ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM (U01 DK60995)

UPDATE REPORT (September 2001 –January 2004)

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ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM (U01 DK60995)

PART A:

PRINCIPAL INVESTIGATOR'S SUMMARY

Program Overview and Aims of Original Proposal (taken from grant application):

Rationale and Proposed Approaches

Diabetic nephropathy (DNP) is well studied in humans and several candidate pathways involved in initiation and/or progression of DNP have been identified. Hyperglycemic damage is limited to those cell types that develop *intracellular hyperglycemia due to a failure to downregulate glucose transport when exposed to extracellular hyperglycemia*. Intracellular hyperglycemia induces *overproduction of superoxide by the mitochondrial electron transport chain*. Overproduction of superoxide by the mitochondrial electron transport chain activates a number of downstream signaling pathways such as protein kinase C, the polyol pathway, the hexosamine pathway, NFκB and advanced glycation endproduct formation, which lead, which lead in part to *activation of the TGF-b signaling pathway*, a central mediator of cellular hypertrophy, apoptosis and fibrosis. Structural damage to and *depletion of glomerular podocytes* is also required for development of progressive sclerotic lesions in glomeruli and is enhanced by defects in cytoskeletal proteins. Our group has brought together leading experts in these important pathogenetic steps presumably involved in diabetic nephropathy. These steps are being attacked to generate valid mouse models of human diabetic nephropathy by combining existing mouse diabetes models and new strains with transgenes and/or targeted gene deletions.

Table 1. Proposed Genetic Approaches useful to Derive New Mouse Models of Human DNP (taken from original application).

1. *db/db* mut in 129Sv/J and C57Bl/6J defined genetic background (available at Jackson Labs)
2. Glut1 transgene expression (to be created)
   a) tie-1 promoter construct for endothelial targeting of Glut1
   a) podocin promoter construct for targeting of transgene expression in podocytes
3. Decorin deficiency (available at TJU)
4. Synaptopodin deficiency (available at AECOM)
5. Mitochondrial superoxide dismutase deficiency (available at AECOM)

We will propose new standards for validating these models of diabetic nephropathy which rely heavily on advanced histopathology, innovative gene expression profiling and computational analysis. Human anatomic and pathologic information will be used to define and propose standards for histopathologic and functional validation of DNP in existing and newly derived mouse models. Information from ongoing gene expression profiling of these existing models and of planned transcriptome exploration of human diabetic nephropathy will be used to define and propose standards for genomic validation. Computational analysis will ascertain which of the different models mimics transcriptome changes seen in human DNP. Thus, comparative transcriptional profiling will add significant depth to validating mouse models of DNP.

Accomplishments of Program

A brief summary of the progress, accomplishments, and perceived problems of our program since its inception in fall of 2001 is provided here. Detailed illustrations and updates for major projects of our program are provided in PART B of this report.

I. Strain Creation and Background Comparisons

High priority projects

I.1. We completed strain creation, characterization and backcrossing to C57BL/6 background of Tie1-GLUT1 transgenic lines characterized by forced expression of GLUT1 in endothelial cells, including glomerular capillaries (Project leader: Maureen Charron, AECOM).

We had proposed to create new transgenic mouse strains for overexpression of GLUT1 in mouse endothelial cells using established tissue-specific promoter Tie1 promoter. The goal is to determine whether forced glucose uptake in endothelial cells in diabetic mice enhances manifestations of nephropathy according AMDCC Nephropathy criteria. Four Tie1-GLUT1 founder lines (A – D) were generated and characterized. Line C demonstrates best expression profile of GLUT1 transgene in the kidney and glomerular vascular bed. Two lines (C and D) were backcrossed eight generations with C57BL/6. Matings of C57BL/6 Akita type I diabetic and Tie1-GLUT1 transgenic mice are ongoing. In addition, we plan to start experimental model phase subjecting Tie1-GLUT1 mice to the low-dose STZ protocol starting spring 2004.

I.2. We completed creation of new transgenic strain (NPHS2-GLUT1) to force constitutive expression of GLUT1 in podocytes (Project leader: Maureen Charron, AECOM).

We reasoned that sustained, forced glucose uptake in podocytes may be associated with progressive podocyte depletion and enhanced diabetic glomerulopathy. We are creating a new transgenic strain for constitutive podocyte-specific expression of GLUT1 using a 2.5-kb podocin (NPHS2) promoter. We have recently accomplished creation of potential founder lines. Genotyping and characterization of transgene expression is scheduled for spring 2004.

Ongoing low priority projects

I.3. Analysis of kidney phenotypes previously associated with Glut4 +/- mice in 129 inbred background (Project leader: Maureen Charron, AECOM)

Backcross of Glut4 targeted allele from C57BL6 to 129 background is currently in the 8th generation. Glomerular abnormalities of diabetic nephropathy, as observed previously in 129 background, have not been identified so far. This is a low priority and low effort activity and we will terminate further backcrossing and analysis if we find that 8th generation 129/J background does not reveal new and increased renal lesions in Glut4+/- mice.

Completed projects

I.4. We completed a direct comparison of renal phenotypes in C57BL/6J ("sclerosis-resistant" genetic background) and 129/J ("sclerosis-prone" genetic background) mice after STZ low-dose protocol (Project leader: Kumar Sharma, TJU).

While diabetic 129/J mice had increased mesangial expansion scores compared with diabetic C57Bl/6 mice, we did not find qualitatively different lesions and nephropathy phenotype criteria were not significantly enhanced in 129/J background. We concluded and reported to the Steering Committee that 129/J background does not provide significant advantages compared with C57BL/6J to enhance manifestation of characteristic phenotype lesions of diabetic nephropathy in mice.

New project recommended and approved by the Steering Committee and EAC

I.5. We identified and developed Cd36 as a novel candidate mediator of tubulointerstitial disease in diabetic nephropathy and initiated creation of Cd36 transgenic strain for tubuar transgene expression (Project leader: Erwin Bottinger and Katalin Susztak, MSSM)

At the 2003 Steering Committee meetings, we presented our promising findings identifying Cd36 as mediator of AGE and FFA induced apoptosis in proximal tubular epithelial cells. The Steering Committee and the External Advisory Committee (EAC) recommended that our group should initiate a new project to evaluate whether expression of Cd36 in proximal tubules would mediate tubulointerstitial manifestations of diabetic nephropathy, which have not been detected in any of the existing models to date. Quote from a previous EAC report: *"The CD36 story is quite interesting, and it should be pursued as you plan. Creating a proximal tubule CD36 knock-in might be worthwhile to see how it interacts with oxidized LDL; ox-LDL is highly atherogenic and highly cytotoxic to many cell types, including vascular wall cells. CD36 is one of the major scavenger receptors for ox-LDL on many cell types, and the diabetic state is associated with higher oxidation levels and an increase in oxidation of LDL. Thus, there may be increased uptake of ox-LDL contributing to apoptosis and subsequent scarring in these kidneys.*"

We plan to generate mice with transgenic expression of Cd36/FAT specifically in tubular epithelial cells using gammaGT promoter transgenic targeting vector. We hypothesize that when made diabetic, gammaGT-Cd36 transgenic mice may develop progressive tubulointerstitial lesions typically observed in human diabetic nephropathy. Generation of a gGT-Cd36 transgene targeting vector is ongoing and we plan to have gGT-Cd36 transgenic strains available by the end of 2004.

II. Models and Experiments

Ongoing project nearing completion

II.1. Effect of deficiency of decorin, a natural inhibitor of TGF-β, on diabetes

induced nephropathy in mice of C57BL/6 genetic background (Project leader: Kumar Sharma, TJU)

Mice lacking decorin were postulated to be pre-disposed to progressive diabetic nephropathy due to lack of the endogenous inhibitor of active TGF-b. At present, we have 10 mice with diabetes for at least 4 months in dcn+/+, dcn+/-, and dcn-/- groups. Cohorts have now been followed and evaluated using AMDCC Nephropathy standard assays for body weight, glycemia, albuminuria and creatinine clearance at 8, 16, 24, 32, and 40 weeks. STZ-treated Dcn+/- and dcn-/- mice tend to have increased U alb/crea ratios, and decreased Crea clearances by HPLC when compared with STZ-treated dcn+/+ control mice (see also spreadsheet 'einstein report' for detailed data).

Suspended project

II.2. Effect of deficiency of synaptopodin, an actin bundling protein critical for structural homeostasis of podocytes, on diabetes induced nephropathy in mice of C57BL/6 genetic background (Project leader: Peter Mundel, AECOM)

Deficiency of synaptopodin protein in mice is associated with increased susceptibility for glomerulosclerosis induced by diverse glomerular injury models (Peter Mundel, unpublished). The synaptopodin targeted allele was backcrossed to inbred C57BL6 background for 8 generations. This project is currently on hold and resources have been prioritized to generate the gGT-Cd36 transgenic mouse model with approval of the Steering Committee.

III, Standardization and Application of Phenotyping Tools for Model Validation

Completed and published

III.1. We completed the study "Molecular Profiling of Diabetic Mouse Kidneys Reveals Novel Genes Linked to Diabetic Nephropathy, including Hsd3b4 and Cd36" (Project leaders: Erwin Bottinger and Kumar Sharma).

To describe important genetic and gene-environmental interaction during the pathogenesis of diabetic nephropathy, the Bottinger and Sharma laboratories had initiated a collaborative study using microarray analysis (Bottinger) on existing mouse models of diabetic nephropathy (Sharma) in 2000 before inception of the AMDCC. The microarray analysis phase of this project was completed by end of 2001 with funding from other mechanisms. Data analysis performed by Dr. Susztak in Bottinger's group suggested specifically sex hormone synthesis enzyme hydroxysteroid dehydrogenase 3beta isotype 4 (Hsd3b4) and the scavenger receptor Cd36 as new candidate genes involved in diabetic nephropathy.

A manuscript by Susztak et al. describing our findings was published in the March issue of Diabetes [Diabetes. 2004 Mar;53(3):784-794] ; see attached pdf file by Susztak et al.

III.2. We completed development of a new and improved HPLC-based method to determine levels of creatinine in serum and/or plasma of mice, and implemented the method as AMDCC core service for all members (Project leader: Kumar Sharma, TJU).

This project led to a very significant contribution to improve and standardize phenotyping methods for non-invasive, quantitative evaluation of renal function in mice. An HPLC core laboratory was established with supplemental funding at TJU and is now operational. A manuscript by Dunn et al. describing the methodology is scheduled for publication in the May issue of Kidney International. See attached pdf file by Dunn et al.

Ongoing project nearing completion

III.3. AMDCC-standardized, longitudinal phenotype analysis of kidney morphology and function in the db/db model of type II diabetes on BLKS genetic background (Project leaders: Erwin Bottinger and Katalin Susztak, MSSM)

Comprehensive, longitudinal phenotyping of db/db mice in C57BLKS background using AMDCC Nephropathy phenotyping standards represents an essential "benchmark dataset" (BDS), but is not available to date. We completed a longitudinal study to establish a BDS for diabetic nephropathy in db/db C57BLKS mouse. We demonstrate that

- Hyperglycemia is persistent in db/db on C57BLKS background
- Onset of hyperglycemia at 8 wks is associated with GBM thickening, podocyte apoptosis and depletion, and precedes significant albuminuria detectable at 12 wks.
- A trend to glomerular hyperfiltration is consistently detectable at 12 and 20 weeks.
- Tubulointerstitial lesions or apoptosis are absent in db/db throughout the study period up to 28 wks.

These results suggest that the course and manifestations of hyperglycemia induced glomerulopathy in db/db C57BLKS resemble those of diabetes-induced glomerular lesions in humans. However, we have not yet been able to document progressive decline in renal function and tubulointerstitial lesions in this model.

Collaborations with other Groups (Including Core Facilities):

Dr. Sharma has carried out a major collaboration with Dr. Breyer in the AMDCC program at Vanderbilt to evaluate the utility of HPLC-based serum creatinine assays and endogenous creatinine clearance as a measure of renal function in mice. These studies suggest that conventional methods (picric acid) for creatinine measurements in serum or plasma are unreliable and demonstrate a novel and improved way to determine renal function in mice by HPLC-based creatinine assays. In fact, the AMDCC has adopted the methodology developed by Drs. Sharma and Breyer as standard for the consortium and a publication from Dr. Sharma's laboratory as the lead laboratory is IN PRESS scheduled for the May 2004 issue of the journal Kidney International.

Pertinent non-AMDCC Collaborations:

Project leaders: Erwin Bottinger and Katalin Susztak, MSSM:
Upon recommendation of the AMDCC Steering Committee, we have imported flk-1 RAGE transgenic mice from Dr. H. Yamamoto of Kanazawa University in Japan on a collaborative basis in late fall 2003. AMDCC standardized phenotyping and diabetes protocol experiment will be conducted in 2004 and 2005 on behalf of the Consortium.

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PART B:

UPDATE BY PROJECT LEADERS

We present detailed updates on seven completed and ongoing projects.

Responsible Investigators: **Kumar Sharma, M.D.**

Project Title: **Development and validation of new phenotyping standards for mouse models of diabetic kidney disease: A novel HPLC-based plasma creatinine assay and endogenous creatinine clearance to measure renal function**

Project Status: **- Completed -**

A. Rationale and relevance

The use of endogenous plasma creatinine and creatinine clearance as a tool to evaluate renal function in mice has come under scrutiny. Prior studies have reported that the Jaffé alkaline picrate method grossly overestimates true plasma creatinine in mice. As members of the NIDDK Animal Models of Diabetic Complications Consortium (AMDCC), we evaluated the performance and feasibility of alternative HPLC-based methods for standard determination of plasma creatinine and creatinine clearance in mice. Our purpose was to develop a simple HPLC method that provides a reliable, reproducible and sensitive assay for small volumes (< 25 microliters) of mouse plasma.

B. Summary of Accomplishments

A critical question for adequate phenotyping of progression of renal insufficiency was to address how to measure renal function in mice in a non-invasive manner. We obtained HPLC equipment via supplemental funds from NIDDK and optimized a simple, isocratic assay to reproducibly measure creatinine levels in mouse plasma or serum. The assay requires between 10-25 ul of sample and is sensitive down to 0.01 mg/dl of creatinine. Using this assay and comparing to creatinine measurements using the conventional Jaffe reaction and a FITC-inulin clearance technique (developed by Matt Breyer's group at Vanderbilt) we have made the following conclusions:

1) HPLC creatinine levels in mouse plasma are 3-fold lower than creatinine levels as measured by Jaffe in normal C57Bl6/J female and male mice.
2) HPLC plasma creatinine, 1/creatinine, or creatinine clearance is reflective of changes in renal function in mice as induced by a low salt diet with enalapril, or a high salt diet.
3) HPLC plasma creatinine, 1/creatinine, or creatinine clearance is reflective of renal function in streptozotocin-induced diabetic mice.
4) HPLC creatinine clearance correlates significantly with FITC-inulin clearance in conscious mice ($r=0.643$ and $p<0.001$), whereas creatinine clearance based on Jaffe does not ($r=0.372$ and $p=0.075$). This data is included in an ASN abstract submitted for 2003 ASN meeting.

C. Plans for the coming year

Per recommendation of the AMDCC SC and with approval of the Advisory Panel, Dr. Sharma and Mr. Steve Dunn have established and operate a core to provide standardized HPLC creatinine measurements for all members of AMDCC.

D. Significant Achievement

- Developed and validated a new and significantly improved method for plasma creatinine measurement in mice for renal function assay by endogenous creatinine clearance
- Established operational core open for standardized HPLC creatinine measurements for models developed by all members of AMDCC
- Published method and validation paper.

Publications:

A manuscript reporting the assay under the title “Utility of Endogenous Creatinine Clearance as a Measure of Renal Function in Mice” is scheduled for publication in the May 2004 issue of Kidney International with authors/co-authors Stephen R. Dunn[1], Emilio Ciccone[1], Zhonghua Qi [2], Erwin P. Böttinger[3], Matthew D. Breyer[2], Kumar Sharma[1]

See Appendix pdf file Dunn_Kidney Int_ 2004

Responsible Investigators:	**Kumar Sharma, M.D.**
Project Title:	**Effect of deficiency of decorin, a natural inhibitor of TGF-β, on diabetes induced nephropathy in mice of C57BL/6 genetic background**
Project Status:	**Experimental Model and Phenotyping**

A. Rationale and Relevance

The cytokine TGFß is a logical target for the creation of new mouse models of diabetic complications that more realistically mimic human disease. We hypothesized that augmentation of TGFß action in a diabetic model will enhance the speed and complexity of subsequent renal disease.

We proposed to use the decorin-deficient mouse (Danielson, Baribault, et al. 1997) as an indirect approach to increase local TGFß activity to an extent that is pathophysiologically plausible. The proteoglycan decorin is a natural inhibitor of TGF-β. Decorin expression is increased in kidneys of diabetics, possibly as a compensatory response to antagonize local activity of TGFß. A colony of Dcn knockout mice has been available at TJU in Dr. Williams's and Dr. Sharma's laboratories. The basal (*i.e.,* non-diabetic) phenotype of Dcn-/- mice is minimal and appears to be limited to skin fragility.

B. Summary of accomplishments

Mice lacking decorin were postulated to be pre-disposed to progressive diabetic nephropathy due to lack of this endogenous inhibitor of active TGF-b. Since Dcn+/- were in a mixed background at the beginning of the project in the fall of 2001, we needed to backcross to C57BL/6 genetic background for five generations. Next, cohorts of wild type (+/+), heterozygous (+/-), and decorin knockout (-/-) male mice in C57BL/6 background were made diabetic with the multiple low dose strep protocol (50 mg/kg/ip qd for 5 consecutive days) and monitored using AMDCC standardized protocols. Interestingly, decorin KO mice appeared to be resistant to the effects of STZ as it required roughly twice as much STZ to maintain blood glucose values above 300 mg/dl in the decorin KO group as compared to the heterozygous and wild type groups. In the wild type mice, 21/29 (73%) became diabetic (blood glucose > 300 mg/dl), in the heterozygous group 18/30 (60%) became diabetic, and in the knockout group only 14/27 (52%) became diabetic. This interesting observation will be followed up in independent studies. After two months of diabetes 2 mice in each group were sacrificed. No obvious differences were noted in the kidneys of the diabetic or control groups independent of genotype. At present, we have 10 mice with diabetes for at least 4 months in all groups. At 4 months there is a slightly greater degree of albuminuria in diabetic knockout mice than other groups but the increase vs non-diabetic is only 30% (34 □g/24h vs 26 □g/24h, respectively).

Cohorts have now been followed and evaluated using AMDCC Nephropathy standard assays for body weight, glycemia, albuminuria and creatinine clearance at 8, 16, 24, 32, and 40 weeks. Results are presented in detail in Excel spreadsheet format in the document “Einstein report”.

C. Plans for the coming year

Urine will be screened for albumin excretion and creatinine clearance will be determined every two months and mice will be sacrificed when they meet the screening endpoint of the consortium’s goals (see AMDCC Nephropathy Report) or if mice begin to lose weight and appear sickly.

D. Significant achievement and its importance

- Albuminuria tends to be increased in decorin-deficient mice compared to wildtype control mice made diabetic with low-dose STZ (Figure 1).

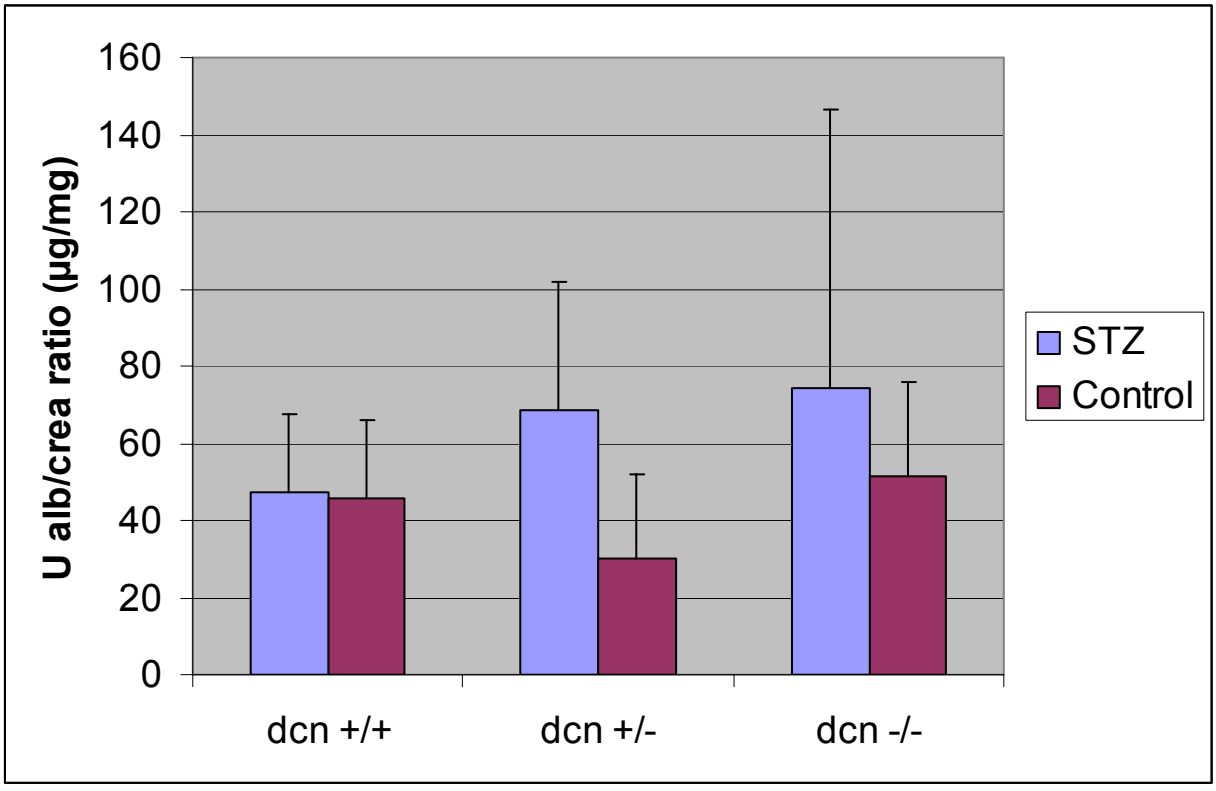

Figure 1. Albuminuria in dcn +/+, dcn +/-, dcn -/- mice at 32 weeks after induction of diabetes with streptozoticin low dose protocol (see AMDCC Nephropathy Report for protocol).

- Creatinine clearance tends to be decreased in decorin-deficient mice compared to wildtype control mice made diabetic with low-dose STZ (Figure 2).

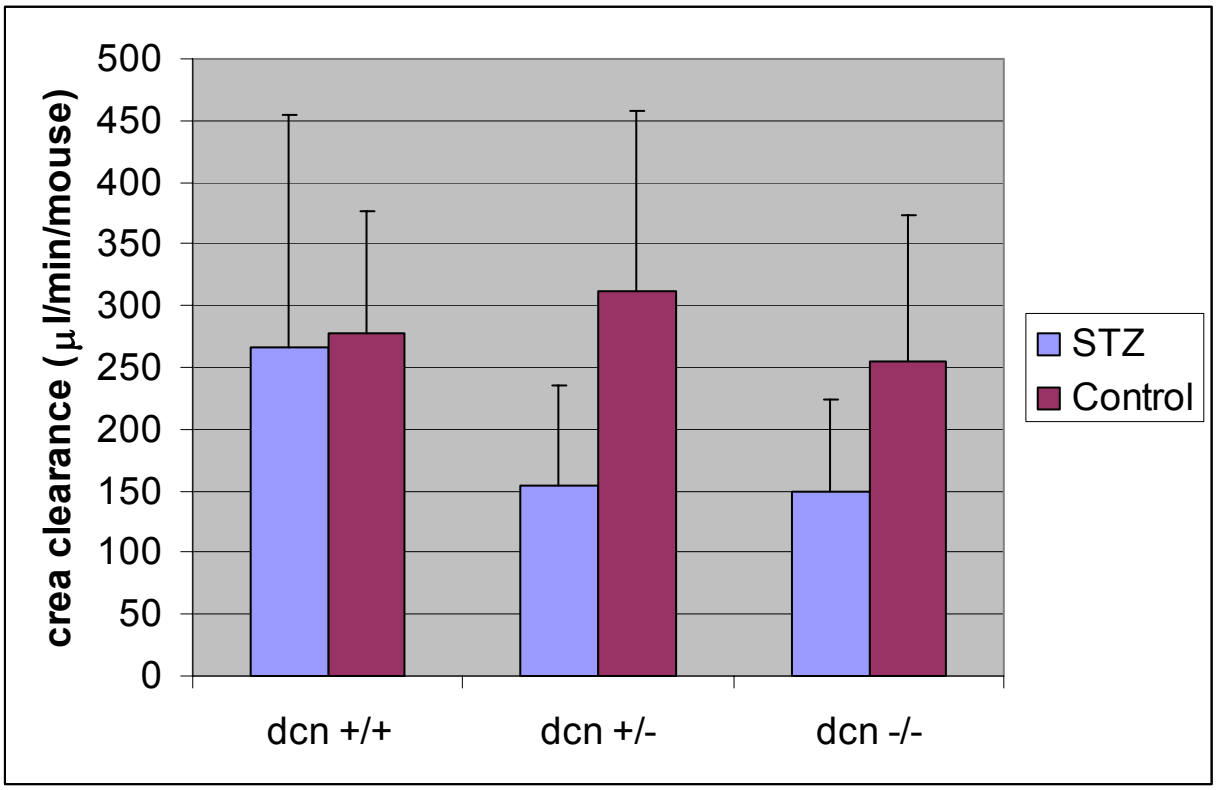

Figure 2. Creatinine clearance in dcn +/+, dcn +/-, dcn -/- mice at 32 weeks after induction of diabetes with streptozoticin low dose protocol (see AMDCC Nephropathy Report for protocol).

Responsible Investigators: **Maureen Charron, M.D.**

Project Title: **Create Tie1-GLUT1 transgenic strains to maximize the effect of increased capillary endothelial GLUT1 expression and glucose uptake on diabetes induced nephropathy in mice**

Project Status: **- Strain Creation Complete –**
- Diabetes Experiment Ongoing –

A. Rationale and Relevance:

Clinical and animal model data indicate that chronic hyperglycemia is the central initiating factor for all types of diabetic microvascular disease. Duration and magnitude of hyperglycemia are both strongly correlated with the extent and rate of progression of diabetic microvascular disease. At present it is not known whether plasma membrane overexpression of GLUT1 *in vivo* could provoke diabetic pathology in a normal animal or accelerate diabetic pathology in a diabetic animal. To determine whether diabetic pathologies could be induced in endothelial cells by increased expression of GLUT1 *in vivo* in the absence or presence of hyperglycemia, we have created/are creating new transgenic mouse strains to overexpress GLUT1 in mouse endothelial cells using established tissue-specific promoter Tie1 promoter.

B. Summary of Accomplishments

We obtained the Tie-1 endothelial cell-specific transgene promoter vector and GLUT1 cDNA was available from Maureen Charron's lab. A Tie1-GLUT1 transgene vector was constructed by standard molecular techniques. Sequence fidelity was verified and transgene expression was verified by transfection experiments. The validated vector was linearized and microinjected in FVB/N oocytes at the AECOM Transgenic Core Facility. Four founder lines have been generated. Founders and F1 offspring appear normal and are fertile. F2 matings have been completed and characterization of transgene expression shows strong transgene expression in kidney. Line C (highest expression in kidney) was backcrossed for eight generations with C57BL/6.

C. Plans for the coming year

We will continue to characterize GLUT1 transgene product expression in renal and non-renal vascular beds and expect to conclude this task by early summer of 2004.
We have begun to monitor blood glucose, urinary albumin exretion, and glucosuria in aging cohorts for the two strains with highest expression levels in the kidney.

To accelerate the process of inducing diabetes in Tie1-GLUT1 tg, we have initiated crossing Akita type I DM mice and Tie1-GLUT1 tg lines B and C, both in C57BL/6 background. We will monitor blood glucose levels, urinary albumin excretion, and glucosuria.

Once characterization of transgene expression is complete, we will select the line with best renal expression (probably line C) for colony expansion. Mice will then be shipped to TJU for STZ injection protocols and monitoring.

D. Most significant achievement.

A new strain of transgenic mice has been created and validated with forced increased expression of GLUT1 specifically in endothelial cells, including glomerular capillaries. This strain will allow for the first time to test whether forced glucose uptake in endothelial cells in mice will enhance manifestations of diabetic glomerulopathy and nephropathy. We anticipate that this new strain can be made available to members of AMDCC by spring/summer 2004.

Responsible Investigators: **Maureen Charron, M.D.**

Project Title: **Create NPHS2-GLUT1 transgenic strains to maximize the effect of increased podocyte-specific GLUT1 expression and glucose uptake on diabetes induced nephropathy in mice**

Project Status: **NPHS2-GLUT1 tg Strain Creation Ongoing**

A. Rationale and Relevance:

We have found that both high ambient glucose induce apoptosis in cultured murine podocytes (Susztak and Bottinger, unpublished). We also showed that podocyte apoptosis occurs with the onset of hyperglycemia in db/db mice at approx. 8 weeks of age and is associated with a decrease of glomerular podocyte counts compared with normoglycemic db/m controls. Interestingly, early podocyte apoptosis is transient and not sustained and decrease in glomerular podocyte counts is not progressive. This adaptation may be due to compensatory downregulation of glucose uptake by surviving podocytes. Thus, we reason that sustained, forced glucose uptake in podocytes may be associated with progressive podocyte depletion and enhanced diabetic glomerulopathy.

B. Summary of Accomplishments

We have isolated a 2.5-kb genomic DNA fragment of the human *NPHS2* gene containing the podocyin promoter region as described by Holzman and coworkers. The *NPHS2* promoter was ligated with GLUT1 cDNA in a transgenic vector cassette. Sequence fidelity of the NPHS2-GLUT transgene cassette was confirmed. The plasmid was transiently transfected in murine podocytes in culture to verify functional transgene expression in podocytes.

After we had confirmed function of the transgene in podocytes, the transgene was microinjected in oocytes in January 2004 at the AECOM Transgenic core facility. Five litters of offspring were obtained in February 2004.

C. Plans for the coming year

We will identify founders and establish multiple transgenic lines. We will characterize transgene expression profiles in all new lines of NPHS2-GLUT1 transgenic strain. Two lines with highest kidney/podocyte expression profiles of GLUT1 will be selected for backcrossing to C57BL/6 and DBA (based on recent data from Dr. Breyer's and Dr. Coffman's groups on genetic background strain comparisons) backgrounds for eight generations.

Responsible Investigators:	**Erwin Bottinger, M.D.** **Kumar Sharma, M.D.**
Project Title:	**Molecular Profiling of Diabetic Mouse Kidneys Reveals Novel Genes Linked to Diabetic Nephropathy, including Hsd3b4 and Cd36**
Project Status:	**- Completed and Published -**

A. Rationale and Relevance:

To describe important genetic and gene-environmental interaction during the pathogenesis of diabetic nephropathy, the Bottinger and Sharma laboratories had initiated a collaborative study using microarray analysis (Bottinger) on existing mouse models of diabetic nephropathy (Sharma) in 2000 before inception of the AMDCC..

B. Summary of Accomplishments

The microarray analysis phase of this project was completed by end of 2001 with funding from other mechanisms. Data analysis performed by Dr. Susztak in Bottinger's group suggested specifically sex hormone synthesis enzyme Hsd3b4 and the scavenger receptor Cd36 as new candidate genes involved in diabetic nephropathy. At the fall 2002 and spring 2003 Steering Committee meetings, the Committee and the External Advisory Committee recommended that our group should initiate a new project to evaluate whether expression of Cd36 in proximal tubules would mediate tubulointerstitial manifestations of diabetic nephropathy, which have not detected in any of the existing models to date.

D. Most significant achievement.

While this study was initiated prior to the start of the AMDCC with independent funding to the Bottinger and Sharma labs, it has led to highly relevant discoveries of the Cd36 gene as a new candidate mediator of tubulointerstitial manifestations of diabetic nephropathy. Upon recommendation by the Steering Committee and the EAC, we have now initiated a new project to create a transgenic strain with proximal tubule specific constitutive overexpression of Cd36 (see below).

Publications

A manuscript by Susztak et al. describing our findings was published in the March issue of Diabetes [Diabetes. 2004 Mar;53(3):784-794] ; see attached pdf file by Susztak_Diabetes_2004.

Responsible Investigators:	**Erwin Bottinger, M.D.** **Katalin Susztak, M.D., Ph.D.**
Project Title:	**Cd36: a candidate mediator of tubulointerstitial disease in diabetic nephropathy**
Project Status:	**- Novel Target Characterization Complete** **- gGT-Cd36 Strain Creation Ongoing**

A. Rationale and Relevance:

We have previously demonstrated that diabetic mice develop glomerular lesions resembling those observed in DNP in humans, but in murine diabetes postglomerular pathology and progressive kidney failure are not observed. In a screen of genes expressed in kidneys of mice subjected to experimental type 1 (streptozotocin) and type 2 (db/db) diabetes, we identified the *Cd36* gene as a robust classifier gene for diabetic animals with albuminuria. *Cd36*, also known as fatty acid translocase (FAT), encodes a class B multi-functional scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism.

B. Summary of Accomplishments

Recently we identified CD36 via microarray screen as a gene with a characteristic expression profile that was able to diagnose the absence or presence of the diabetic phenotype in kidney samples. Immunohistochemical analysis showed that renal tubular epithelial cells express CD36, however, while in the mice it is located in the distal nephron and the collecting ducts, it is expressed in the proximal tubule of humans. Also in sharp contrast to mice, CD36 was markedly increased in human diabetic kidney samples. Similar to the in vivo findings, we found marked differences in the in vivo regulation of CD36 transcript by glucose. While its expression was increased in human proximal tubular cells glucose downregulated CD36 in murine collecting duct cells. We found that CD36 mediates apoptosis in tubular cells in vitro, in the presence of its ligands; glycated albumin, palmitic acid, and thromospondin1. The CD36 mediated apoptosis involved p38 MAPK and caspase3 activation. In agreement with the in vitro findings we found significant tubular apoptosis (in CD36 positive proximal tubular cells) and tubulointerstitial disease in human diabetic nephropathy samples, while this was absent in mice. Our data therefore indicate that tubular CD36 expression and regulation have an important role in mediating tubular apoptosis and injury during the development of diabetic nephropathy. Importantly, the lack of expression of Cd36 in tubules of diabetic mice may protect mice from diabetes associated tubular apoptosis and tubulointerstitial scarring.

C. Plans for the coming year

Upon recommendation of the Steering Committee and the EAC, we plan to create new transgenic strains with constitutive overexpression of Cd36 in renal tubuli under control of a proximal tubule expressing gamma-GT promoter. We have obtained Cd36 cDNA and gamma-GT promoter transgenic vector (gift from Dr. Tom Coffman, Duke). Generation of gGT-Cd36 transgenic vector is ongoing. We anticipate to complete transgenic strain creation by summer of 2004 and characterization of expression by the end of 2004.

Responsible Investigators:	**Erwin Bottinger, M.D.** **Katalin Susztak, M.D., Ph.D.**
Project Title:	**AMDCC-standardized, longitudinal phenotype analysis of kidney morphology and function in the db/db model of type II diabetes on BLKS genetic background**
Project Status:	**- Phenotyping Completed –** **- Molecular Fingerprinting Ongoing -** **(see also spreadsheet 'einstein report')**

A. Rationale and Relevance:

A major accomplishment of the first two funding periods of the AMDCC nephropathy group was the standardization of assays, criteria and tools of morphological and functional phenotyping of kidney in mouse models. The db/db mouse model currently is considered the most robust model of hyperglycemia-induced glomeropathy, including mesangial matrix expansion and albuminuria, in mouse. Recent observations from Dr. Breyer's group indicate that renal lesions may be enhanced in mixed C57BLKS background when compared to inbred C57BL/6. To analyze the phenotype of db/db mutants in C57BLKS background in a longitudinal analysis applying for the first time AMDCC Nephropathy standards (see AMDCC Nephropathy Report), we conducted the following study.

B. Summary of Accomplishments

We have completed the experimental phase of this study. Blood, urine, and kidneys were harvested from db/db (BLKS) and db/m (BLKS) mice (N=10 per group) at 4, 8, 12, 20, and 28 week-of-age. Ongoing phenotype analysis is performed applying standard AMDCC nephropathy phenotyping protocols. Hyperglycemia (~ 500 mg/dl db/db vs ~ 150 mg/dl db/m) was detected at 8 wks and persisted at similar levels at all subsequent time points. Albuminuria (~ 200 µg/24hr db/db vs. ~ 30 µg/24hr db/m) was first detected at 12 wks of age and persisted at all subsequent time points. Serum creatinine by HPLC showed decrease in db/db compared to controls at 20 wks. Conversely, calculated creatinine clearance was increased at 20 wks. 28-wk creatinine results have been highly variable so far and we are collecting additional data. Glomerular basement membrane measurements performed by Dr. Steffes and John Basgen at U. Minnesota demonstrated significant increase in GBM width starting at 8 weeks-of-age. Analysis of immersion-fixed and perfusion-fixed kidney sections demonstrated mesangial matrix expansion beginning at 12 wks. At 20 wks, severe matrix expansion and occasional glomerulosclerosis were detected. Severe glomerulosclerosis was found at 28 wks. Preliminary analysis found neither vascular lesions nor tubulointerstitial abnormalities. Podocyte apoptosis rates were significantly increased early in db/db compared to db/m at the onset of hyperglycemia at 8 wks, but returned to control group levels at later

ages. Podocyte counts per glomerular section were decreased at 8 wks, coincident with the onset of hyperglycemia and peak of podocyte apoptosis in db/db compared to db/m.

In summary, comprehensive, longitudinal phenotyping of db/db mice in C57BLKS background using AMDCC Nephropathy phenotyping standards revealed for the first time that

- Hyperglycemia is persistent in db/db on C57BLKS background
- Onset of hyperglycemia at 8 wks is associated with GBM thickening, podocyte apoptosis and depletion, and precedes significant albuminuria detectable at 12 wks.
- A trend to glomerular hyperfiltration is consistently detectable at 12 and 20 weeks.
- Tubulointerstitial lesions or tubular apoptosis are absent in db/db throughout the study period up to 28 wks.

We propose that course and manifestations of hyperglycemia induced glomerulopathy in db/db C57BLKS resemble those of diabetes-induced glomerular lesions in humans. However, we have not yet been able to document progressive decline in renal function and tubulointerstitial lesions in this model.

Creation of standards for molecular phenotype validation

To develop standardized molecular validation tools in mouse and human kidneys, as mandated in the RFA, we have developed a new method allowing rapid separation of glomerular and tubular preparations at high purity using Fe-beads and magnetic capture of glomeruli. RNA prepared from these samples is of excellent quality and sufficient quantity for Affymetrix 430PLUS GeneChip analysis. To establish characteristic molecular “fingerprints” of glomeruli in non-diabetic control db/m and diabetic db/db kidneys, we have conducted a genome-wide screen of gene expression using Affymetrix GeneChips in three animals per genotype and age group. Data analysis is in progress to identify stage-specific molecular fingerprints of diabetic glomerulopathy in mice that will be useful for molecular validation of new and advanced mouse models of diabetic nephropathy that are under development by the AMDCC, and for molecular validation of human diabetic nephropathy.

Creation of phenotyping standards for morphological model validation.

In addition, to compare directly the effect of perfusion fixation vs immersion fixation on glomerular histopatholoy we have subjected three mice in each group to perfusion fixation using the protocol developed by Peter Mundel from our group at AECOM. The protocol has been approved by AMDCC Nephropathy Subcommittee as standard AMDCC protocol for perfusion fixation. The protocol can be viewed on the AMDCC website https://www.amdcc.org/members/shared/showFile.asp?docTypeID=3&docID=24 and can be downloaded as pdf file. Perfusion-fixed kidney samples were also embedded in plastic for electron microscopy analysis.

C. Plans for the coming year

- comparison of glomerular histopathology and morphometry between immersion- and perfusion-fixed samples will be finalized by March 2004
- quantitative image analysis is ongoing to compare standard PAS staining with immunohistochemical analysis for collagens I and III to develop standards for quantitative analysis of mesangial and interstitial matrix by light microscopy – expected completion March 2004
- whole genome gene expression data will be used to develop glomerular molecular fingerprints for molecular phenotype validation of new mouse models and human disease – expected completion summer 2004.

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