eNOS deficiency (eNOS KO-*db/db*), glomerular COX-2 expression was further increased. Administration of a COX-2 specific inhibitor, SC 58236 (6mg/L in drinking water) to eNOS KO-*db/db* and age matched (26 wks) eNOS KO mice for 4 weeks accelerated renal injury in eNOS KO-*db/db*, indicated by more marked mesangial expansion and nodular sclerosis and further declines in GFR (from 188±31 to 132±7 ul/min/mouse in eNOS KO-*db/db* vs. 244±27 to 175±6 in eNOS KO). Therefore, these studies indicate that eNOS uncoupling and impaired phosphorylation at Ser 1179 progressed after 26 wks of age in *db/db* (BKS) mice. The upregulated COX-2 in mesangium and endothelium and eNOS deficiency in aged *db/db* mice, along with the detrimental effect of COX-2 inhibition to eNOS KO-db/db mice, suggest that increased COX-2 may play a compensatory role in the face of eNOS dysfunction as diabetic nephropathy progresses.

We have also 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. Diabetic nephropathy (DN) increases podocyte COX-2 expression, and COX-2 inhibition reduces proteinuria and glomerular injury in animal models of diabetes. To investigate if COX-2 plays a pathogenic role in diabetic glomerular injury and examine interaction with local RAS

activation, we used a low dose streptozotocin model of diabetic mellitus in wild type (Wt) and nephrin promoter driven COX-2 transgenic mice (tg), which selectively over-express COX-2 in podocytes. Age and gender matched mice were divided into five groups: 1. Wt (citrate buffer only); 2. Wt+STZ; 3. Tg control (Tg); 4. Tg+STZ; 5. Tg+STZ treated with the COX-2 selective inhibitor, SC58236. STZ induced hyperglycemia equally, and blood pressure and GFR were not different among groups. Animals were sacrificed after 16 weeks of diabetes. Progressive albuminuria developed only in diabetic transgenic mice (Tg+STZ vs Wt+STZ: 97.1±9.1 vs 28.3±5.3 μg alb/mg Cr n=5-6: tg control (24.3±5.3, n=12). Electron microscopy indicated significant foot process effacement (FPE) (70±10%), moderate mesangial expansion and segmental (30%) GBM thickness in Tg+STZ; while minimal FPE (12±2%) and mesangial expansion and no increase in GBM thickness were detected in Wt+STZ. Elevated COX-2 was detected in Tg control mice, and STZ further up-regulated its expression. Increased glomerular COX-2 was largely restricted to podocytes. COX2 inhibitor treatment reduced albuminuria (to 49.8±4.3), decreased FPE to 40±2% and inhibited mesangial matrix expansion. Real time PCR demonstrated that prorenin receptor (PRENr) mRNA increased in glomeruli from tg mice (2.3±0.3 fold wild control) and STZ further stimulated expresion (to 3.9±0.2 fold). In situ hybridization and immunohistochemistry indicated that the increased PRENr localized to podocytes and mesangial cells. The COX-2 inhibitor partially inhibited PRENr mRNA up-regulation (from 3.9±0.2 to 2±0.6 fold control) in the diabetic tg mice. These studies demonstrate that increased podocyte COX-2 expression predisposes to diabetic glomerular injury and suggest that glomerular prorenin receptor up-regulation and subsequent RAS activation might play a role.

As noted in our previous progress report, we 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. One protein of interest that was selectively decreased in the eNOS-/- db/db mice was peroxiredoxin 6. Both RT-PCR and immunoblotting confirmed decreased glomerular peroxiredoxin 6 expression. We have obtained the peroxiredoxin knockout mice from AB Fisher at the University of Pennsylvania and have expanded our colony. We are currently studying whether there is accelerated nephropathy in mice in which diabetes was induced by the low dose STZ protocol.

B) Characterization of the role of superoxide dismutase-1 in the 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. These studies demonstrate an important role for SOD1 isoenzyme in preventing renal injury under chronic hyperglycemic condition. Based on the finding, we have backcrossed nephropathy-resistant C57BL/6-strain *Ins2+/C96Y(Akita)* mouse (C57BL/6-Akita) to the nephropathy- prone KK/Ta strain mouse, and successfully generated a new congenic diabetic strain of the Akita mutation, KK/Ta-Akita mouse, that exhibits progressive DN. By comparative analysis of these two models, we have found that renal expression of superoxide dismutase (SOD) isoenzymes, SOD1 and SOD3 , but not SOD2, is prominently down-regulated in KK/Ta-strain Akita mouse which exhibits progressive DN, whereas their expression was unaltered in DN-resistant C57BL/6-Akita mouse. Given the fact that SOD enzymes serve as a major defense system against superoxide overproduction which plays a central role in diabetic vascular cell injury, the finding suggests that SOD1 and SOD3 down-regulation may be a key mechanism in the development of advanced DN.

C) Characterization of additional murine models of diabetic nephropathy

1) We have backcrossed the eNOS-/- mice onto both the DBA2 strain and the KK/HIJ strain. In preliminary studies, we have made the mice diabetic with th low dose STZ protocol. Both strains of mice exhibited accelerated nephropathy (urine ACR is in the 700-1000 µg/mg range and there is advanced glomerular pathology); however, to date we have only studied two mice per strain. We will study more of these mice in the upcoming year to determine if the glomerular lesions resemble that of human nephropathy than what we have reported in the B6 STZ-eNOS-/- mice.

2) We are also trying to generate DBA2-Akita eNOS-/-, KK/HIJ-Akita eNOS-/- mice. However, we are having difficulty expanding the KK/HIJ-Akita colony.

3): The C57BLKS SOD1 +/- mouse strain is now on the N9 generation. We are going to cross this mouse with db/m mouse to generate db/wt SOD1 +/- mouse.

4) Long term follow-up of JAX's DBA2-Akita mice (n=10, until 35 weeks of age): Our data demonstrates that this Akita strain develops nephropathy at 15-20 weeks of age, and the nephropathy progresses slowly, the mice are susceptible to UTI (infection). In additional studies, we are currently studying whether unilateral

nephrectomy will accelerate development of diabetic nephropathy in this model, as well as in DBA2 mice made diabetic with the low dose STZ protocol.

5) We have also backcrossed the Akita mutation onto SOD-3-/- mouse, which did not develop nephropathy and are currently producing an SOD1-/- xSOD3-/- mouse with the Akita mutation.

Collaboration:

With Jax: The development of the floxed eNOS mouse was a collaboration with Jackson Labs. 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.

Responses to Previous EAC comments

1) The Vanderbilt group remains highly productive. To date, this pathobiology site has developed the model thought to be the best phenocopy of human diabetic nephropathy. A number of standard and conditionally targeted allele carrying strains have been/are being generated to study the role of vascular wall Ptgis and Nos3 in diabetic complications.

As we indicate in our progress report, we have made significant progress in our major goal to target the eNOS gene (the project that the AMDCC selected as our first priority). As we indicate, the ES injections performed at Jackson laboratory led to successful germline transmission. We have subsequently been able to demonstrate successful deletion of the Neo cassette by crossing with 129-flpe Cre mice. We are currently crossing these offspring with Sox2-Cre mice to determine if we will have selective eNOS deletion from the vasculature. With the help of Jackson Labs, we will then backcross these to nephropathy-sensitive backgrounds. As noted above, we are also pursuing development of the PGIS floxed mice. Our first round of ES injections did not produce any mice that went germline so we have undertaken another round of injections and are awaiting the results.

2) The follow-ups to the proteomic studies targeting peroxiredoxin are interesting. It would be interesting to test whether this protein is reduced in other models of accelerated diabetic injury such as the bradykinin receptor KO.

As indicated, we are in the process of testing whether there is an acceleration of diabetic nephropathy in the peroxiredoxin 6 null mice. We plan in the near future to test whether there is any alteration in expression of peroxiredoxin 6 in STZ models of diabetes as well as SOD-1-/- models of diabetic nephropathy.

3) The SOD KO results are interesting. How will they be pursued? Is there anything to be learned from the earlier studies on SOD from the Brosius laboratory?

The Brosius laboratory undertook extensive studies of diabetic nephropathy in mice with selective SOD-2 gene deletion and were not able to show any increase in diabetic nephropathy. It was interesting for us to find that in the nephropathy-sensitive strain, KK/TA-Akita, there was a relative decrease in renal expression of the SOD isoenzymes -1 and -3 but not of SOD-2. Our studies with gene deletion of SOD-1 in the DN-resistant C57BL/6-Akita mouse indicated that there is increased nephropathy, suggesting that SOD-1 rather than SOD-2, may be involved. We were somewhat surprised that SOD-3-/- mice did not develop diabetic nephropathy. However, we are in the process of making SOD-1-/- x SOD-3-/- mice with the Akita mutation in order to determine if there is further acceleration of nephropathy.

Recent Publications

Tchekneva, EE, Kjuchua, Z, Davis, LS, Kadkina, V, Dunn, SR, Bachman, S, Ishibashi, K, Rinchik, EM, Harris, RC, Dikov, MM and Breyer, MD. A newly identified ENU-induced single amino acid mutation in aquaporin-11 resulting in perinatal kidney failure in mice. JASN 19:1955-64, 2008

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

Fujita, H, Fujishima, H, Chida, S, Takahashi, K, Qi, Z, Kanetsuna, Y, Breyer, MD, Harris, RC, Yamada, Y and Takahashi, T. Reduction of renal superoxide dismutase in a murine model of progressive diabetic nephropathy. JASN In Press

Cheng, H, Fan, X, Guan, Y, Moeckel, GW, Zent, R and Harris, RC. The role of prostanoids in podocyte injury. JASN In Press

Takahashi, T and Harris, RC. Endothelial Dysfunction in Diabetic Nephropathy. In Advances in the Pathogenesis of Diabetic Nephropathy, SS Prabhakar, ed. In Press.

Brosius, F, Alpers, C, Bottinger, E,, Breyer, M, Coffman, T, Harris, R, Kakoki, M, Kretzler, M, Leiter, E, Levi, M, McIndoe, R, Sharma, K, Smithies, O, Susztak, K, Takahashi, N and Takahashi, T. Mouse Models of Diabetic Nephropathy. JASN In Press

Fujita, H, Fujishima, H, kagaya, K, Morii, T, Takahashi, K, Qi, Z., Shimizu, T, Shirasawa, Breyer, MD, Harris, RC, Yamada, Y and Takahashi, T. Deficiency of CuZn-superoxide dismutase (SOD1) accelerates renal injury in C57BL/6-Ins2^{Akita} diabetic mice. Submitted