Animal Models of Diabetic Complications Consortium
(U01 DK60995)

Annual Report
(2005)

"Mouse Models of Diabetic Nephropathy"

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(Bottinger)

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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 RAGE pathways in endothelial cells, and a new putative pathway for tubulointerstitial progression of human DNP recently described by our group,

- RAGE transgenic mice with overexpression of RAGE under control of a flk1 promoter specifically in endothelial cells were imported from Japan, kindly provided by Prof. Yamamoto.
- amely 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 2005 are:

Models:

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

- 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.

- Completed analysis of transgene expression in twelve founder lines for transgenic expression of CD36 in proximal tubules under control of the gamma glutamyl transferase (gGT) promoter.

- Generated iL1-sglt2-CD36 transgene construct and completed oocyte injections to use alternate promoter for tubular expression of CD36

- Completed 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).

- Completed 20 week low-dose STZ model in Cd2ap+/- mice

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 1000 samples for plasma creatinine measurements for Consortium members in 2005.

- 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 have been deposited in the AMDCC web-accessible database to support model development and validation by Consortium members.

Publications

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*. 2(2):152-161, 2005.

Susztak K, Raff AC, Schiffer M, and Böttinger EP. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes.* 55(1):225-33, 2006.

Our major experimental and phenotyping focus during the remainder of the funding period until September of 2006 will be:

- Decorin deficient model (details see Project Report #2):
  - Publication of completed studies of low-dose STZ induced nephropathy
  - 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):
  - Establish new founder lines with sglt2-CD36 transgene expression in proximal tubules, as twelve gGT-CD36 lines failed to show expression in kidney.
  - Establish and phenotype cohort for gGTCD36 transgenic FVB/N-Lepr^{db} (available at Albert Einstein, Katalin Susztak) type II model with emphasis on tubulointerstitial lesions and/or renal progression

- Endothelial RAGE transgenic model (details see Project Report #7)
  - Complete renal phenotyping studies of low-dose STZ type I cohort
  - Complete renal and metabolic phenotyping of cohort of flk-RAGE-Ins2^{Akita} type I transgenic model
  - Provide mice and/or tissue from flk1-RAGE-Ins2^{Akita} cohort to 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 (Project #5)
  - Test the functionality and reliability of data analysis after deposition in the microarray database on AMDCC website.
  - Publish glomerular transcriptome data and deposit in public databases for public access

2. Collaboration within our group:

- Completed Project #2 for joint publication of manuscript between TJU (Sharma/Dunn/Williams/McCue) and MSSM (Bottinger).
- Complete microarray studies and prepare for publication between AECOM (Susztak) and MSSM (Bottinger)
- Continue use of HPLC creatinine core services between TJU (Dunn) and MSSM (Bottinger)

3. Collaboration with other AMDCC groups:

- Rockefeller/Columbia (Breslow/Goldberg/Dansky/Stoffel): MSSM (Bottinger) is performing nephropathy screening for models in progress by this cardiovascular group.
- MSSM (Bottinger) has sent flk1-RAGE mice to UMich (Feldmann) for neuropathy phenotyping.
- MSSM (Bottinger) and Bioinformatics Group (McIndoe) are validating and testing AMDCC Microarray Web database and analysis tools using MSSM data (Bottinger) (see project 5)
- MSSM (Bottinger) will provide Uropathy Core (Daneshgari) with bladders and Retinopathy Core (Kern) with eyeballs and Neuropathy Core (Feldmann) with peripheral tissues from ongoing cohort study of flk1-RAGE-Ins2^{Akita} at 24 wks, 36 wks, 48 wks of diabetes.

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 have completed a 20 week nephropathy analysis of flk-RAGE transgenic low-dose STZ type I model as part of this collaboration. Cohort phenotype analysis of CD1-flk1-RAGE / C57BL6-Ins2^{Akita} is in progress.

5. Address previous EAC comments:

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

Response

1. flk1-RAGE-Ins2^{Akita}: retinopathy, uropathy, and neuropathy phenotyping arrangements have been/will be made to phenotype all organs in this model.

2. Male / female mice: we are now only including male mice in our experiments.

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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: **- completed -**

- HPLC-based method for plasma creatinine measurement in mice for renal function assay has been established and is available to all investigators as core service
- Published method and validation paper in 2004.

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: **- completed -**

- 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:

Kevin Jon Williams[1], Gang Qiu[2], Stephen R. Dunn[2], Peter McCue[3], Erwin Bottinger[4], Renato Iozzo[3], Kumar Sharma[2‡]. **Deficiency of decorin produces an advanced and lethal nephropathy in diabetic mice (manuscript in preparation)**

To determine the role of Dcn in the progression of diabetic kidney disease, we used low-dose streptozotocin to induce type 1 diabetes in Dcn-deficient (-/- and +/-) and wild-type (+/+) C57BL/6J mice. Decorin gene dose had no effect on severity of diabetes, but the *Dcn*-/- diabetic mice were the only diabetic group to exhibit a significantly accelerated mortality – nearly 60% dead by 12 months – over wild-type non-diabetic controls. At the 10-month timepoint, wild-type diabetic mice failed to develop significant increases in albuminuria, renal failure, or mesangial matrix expansion, whereas *Dcn* -/- diabetic mice developed a significant 220% increase in albuminuria, a nearly 50% increase in plasma creatinine levels (by HPLC), and significantly

advanced glomerular pathology (diffuse mesangial matrix expansion, glomerular type I collagen accumulation, fibrin caps). Dcn-deficient (+/- & -/-) diabetic mice exhibited low plasma adiponectin levels at 6 and 8 months of diabetes, and all Dcn-deficient mice showed increased renal NADPH oxidase (*Nox4*) gene expression. Mortality in diabetic mice was preceded by increased plasma creatinine and decreased plasma adiponectin levels. We conclude that decorin is a significant endogenous protective factor against diabetic nephropathy, the major cause of ESRD in the developed world. Decorin-deficient diabetic mice will be a useful model to study advanced diabetic kidney disease and accelerated mortality.

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: **- line generation ongoing -**

PROJECT 4

Responsible Investigator: Katalin Susztak, MD, PhD

Project title: **Cd36 transgenic strains for tubular epithelial expression of Cd36"**

Project Status: **- alternate transgenic approach ongoing -**

- Transgenic lines were established from twelve transgenic founders
- Expensive RNA and protein analyses suggested that the transgene was not expressed in kidney of any of the twelve lines, indicating that the gGT promoter is not sufficient to drive expression.
- As an alternate approach, Dr. Susztak has obtained a strong transgene promoter from the sglt2 gene which has been proven to direct strong transgene expression in proximal tubular epithelia in mice. Transgene vector has been constructed and prepared for oocyte injections.

PROJECT 5

Responsible Investigator: Bottinger, Erwin

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: - completed -

- Phenotyping manuscript has been published (see below)
- Age-matched microarray analysis of glomerular RNA from db/db and Akita mice has been completed
- Microarray data has been uploaded to AMDCC Microarray Database. AMDCC Webportal search and analysis tools for microarray data mining are being tested and validated prior to release of dataset on the production server (collaboration with McIndoe (AMDCC Website).

Publications

Susztak K, Raff AC, Schiffer M, and Böttinger EP. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes.* 55(1):225-33, 2006.

Diabetic Nephropathy is the most common cause of end stage renal disease in the US. Recent studies demonstrate that loss of podocytes is an early feature of DNP that predicts its progressive course. Cause and consequences of podocyte loss during early DNP remain poorly understood. Here we demonstrate that podocyte apoptosis increased sharply with onset of hyperglycemia in $Ins2^{Akita}$ (Akita) mice with T1DM and $Lepr^{db/db}$ (db/db) mice with obesity and T2DM. Podocyte apoptosis coincided with the onset of urinary albumin excretion (UAE) and preceded significant losses of podocytes in Akita (37% reduction) and db/db (27% reduction) mice. Increased extracellular glucose (30 mM) rapidly stimulated generation of intracellular reactive oxygen species (ROS) through NADPH oxidase and mitochondrial pathways, lead to activation of proapoptotic p38 mitogen-activated protein kinase (MAPK), caspase 3 and apoptosis of conditionally-immortalized podocytes in vitro. Chronic inhibition of NADPH oxidase prevented podocyte apoptosis and ameliorated podocyte depletion, UAE, and mesangial matrix expansion in db/db mice. In conclusion, our results demonstrate for the first time that glucose-induced ROS production initiates podocyte apoptosis and podocyte depletion in vitro and in vivo and suggest that podocyte apoptosis/depletion represent novel early pathomechanism(s) leading to DNP in murine T1DM and T2DM models.

Susztak K, Khitrov G, Zhang W, and Bottinger EP. Genome-wide glomerular gene expression patterns during evolution of diabetes-induced glomerulopathy in type 1 and type 2 diabetic mice. (Manuscript in preparation).

PROJECT 6

Responsible investigator: Bottinger, Erwin

Project title: "Effect of deficiency of CD2-associated protein (Cd2ap), a podocyte survival gene, in type I diabetes in Ins2^{Akita} mice"

Project status: - phenotyping cohorts established -

- Phenotyping screen for nephropathy in 20 wk old Cd2ap+/- made diabetic with low-dose STZ protocol did not reveal evidence for effect of Cd2ap heterozygosity on UAE, mesangial expansion, and diabetes.
- Cohorts of 129-Cd2ap+/- / C57BL6-Ins2^{Akita} mice were established for phenotype analysis at 24, 36, 48 wks and survival study. Preliminary results at 24 wk of age indicated increased mesangial expansion and glomerular injury.

PROJECT 7

Responsible investigator: Bottinger, Erwin

Project title: "Effect of endothelial flk1-RAGE overexpression in type I diabetes induced Ins2^{Aktia}"

Project status: - phenotyping cohorts established –

- Nephropathy screening study with flk1-RAGE and low-dose STZ model did not reveal an effect of endothelial RAGE transgene on STZ-induced UAE and mesangial expansion.
- We have established cohorts of CD1-flk-RAGE / C57BL6-Ins2^{Akita} mice to analyze nephropathy (Bottinger), uropathy (Daneshgari), neuropathy (Feldmann), cardiovascular (Breslow), retinopathy (Kern), and cardiomyopathy (Abel) at 24, 36, 48 wk and survival outcomes.