Animal Models of Diabetic Complications Consortium
(U01 DK60995)

Annual Report
(2004)

"Mouse Models of Diabetic Nephropathy"

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Table of Contents

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

The overall goal is to develop a valid model of diabetic nephropathy in mouse that recapitulates functional, morphological and molecular features of the natural history of diabetic nephropathy in humans.

Our main strategy is to use genetic engineering in mouse strains to enhance manifestation of key features of diabetic nephropathy that are missing in currently available models when compared to human DNP. Broadly, we are focusing on known pathways that are thought to play major roles in DNP and on a new pathway that we discovered in studies supported in part by the AMDCC.

Engineered modifications in known pathways aim to:

- Enhance activity of transforming growth factor beta (TGFb) through targeted deletion of a natural endogenous inhibitor, the proteoglycan decorin.
- Enhance podocyte injury through targeted deletion of a podocyte survival gene, CD2-associated protein.
- Enhance glucose uptake and toxicity in endothelial cells and podocytes through transgenic expression of GLUT1 under control of endothelial cell (Tie1) and podocyte (NPHS2) specific promoters.

In addition, we were encouraged by EAC to enhance activity of a new putative pathway for tubulointerstitial progression of human DNP recently described by our group,

- namely CD36 dependent activation of p38 MAPK and apoptosis in proximal tubules. We recently discovered a novel functional requirement for the scavenger receptor CD36 to mediate apoptosis of proximal tubular epithelial cells induced by glycated albumin or fatty acids. Since CD36 expression was upregulated specifically in proximal tubules in human DNP, we propose that increased CD36 may initiate tubulointerstitial progression of DNP.

Our major accomplishments during 2004 are:

Models:

- Finalization of functional, morphological and molecular (microarray) phenotyping of natural history of diabetes induced lesions in db/db type II mice on permissive BLKS genetic background (completed) and in Ins2-Akita type I mice on C57BL/6J background (near completion).

- Completed functional and morphological phenotyping of cohorts of type I diabetes (low-dose STZ) induced renal lesions up to 48-56 weeks in mice carrying heterozygous or homozygous deletions of the TGF-b inhibitor decorin.

- Created twelve founder lines and begun characterization for transgenic expression of CD36 in proximal tubules under control of the gamma glutamyl transferase (gGT) promoter.

- Near completion of 20 week low-dose STZ model in flk-RAGE transgenic mice characterized by endothelial-specific overexpression of RAGE (mice provided as AMDCC collaboration by Dr. Y. Yamamoto, Kanazawa University, Japan; see "Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice", J Clin Invest 108:261, 2001).

Phenotyping cores:

- TJU (Sharma/Dunn). After taking the lead for AMDCC on development of improved and reliable HPLC assays for measurement of creatinine concentration in mouse plasma, we were functioning as a standardized HPLC creatinine core facility and have now processed over 500 samples for plasma creatinine measurements for Consortium members in 2004.

- MSSM (Bottinger/Susztak). We have led the way for the AMDCC to develop and validate robust methods for microarray molecular phenotyping of glomerular or tubular transcriptomes and generated glomerular gene expression profiles at different stages of diabetic glomerulopathy in type I (Akita) and type II (db/db) diabetic mice. These complete molecular portraits of DNP are a truly unique resource of AMDCC and are being deposited in the AMDCC web-accessible database to support model development and validation by Consortium members.

Publications

Dunn, S., Qi, Z., Bottinger, E.P., Breyer, M.D., **Sharma, K.** Utility of endogenous creatinine clearance as a measure of renal function in mice. ***Kidney International***, 65:1959-1967, 2004.

Susztak K*, Bottinger EP*, Novetsky A, Liang D, Zhu Y, Ciccone E, Wu D, McCue P, Sharma K. Molecular Profiling of Diabetic Mouse Kidneys Reveals Novel Genes Linked to Glomerular Disease. ***Diabetes*****,** 2004, 53(3):784-794.
*both authors contributed equally

Yuen, P., Dunn, S., Miyaji, T., Yasuda, H., **Sharma, K**., Star, R. A simplified method for HPLC determination of creatinine in mouse serum. ***American Journal of Physiology, Renal Fluid and Electrolyte,*** 286:F1116-F1119, 2004

Breyer, M., Bottinger, E.B., Brosiun, F., Coffman, T., Harris, R., Heilig, C., **Sharma, K.** Mouse models of diabetic nehropathy. ***Journal of the American Society of Nephrology*****,** Jan;16(1):27-45, 2005.

Susztak K, Ciccone E, McCue P, Sharma K, Böttinger EP. Multiple metabolic hits converge on scavenger receptor CD36 as novel mediator of tubular epithelial apoptosis in diabetic nephropathy. ***PLoS Med.*** 2005 Feb;2(2):152-161.

Our major experimental and phenotyping focus during 2005/2006 will be:

- Decorin deficient model (details see Project Report #2):
  - Complete studies of low-dose STZ induced nephropathy
  - Confirm and extend low-dose STZ results using DBA2J-Ins2-Akita model
  - Continue and extend phenotyping collaboration with other members of the AMDCC to evaluate retinopathy, neuropathy, uropathy and cardiovascular lesions.

- Tubular CD36 transgenic model (details see Project Report #4):
  - Complete characterization of founder lines and identify high tubular expressor lines
  - Generate high expressor gGTCD36 FVB/N and DBA/2J or BLKS intercrosses (N1, N2, etc.) to take advantage of dominant renal disease susceptibility conferred by DBA/2J and BLKS inbred alleles
  - Establish and phenotype cohort for gGTCD36 transgenic low-dose STZ type I model with emphasis on tubulointerstitial lesions and/or renal progression
  - Establish and phenotype cohort for gGTCD36 transgenic FVB/N-Lepr^{db} and C57BLKS/J-Lepr^{db} type II model with emphasis on tubulointerstitial lesions and/or renal progression
  - Provide CD36 transgenic model and establish collaborations with other Consortium members if gGTCD36 transgenesis mediates tubulointerstitial progression of DNP.

- Endothelial RAGE transgenic model (details see Project Report #7)
  - Complete renal phenotyping studies of low-dose STZ type I cohort
  - Establish and analyze cohort of flk-RAGE-Ins2^{Akita} type I transgenic model
  - If screening results are promising, consider to establish and analyze cohort of flk-RAGE-Lepr$^{db/db}$ type II transgenic model
  - Establish collaborations with Consortium members and cores for retinopathy, neuropathy, and cardiovascular phenotyping.

- CD2AP podocyte survival gene deficient model (details see Project Report #6)
  - Complete screening experiment of low-dose STZ type I in 129/SvJ-CD2AP+/- mice
  - Expand breeding for Ins2-Akita/CD2AP+/- intercrossing to establish cohort if STZ screening provides promising results

- Glomerular transcriptome database of stage-specific gene expression in type I (Akita) and type II (db/db) models
  - finalize data processing and analysis for differentially-expressed genes in glomeruli of db/db and Akita at onset of i) hyperglycemia and podocyte injury; ii)

albuminuria and mesangial activation; and iii) podocyte depletion and mesangial activation

- establish molecular validation database on AMDCC web database for access and use by AMDCC members (Step I) and later general public(Step II).

Major achievements have been:

Project 1: HPLC-based plasma creatinine assay core open and active

- Further validated the HPLC-based method for plasma creatinine measurement in mice for renal function assay
- Established a functional operational core for standardized HPLC creatinine measurements for models developed by all members of AMDCC. Over 500 such assays have been performed for AMDCC members outside our Sinai/TJU/Einstein group
- Published method and validation paper.

Project 2: Decorin deficient cohort in low-dose STZ develops advanced Glomerular lesions compared to wildtype control cohort

- Our initial results show increased albuminuria, increased plasma creatinine, and increased mesangial matrix accumulation in diabetic dcn -/- mice.
- Our data supports the view that an important function of decorin is to compensate for the stimulation of matrix accumulation, possibly by antagonizing TGF-b

Project 4: Identified CD36 as likely mediator of tubulointerstitial progression of human DNP and generated founder lines of gGTCD36 transgenic strains

- Identified striking species-specific upregulation of CD36 protein in proximal tubules in human DNP while CD36 is not expressed in proximal tubules in longterm diabetic mice (db/db)
- Demonstrated that CD36 mediates AGE and FFA induced tubular apoptosis in CD36-positive human DNP and tubular epithelial cells, but not in CD36-negative murine DNP and tubular epithelial cells
- Demonstrated that CD36 is essential and sufficient to mediate tubular apoptosis induced by AGE and/or FFA
- Published CD36 validation findings in paper entitled “Multiple metabolic hits converge on CD36 as novel mediator of tubular epithelial apoptosis in diabetic nephropathy” by Susztak et al., PLoS Med. 2005 Feb;2(2):152-161.
- Generated and expanded ten founder lines for gGTCD36 transgenic strains with the goal to overexpress CD36 in proximal tubular epithelium.

Project 5: Completed systematic, longitudinal phenotyping of existing models based on AMDCC protocols and reporting standards

- In both, db/db and in Akita mice podocyte injury peaks with onset of hyperglycemia and leads to significant loss of podocytes, validating podocyte depletion observed in human DNP

- We defined the time sequence of phenotype manifestations as follows: hyperglycemia onset > podocyte injury > albuminuria > glomerular hypertrophy/mesangial expansion/GBM thickening
- Stage-specific transcriptome maps have been generated to facilitate molecular model validation

2. Collaboration within your group:

In brief, we have established the following ongoing collaborations (details see individual project reports):

- TJU (Sharma/Dunn/McCue) performed HPLC plasma creatinine assays and glomerular morphometry for all 2004 models and experiments of MSSM (Bottinger/Susztak).
- MSSM (Bottinger) performs molecular profiling for TJU (Sharma/Dunn/McCue) experiments.

3. Collaboration with other AMDCC groups:

In brief, we have established collaborations with other AMDCC members and cores for the following experiments/models (details see Project Reports):

- Rockefeller/Columbia (Breslow/Goldberg/Dansky/Stoffel): MSSM (Bottinger) is performing nephropathy screening for models in progress by this cardiovascular group.
- TJU (Sharma) has sent eyeballs from Decorin deficient low-dose STZ cohort to retinopathy cores (Kern) and is arranging for on site neuropathy phenotyping (Feldmann).
- Plans for retinopathy, neuropathy, and uropathy phenotyping for Flk-RAGE transgenic low-dose STZ model for 2005.
- Sharing of gGT-CD36 tansgenic strain with other nephropathy groups and of Tie1-GLUT1 transgenic strain with other nephropathy/cardiovascular/neuropathy groups.

4. Pertinent non-AMDCC Collaboration:

On behalf of the AMDCC and with EAC recommendations we entered a collaboration with Dr. Y. Yamamoto, Kanazawa University, Japan, to analyze the flk-RAGE transgenic strain subjected to AMDCC standardized type I and type II models. We are completing a 20 week nephropathy analysis of flk-RAGE transgenic low-dose STZ type I model as part of this collaboration.

5. Address previous EAC comments:

This group wishes to thank the EAC for their continued valuable input to enhance our progress.

Response to General Comments

1. Analysis of existing models

The October 2004 EAC comments and recommendations state that "[the AMDCC] could be asked to evaluate the currently available models based on the data generated. This might help to stimulate ideas about continuing needs for animal models of diabetic complications, and future goals of the consortium. Questions to consider include: (1) are the existing models sufficient? (2) should existing models be further modified, if so, how and why? (3) Are new and/or additional models still needed?". Our groups has made a large, systematic effort to define precisely the evolution of functional, morphological and molecular phenotype parameters in C57BLKS/J db/db (type II) and C57BL/6J Ins2Akita mice based on strict application of AMDCC protocols (see Project #5). The experiments are completed (db/db) or near completion (Akita) and provide appropriate data and a foundation to address these general questions of the EAC.

2. Call for continued efforts to standardize protocols and develop assays

We appreciate that EAC recognizes the importance of "these workproducts [as] critical measures of success". Under the leadership of Dr. Sharma, our group continues to improve robustness of HPLC creatinine measurements (see Project Report #1). The HPLC creatinine core lab at TJU processed over 500 samples from other members of the consortium in 2004. Along these lines, we have developed a robust protocol for microarray analysis of minute tissues, such as glomeruli, and established data analysis protocols based on the Affymetrix platform under the leadership of Dr. Bottinger (see Project Report #5). We believe that this resource may be of tremendous value as phenotyping efforts in different complications proceed vigorously and we propose to establish a molecular phenotyping core (microarray and real-time PCR) for AMDCC at MSSM, led by Dr. Bottinger.

Response to site-specific comments

1. McIndoe – question concerning validation and standardization of microarray data?

We are working with McIndoe to standardize raw and processed microarray data formats for the AMDCC website/database. We are routinely validating interesting and potentially important array findings with high-throughput real-time PCR (ABI 7900HT, Taqman) and propose this should be standard procedure.

2. Bottinger – should measure BP in decorin -/- mice

We agree. Dr. Sharma's group is implementing infrastructure for tailcuff BP measurement on these mice at TJU.

3. Bottinger – cause of excess mortality of decorin -/- mice ? collaborate with Utah site...

We agree. The mice that died had higher plasma creatinines and albuminuria than the survivors. To investigate the cause of death we will induce diabetes in a new cohort of decorin deficient mice (by crossing with Akita mice) and harvest tissues at earlier time points. We will send cardiac and aortic tissue to the Utah and Rockefeller group for analysis to determine if

cardiovascular pathology is contributing to death. In addition, more extensive analysis of blood chemistries will be performed as well as full autopsies to determine the cause of premature death.

4. Bottinger – provide 24 hr collection and ACR data
We are now reporting both, 24 hr UAE and ACR data.

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Part B:

Update by Project Leaders

PROJECT 1

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

Project Title: **Validation of HPLC-based plasma creatinine assay and endogenous creatinine clearance to measure renal function**

Project Status: **- Ongoing -**

A. Rationale and relevance

We have previously reported that the use of the Jaffé alkaline picrate method grossly overestimates true plasma creatinine in mice leading to difficulties with reproducibility and accuracy. We have established the performance and feasibility of alternative HPLC-based methods for standard determination of plasma creatinine and creatinine clearance in mice. We have developed a simple HPLC method that provides a reliable, reproducible and sensitive assay for small volumes (< 25 microliters) of mouse plasma and is available as a "core" facility for AMDCC members. The prior Steering Committee members and EAC recommended that the use of this measurement be further validated in diabetic mice.

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.

This data is now published in Kidney International. Inclusion of the implication of this data has also been included in the Position paper published by the AMDCC nephropathy group in JASN.

We have recently found an excellent correlation with FITC-inulin clearance using the single shot method developed by Matt Breyer.

Functioning as a core facility we have now processed over 500 samples for plasma creatinine measurements for Consortium members.

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 will continue to operate this core to provide standardized HPLC creatinine measurements for all members of AMDCC. Per recommendation of the AMDCC SC and with approval of the Advisory Panel, Dr. Sharma, Steve Dunn and Matt Breyer's group will further validate HPLC-based plasma creatinines and creatinine clearance with single shot FITC inulin clearance in different models of diabetic mice.

D. Significant Achievement

- Further validated the HPLC-based method for plasma creatinine measurement in mice for renal function assay
- Established a functional operational core for standardized HPLC creatinine measurements for models developed by all members of AMDCC. Over 500 such assays have been performed for AMDCC members outside our Sinai/TJU/Einstein group
- Published method and validation paper.

Publications:

Dunn, S., Qi, Z., Bottinger, E.P., Breyer, M.D., **Sharma, K.** Utility of endogenous creatinine clearance as a measure of renal function in mice. ***Kidney International***, 65:1959-1967, 2004.

Yuen, P., Dunn, S., Miyaji, T., Yasuda, H., **Sharma, K**., Star, R. A simplified method for HPLC determination of creatinine in mouse serum. ***American Journal of Physiology, Renal Fluid and Electrolyte,*** 286:F1116-F1119, 2004

Breyer, M., Bottinger, E.B., Brosiun, F., Coffman, T., Harris, R., Heilig, C., **Sharma, K.** Mouse models of diabetic nehropathy. ***Journal of the American Society of Nephrology*****,** in press 2005.

PROJECT 2

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 (1) 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. 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. The degree of hyperglycemia and susce[ptibility to strep was similar in all three groups. We initially found less susceptibility to strep in dec -/- mice but this was not borne out when more mice were injected. 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 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). At months of diabetes, we began to see significantly differences in albuminuria and plasma creatinine values among the diabetic mice with the dcn -/- mice having the greatest degree of albuminuria and the highest plasma creatinines. This trend continued in mice with diabetes for 10-12 months of diabetes with dcn -/- mice having creatinine values of 0.286 mg/dl, almost twice of the non-diabetic mice. Interestingly we found that many mice in all the diabetic groups began to die spontaneously around this time frame. So far pathology scoring has been completed in 4-16 mice in 6 groups.

Our preliminary results demonstrate a marked increase in stage IV diffuse mesangial matrix expansion in the dcn -/- diabetic group, as compared to the other diabetic 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, 40, and 48-56 weeks. Results are presented in detail in Excel spreadsheet format in the document "Einstein report".

C. Plans for the coming year

The pathologic scoring will be completed in all mice. Qunatitive analysis of mesangial matrix accumulation will be performed by image analysis software. Immunohistochemistry will be performed on diabetic mice to verify accumulation of mesangial matrix constituents. EM will be performed on a limited sample to assess for GBM thickening.

The initial results with diabetic dcn -/- mice are extremely promising as they exhibit increased albuminuria, increased plasma creatinines, and increased stage IV mesangial matrix accumulation. However it requires 40-56 weeks of diabetes before significant changes are observed. In addition, many mice die during the development of diabetic kidney disease. Therefore we would like to determine if the disease process can be accelerated by introducing other abnormalities encountered in diabetes. Firstly, we will introduce LDL receptor deficiency in the dcn -/- mice to increase lipid abnormalities. Secondly, the mice will be made diabetic via crossing with Akita mice. This approach will obviate the need for strep and possibly accelerate the nephropathy phenotype.

D. Significant achievement and its importance

- Our initial results show increased albuminuria, increased plasma creatinine, and increased mesangial matrix accumulation in diabetic dcn -/- mice.
- Our data supports the view that an important function of decorin is to compensate for the stimulation of matrix accumulation, possibly by antagonizing TGF-b

Publications:

Qiu, G., Dunn, S., Iozzo, R., Bottinger, E.P., Williams, K., M.D., **Sharma, K.** Decorin deficiency and diabetic nehropathy. ***Journal of the American Society of Nephrology*****,** abstract 2004.

PROJECT 3

Responsible Investigator:	**Maureen Charron, PhD**
Project title:	**"Create and analyze Tie1-GLUT1 and NPHS2-GLUT1 transgenic strains for capillary endothelial and podocyte-specific GLUT1 overexpression, respectively"**
Project status:	**transgenic strain generation**

Generation of Podocin/GLUT1 (PG1) Transgenic Mice: The murine podocin promoter was used to drive expression of the rat GLUT1 cDNA and the SV40 Poly A sequences containing a spliced exon was used to stabilize the transgene. Following FVB pronuclear microinjections, 37 potential founder mice were screened for the presence of the PG1 transgene. Eleven mice were determined to be transgenic by both PCR and Southern blot analysis. Of the 11 potential founders, 8 have successfully mated and produced offspring. Three potential founders had fertility issues that are unresolved. One of the three expressed high levels of GLUT1 in kidney, produced only one transgenic pup and died. Five potential founders have been further evaluated. Fertility problems resulting in fewer transgenic offspring than expected, has been a lingering problem for the other 3 potential founders. This transgene is being mated at present onto the C57Bl6/J strain. It is unclear whether the persistent fertility problems are due to strain incompatibilities.

Semi-Quantitative (Poly-A/β-Actin) RT-PCR Analysis: All 5 lines screened thus far exhibit significant expression of the transgene in kidney. [(PG-17) highest, (PG-15) lowest, and (PG-13, -11 and -16) were exhibit intermediate levels.

GLUT1/β-actin Immunoblot Analysis: Immunoblot analysis using whole kidney extracts proved to be inconsistent. Due to unexpected low levels of GLUT1 over-expression in whole kidney preparations, glomerular and tubular enriched-preparations were made using 1st and 2nd generation offspring of the 5 potential founders. After several attempts at immunoblotting with pooled versus non-pooled preparations, it appears that several lines (PG-13 and PG-15) overexpress GLUT1 in glomeruli. Additional preparations are required to confirm the overexpression levels in these lines as well as the remaining others.

Generation of Tie-2/GLUT1 Transgenic Mice: Due to the disappointing outcome of the Tie-1/GLUT1 transgenic mouse study, a new endothelial cell overexpressing GLUT1 transgene is being prepared. For this we have obtained the Tie-2 promoter which will be used to drive expression of the rat GLUT1 cDNA with SV40 Poly A sequences (splicing and Poly A sites included) as successfully used by us and others to target GLUT1 overexpression. The transgene will be excised from the plasmid and microinjected into FVB pronuclei at the Albert Einstein College of Medicine Transgenic facility. Caution will be used in breeding the transgene as we have encountered unexplainable fertility and transgene transmission/inactivation problems upon mating FVB founders with C57Bl6/J mice.

Publications related to project:

Schiffer, M., K. Susztak, M. Ranalletta, E.P. Bottinger, and **M.J. Charron**. Localization of the GLUT8 glucose transporter in the kidney and regulation *in vivo* in non-diabetic and diabetic conditions. (*Am. J. Physiol.,* in press).

PROJECT 4

Responsible Investigator: Katalin Susztak, MD, PhD

Project title: "Create gGT-Cd36 transgenic strains for tubular epithelial expression of Cd36"

Project Status: Experimental Model Derivation

A. Rationale and Relevance

We found that as opposed to human diabetic nephropathy the currently examined murine diabetic animal models (db/db and STZ diabetes in C57B6J and 129SvJ) do not develop significant tubulointerstitial fibrosis. We found that expression of scavenger receptor CD36 coincided with proximal tubular epithelial cell apoptosis and tubulointerstitial fibrosis in human DNP. CD36 expression was necessary and sufficient to mediate proximal tubular apoptosis induced by glycated albumins and free fatty acid palmitate through sequential activation of src kinase, and proapoptotic p38 MAPK and caspase3. In contrast, paucity of expression of Cd36 in PTEC in diabetic mice with diabetic glomerulopathy was associated with absence of tubular apoptosis and normal tubular epithelium. Mouse PTEC lacked Cd36 and were resistant to glycated albumin induced apoptosis. Recombinant expression of CD36 in mouse proximal tubular epithelial cells conferred susceptibility to glycated albumin induced apoptosis. Our findings suggest a that CD36 is as essential mediator of proximal tubular apoptosis in human DNP.

B. Summary of accomplishments

We did not observe proximal tubular expression of CD36 in murine diabetic models (BLK db/db and STZ treated C57BL/6J and 129SvJ) in addition CD36 was downregulated in these diabetic animal models compared to controls. Therefore we have generated mice with proximal tubular tubular overexpression of CD36 under the GGT promoter. The targeting construct has been injected into C57B6/CH3 oocytes. Currently we have identied 12 founder lines by tail PCR.

C. Plans for the coming year

We will complete the characterization of the founder lines and the highest proximal tubular overexpressing line will be selected by Western blotting and immunohistochemistry. This line will be back-crossed into C57BL/6J background and to BLKS background. Tubulointerstitial fibrosis and renal disease progression will be characterized in GGTCD36/ Ins2Akita and GGTCD36 db/db mice and will be compared to appropriate controls. In addition the renal phenotype (following the AMDCC Nephropathy group standards) will be characterized in STZ induced GGT/CD36 mice. If we fail to observe significant proximal tubular CD36 expression in the GGT/CD36 transgenic mice

we will use the sglt2 (J Am Soc Nephrol. 2004 Aug;15(8):2050-6) promoter for proximal tubular CD36 overexpression.

D. Significant achievement and its importance

Tubulointerstitial fibrosis is an important feature of human DNP and the degree of tubulointerstitial fibrosis correlates well with renal disease progression in human DNP. We hope to see similar degree of tubulointerstitial fibrosis and progressive diabetic nephropathy in the murine diabetic models as seen in human DNP.

Publications:

1. Susztak K*, Bottinger EP*, Novetsky A, Liang D, Zhu Y, Ciccone E, Wu D, McCue P, Sharma K. Molecular Profiling of Diabetic Mouse Kidneys Reveals Novel Genes Linked to Glomerular Disease. ***Diabetes,*** 2004, 53(3):784-794.
*both authors contributed equally

2. Susztak K, Ciccone E, McCue P, Sharma K, Böttinger EP. Multiple metabolic hits converge on scavenger receptor CD36 as novel mediator of tubular epithelial apoptosis in diabetic nephropathy.***PLoS Medicine 2005,***

PROJECT 5

Responsible Investigator: Bottinger, Erwin X

Project title: "Natural history of functional, morphological and molecular phenotypes of diabetes induced renal lesions in AMDCC standard murine type I and type II diabetes "

Project status: phenotyping completed

A. Rationale and Relevance:

The existing data on evolution of manifestations of diabetes induced renal lesions in db/db mice is incomplete and conflicting, in part because different background strains modify hyperglycemic and renal tissue responses (C57BL/6J, resistant vs. C57BLKS/J permissive). Furthermore, Dr. Coffman's and Dr. Breyer's groups evaluated the recently described insulin-2 *Akita* ($Ins2^{Akita}$) mouse mutant model of type 1 diabetes for diabetic nephropathy and found that Akita mice are an attractive alternative to STZ models for type I diabetes and renal lesions.

We analyzed the phenotype of db/db mutants in C57BLKS background in a longitudinal analysis applying strictly AMDCC Nephropathy standards (see AMDCC Nephropathy Report).

B. Summary of Accomplishments

Db/db

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. 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, indicating hyperfiltration. Glomerular basement membrane measurements performed by Dr. Steffes and John Basgen at U. Minnesota demonstrated a trend to increase in GBM width starting at 8 weeks-of-age and statistically significant increase of GBM thickness at 20 wks. Analysis of immersion-fixed and perfusion-fixed kidney sections demonstrated statistically significant mesangial matrix expansion by 12 wks. At 20 wks, severe matrix expansion and occasional glomerulosclerosis were detected. Severe glomerulosclerosis was found at 28 wks. Vascular lesions or tubulointerstitial abnormalities were not detected. Podocyte counts per glomerular section tended to decrease at 8 wks, coincident with the onset of hyperglycemia, and were statiscally significantly reduced at 12 wks and 20 wks. Interestingly, the finding of early podocyte injury and loss is entirely consistent with reports from human DNP in Pima Indian populations (2), validating a manifestation of human DNP in type II DM in db/db mouse kidneys. In summary, we propose that course and manifestations of hyperglycemia induced glomerulopathy in db/db C57BLKS resemble those of diabetes-induced glomerular lesions in humans with the exception of KW lesions which have not

been detected in db/db mice. In addition, However, we have not yet been able to document progressive decline in renal function, and vascular or tubulointerstitial lesions in this model.

Akita

Male C57BL/6J Ins2-Akita develop hyperglycemia already at 2 wk-of-age, but albumin excretion is not different from C57BL/6J controls. Also, female Akita are normoglycemic at 2 wks. At all ages examined (4, 10, 20, 27 wks), male Akita had more significantly more severe hyperglycemia compared with females. UAE (alb/crea ratio by 24 hr collection) tended to increase at 4 wk in male Akita and was significantly increased (~ 5-fold) at 27 wk. Serum creatinine levels by HPLC were comparable between male Akita and non-diabetic C57BL/6J. Morphological analysis is in progress. Quantitation of podocyte injury and numbers per glomerular section is in progress. Glomeruli have been isolated starting at 4 wks for transcriptome analysis which is also pending. In summary, male Akita have more severe hyperglycemia compared with female (confirming previous observations) and develop hyperglycemia as early as 2 wks of age and increased albumin excretion as early as 4 wks of age. Fasting blood glucose levels were comparable to db/db mice.

Creation of standards for molecular phenotype validation

We have established 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. We have conducted a genome-wide screen of gene expression using Affymetrix GeneChips in glomeruli from five animals per genotype and age group. Data analysis is completed for db/db model where we identified (and validated) differentially-expressed genes in glomeruli characteristic of i) hyperglycemia and podocyte injury; ii) albuminuria and mesangial activation; and iii) podocyte depletion and mesangial expansion. Data is MIAME-compliant and stored in web-accessible GeneTraffic database. Microarray analysis of glomerular samples from Akita and control mice is pending.

C. Plans for the coming year

- Complete functional and morphological phenotyping for Akita model
- If morphological phenotyping demonstrates glomerular lesions consistent with human DNP we will proceed with microarray analysis
- establish molecular validation database on AMDCC web database for access and use by AMDCC members (Step I) and later general public(Step II).

PROJECT 6

Project title: "Effect of deficiency of CD2-associated protein (Cd2ap), a podocyte survival gene, in type I diabetes induced by low-dose STZ"

Responsible investigator: Bottinger, Erwin

Project status: screening experiment ongoing

A. Rationale and Relevance:

Our results from Project 5 demonstrate that podocyte injury is the earliest detectable glomerular lesion coincident with onset of hyperglycemia and leads to subsequent podocyte depletion. Thus, we have validated the podocyte defect observed in human DNP in type I and type II diabetics (2;3). However, while podocyte depletion is progressive in our non-diabetic murine models of progressive glomerulosclerosis leading to more than 50% reduction of podocyte numbers, podocyte depletion is not progressive in db/db (and Akita) mice, leading to ~ 25% reduction of podocyte numbers. Results from studies conducted in the Bottinger lab independent of AMDCC have recently demonstrated that CD2-associated protein CD2AP functions as survival gene in podocytes in vitro and in vivo (4). Preliminary studies using non-diabetic models of progressive glomerulosclerosis indicate that haploinsufficiency of CD2AP causes significantly increased podocyte injury and apoptosis in vivo. We propose the hypothesis that CD2AP deficiency will enhance podocyte injury induced in murine diabetes and cause more severe, progressive podocyte depletion and increased diabetic glomerulopathy.

B. Summary of Accomplishments

We have initiated and partially completed a screening experiment using 129/SvJ control and 129/SvJ CD2AP+/- littermate mice for low-dose STZ diabetes induction. Type I diabetes was induced in at least 10 male mice in each group at 8 wks. FBS are > 400 mg/dl at wk 12 in all animals.

C. Plans for the coming year

- UAE will be monitored. Animals will be followed to age 20 wks when we will collect blood, urine and kidney for functional and morphological measurements following AMDCC protocols and standards. Podocyte injury and number per glomerular section will be determined.
- Data from control and CD2AP+/- animals will be compared to determine whether CD2AP-deficiency results in advanced lesions of diabetic glomerulopathy.

PROJECT 7

Responsible investigator: Bottinger, Erwin

Project title: "Effect of endothelial RAGE overexpression in type I diabetes induced by low-dose STZ"

Project status: phenotyping ongoing

A. Rationale and Relevance

Mice tansgenic for endothelial-specific expression of the receptor for advanced glycation endproducts (RAGE) under control of the flk receptor promoter were reported to develop glomerular lesions of human DNP and renal failure when subjected to experimental diabetes induced transgenically by islet cell expression of eNOS (5). Dr. Yamamoto presented these data at an AMDCC workshop in Bethesda in 2003. The SC and EAC recommended that the flk-RAGE mice should be evaluated for the AMDCC. We obtained flk-RAGE mice from Dr. Yamamoto as collaborative effort and agreed to phenotype diabetes-induced renal lesions in this strain.

B. Summary of Accomplishments

We have recently completed a screening experiment where flk-RAGE transgenic and non-transgenic control littermates were subjected to type I diabetes by low-dose STZ model starting at 8 wks. Induction of hyperglycemia and UAE were monitored by standard AMDCC assays. Animals were sacrificed at 20 wks of age. There was no significant difference in Ualb/crea ratio (approx. 10x increased compared to non-diabetic controls) in 24 hr collection and serum creatinine between non-transgenic controls and flk-RAGE mice after STZ diabetes. Morphological phenotyping including mesangial matrix expansion and histopathology is in progress.

C. Plans for the coming year

- Complete renal phenotyping studies of low-dose STZ type I cohort
- Establish and analyze cohort of flk-RAGE-$Ins2^{Akita}$ type I transgenic model
- If screening results are promising, consider to establish and analyze cohort of flk-RAGE-$Lepr^{db/db}$ type II transgenic model
- Establish collaborations with Consortium members and cores for retinopathy, neuropathy, and cardiovascular phenotyping.

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