Animal Models of Diabetic Complications Consortium
(U01 DK060905-08)

Annual Report
(2007)

"Role and Mechanisms of Epithelial Injury in Diabetic Nephropathy"

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Hypotheses

Observations from our laboratories provide a compelling rationale to focus our research program on investigating the emerging role and mechanisms of epithelial cell injury in diabetic nephropathy (DN). Specifically, **we will test two novel hypotheses:** 1. The peroxisomal membrane proteins Mpv17l and/or Mpv17 are essential regulators of antioxidant defenses in glomerular podocytes and protect against diabetes-induced podocyte apoptosis and podocyte depletion (Bottinger, Mount Sinai); and 2. CD36 scavenger receptor for AGE and/or FFA is an essential mediator of AGE and/or FFA-induced tubular epithelial injury/apoptosis and tubulointerstitial progression of DN (Susztak, AECOM).

Recent Progress and Major Accomplishments

A. Studies of Mpv17-family proteins in DN (Aims 1 and 2; PI: Bottinger)

Aim 1: whether peroxisomal membrane proteins Mpv17 and Mpv17l regulate antioxidant defense mechanisms in podocytes and protect against ROS-mediated podocyte injury/apoptosis induced by diabetes

We found that Mpv17l is a mitochondrial protein and its expression is regulated by ROS. In the kidney, Mpv17l is expressed in proximal tubular cells and is downregulated by various ROS-inducing stimuli. Overexpression and silencing experiments reveal that Mpv17l protects against ROS induced mitochondrial membrane depolarization ($\Delta\Psi_m$) and apoptosis by lowering ROS levels. Furthermore, we show that the PDZ binding motif of Mpv17l interacts with the mitochondrial serine protease Omi/HtrA2 , which regulates Omi/HtrA2 protease function that is essential for its protective role against mitochondrial oxidative stress and apoptosis. The interaction of Mpv17l and Omi/HtrA2 in mitochondria provides a molecular mechanism for a mitochondrial oxidative stress sensor and effector complex thereby protecting cells from ROS-induced mitochondrial dysfunction.

Aim 2: whether Mpv17-deficiency accelerates and increases podocyte apoptosis and depletion leading to progressive glomerulosclerosis and/or nodules in diabetic mouse models

Mpv17-/- mice imported from Jackson Laboratory are completely normal in term of growth, body weight, blood glucose, proteinuria, and reproduction, lifespan. The kidney phenotype has essentially disappeared in the stocks of multiple laboratories (Spinazolla A, 2006, Nat. Genet., 38: 570-575). This seems to be the case in our Mpv17-/- mice that were imported from Jackson Laboratory, only two male mice (out of 10 examined) were found to have developed proteinuria after one year old, indicating lack of Mpv17 does not cause overt physiological problems, especially in young age.

When Mpv17-/- mice were made diabetic, we observed the surprising and novel result that Mpv17 deficiency protects mice against type 1 diabetes. Significantly reduced hyperglycemia

and proteinuria was observed in two models of type 1 diabetes, multiple low dose streptozotocin (MLDSTZ), and Akita Ins2. Further studies will thus provide opportunity to reveal novel mechanisms or pathways in Type 1 diabetogenesis.

B. Studies of role of Cd36 scavenger receptor expression in tubular epithelia in DN (Aims 3 and 4; Co-PI: Susztak)

Aim 4: whether proximal tubular overexpression of AGE binding protein CD36 leads to increased tubular epithelial injury/apoptosis and tubulointerstitial progression of DN in mice.
We have successfully created transgenic animals with diabetes and/or glucose/sodium inducible CD36 overexpression in the renal tubules under the control of the sgtl2 sodium/glucose cotransporter promoter elements. When exposed ot diabetes, albuminuria was highly increased in CD36 transgenic diabetic animals compared to wild type (FvB) diabetic animals (1720 μg/mg creat vs. 60 μg/mg creat, respectively). Histological analysis (PAS staining) showed dilated tubules with tubular epithelial degeneration and increased interstitial infiltration. Tubular apoptosis rate (quantified by TUNEL stain) in the kidney cortex was also significantly increased (0.373% vs. 1.5%) in transgenic diabetic animals compared to wild type diabetic animals. Oil Red-O staining confirmed the enhanced tubular lipid accumulation in transgenic diabetic animals. Thus, we conclude that increased proximal tubular CD36 expression plays an important role in diabetic tubular degeneration and renal disease progression, supporting our hypothesis

Plans for the Upcoming Year

- We will generate and characterize a conditional (floxed) Mpv17l allele in mice to enable deletion of Mpv17l using LoxP/Cre-recombinase technology in mice. For example, Mpv17l will be deleted in tubular epithelia of mouse kidney using intercrossing of Ksp1-Cre (proximal and distal tubular promoter) and/or Pepck-Cre transgenic (proximal tubular promoter) and Mpv17l-flox/flox mice. We anticipate that loss of Mpv17l mitochondrial proteins will sensitize to mitochondrial ROS stress in tubular epithelia at baseline, and when mice are challenged with experimental diabetes.

- We plan to explore whether the function of Mpv17l orthologs can be analyzed in experimental diabetes in genetically modified strains of Drosophila melanogaster (fruitfly).

- We will complete phenotype characterization of diabetic sgtl2-Cd36 transgenic mouse models. We anticipate to demonstrate that Cd36 is a critical mediator of tubulo-interstitial progression of diabetic nephropathy in mice and humans. Upon completion of initial phenotype analysis, these mice will be transferred to JAX laboratory

- We will continue to examine why Mpv17-/- mice are protected against STZ and Akita Ins2 mutation induced type 1 hyperglycemia. Based on our initial islet

phenotyping results, we will consider opportunities for phenotyping collaborations with appropriate MMPC partners.

Preliminary Milestones for 2009 and Beyond

- Complete analysis of conditional Mpv17l deletion in tubular epithelia and potential role of Mpv17l in DN (2009/2010). Mpv17l-flox/flox mice may then be deposited with JAX core laboratory (2009/2010).
- Detailed, molecular and physiologic phenotyping of sgtl2-Cd36 transgenic mice and distribution of the model to AMDCC/MMPC collaborating partners (2009)
- Completion of studies to identify mechanisms by which Mpv17-/- mice are protected against type 1 diabetes (2009).

2. Collaboration:

With other AMDCC PIs

Kumar Sharma, UCSD, is analyzing serum creatinine levels in our various mouse models

Jim Kern is analyzing eyeballs from Akita/Cd2ap+/- lesions of diabetic retinopathy

With Jax

We will provide sgtl2-Cd36 transgenic lines to JAX.

With the MMPCs

None

With other non-AMDCC PIs

None

3. Address previous EAC comments:

NOT APPLICABLE THIS YEAR

4. Publications:

NONE THIS YEAR

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Part B:

Update by Individual Project Leaders

Project 1: "Role Mpv17-family members in DN"

Responsible Investigator: Bottinger, Erwin

1. Detailed Project Accomplishments:

Aim 1. Mpv17l in kidney (PI: Bottinger, Mount Sinai School of Medicine)

Mpv17l is a new and poorly characterized member of the Mpv17/PMP22 protein family which shares high sequence homology with the kidney disease gene Mpv17 (Iida et al., 2001). Deletion of Mpv17 in mice caused glomerulosclerosis and nephrotic syndrome (Weiher et al., 1990). Previous reports indicate that two alternate splice variants, Mpv17l long (l-Mpv17l) and Mpv17l short (s-Mpv17l) may be localized to peroxisomes and cytosol, respectively, where both may be involved in reactive oxygen species (ROS) metabolism.

Microarray expression studies in mouse models of kidney disease, performed in our laboratory, demonstrated that Mpv17l mRNA was reduced dramatically from the onset of renal manifestations. In normal kidneys of control mice, Mpv17l mRNA and protein were abundant proximal tubules, but were dramatically reduced in experimental models. Treatment of proximal tubular epithelial cell lines (PTEC) with ROS-generating, injurious stimuli, including high glucose, TGF β1, and CoCl2 (hypoxia mimetic), consistently down-regulated Mpv17l mRNA and protein. Mpv17l downregulation was attenuated by ROS scavengers, indicating that Mpv17l is a common target of diverse ROS-generating extracellular signals. In contrast with previously reported peroxisomal localization, our immunofluorescence and immunogold electron microscopy analyses demonstrated that both Mpv17l isoforms were localized in the inner mitochondrial membrane.

Silencing of Mpv17l expression by RNA interference caused increased mitochondrial ROS generation, depolarization of the inner mitochondrial membrane potential (MMP), and increased apoptosis at baseline in PTEC. Reduction of MMP and apoptosis induced by high glucose, TGF-b, or CoCl2 were significantly attenuated in PTEC with stable overexpression of either Mpv17l splice variant, compared with controls. These protective effects of Mpv17l proteins were associated with retention of mitochondrial serine protease Omi/HtrA2, Smac/DIABLO, and cytochrome c in the mitochondrial intermembrane space. Co-immunoprecipitation studies demonstrated that Mpv17l interacts directly with the PDZ-domain of Omi/HtrA2 through a C-terminal PDZ binding motif. This interaction was required for the antiapoptotic function of Mpv17l.

In conclusion, we demonstrate for the first time that Mpv17l is a mitochondrial membrane protein that interacts with Omi/HtrA2, thereby protecting against ROS-induced MMP, release of Omi/HtrA2, Smac/DIABLO and cytochrome c in the cytosol leading to apoptosis in proximal tubular cells. Therefore, Mpv17l could represent a sensitive marker and protector of tubular injury which is downregulated early in disease thereby leaving the PTEC unprotected against ROS-induced damage.

Based on these novel in vitro observations, we have constructed a targeting vector for generating a conditional Mpv17l allele with loxP-flanked exon3. Using this vector, we generated successfully three embryonic stem cell lines carrying targeted alleles after homologous recombination. Two ES clones were injected in blastocysts to generate chimeric founder mice. We obtained 6 founders with 35 to 95% agouti coat color percentage. These founder are currently in matings to generate F1 offspring for verification of germline transmission of the targeted Mpv17l allele. We have detected germline transmission in one founder line so far.

Aim 2. Type 1 diabetes models in Mpv17-/- mice (PI: Bottinger, Mount Sinai School of Medicine)

Mpv17 is one of the four members of Mpv17/PMP22 gene family (the other three are Mpv17-like, FKSG24, and PxmP2). Inactivation of this gene resulted in glomerulosclerosis in mice. In addition, Mpv17 and Mpv17-like have been shown to be involved in ROS metabolism (Zwacka R, 1994, EMBO J., 13:5129-34; Binder C, Am J Path. 154:1067-75; Wagner G, ,2001, Biol Chem, 382:1019-25). In addition, lack of Mpv17 results in mitochondrial DNA loss (Trott A, et al, 2004, Eukaryot Cell, 3:620-632; Spinazzola A, et al, 2006, Nature Genetics, 38:570-575.). Mpv17/family is ancient in evolution, and its ortholog in yeast, Sym1, is involved in cellular response to stress (Trott A, et al, 2004, Eukaryot Cell, 3:620-632.).

In an effort to elucidate the role of ROS in diabetic nephropathy, we performed multiple low-dose streptozotocin treatment (MLDS) on Mpv17-/- mice to induce diabetes in order to test if elevated ROS level in kidney due to Mpv17 deficiency (Wagner G, ,2001, Biol Chem, 382:1019-25) would accelerates renal especially glomerular damage by hyperglycemia (diabetes). We also bred Mpv17-/- to Akita mice, a type 1 diabetes mouse model carrying C96Y point mutation on insulin 2 that leads to misfolding of the protein, beta-cell destruction and diabetes. Unexpectedly, both Mpv17-/-:MLDS and Mpv17-/-:Akita mice exhibited significant resistance to diabetes. M

In an effort to explore the role for reactive oxygen species (ROS) in the development of diabetic nephropathy, we utilized Mpv17 null mice as a model which has elevated ROS in kidney, had predicted that Mpv17 deficiency (increased ROS) would result in a more severe diabetic nephropathy. When we treated the first nine Mpv17-/- mice and five control mice by MLDS, the Mpv17-/- mice, to our surprise, exhibited extremely strong resistance to MLDS-induced diabetes with only one developed diabetes, while the other 8 mice were completely protected. In contrast, all 5 control mice developed diabetes. This experiment was repeated for several times using mice with different genetic background and using both fasting and non-fasting blood glucose measurement. And the results were consistent. This observation suggests that Mpv17 is implicated in the autoimmune diabetogenesis.

However, this protection of Mpv17 null mice against MLDS might have derived from an increased resistance of beta-cells/islets to non-specific toxicity of STZ instead of an impaired autoimmune process. We performed high-dose STZ treatment (200 mg/kg) on ten Mpv17-/- mice and five control mice. We found that the blood glucose change in these two group of mice, and the difference were greatly reduced compared to MLDS, indicating Mpv17-/- islets are

similarly sensitive to STZ toxicity and the strong resistance of Mp17-/- mice to MLDS was due to their increased resistance to autoimmune attack by immune cells infiltrated.

We also bred Mpv17-/- to Akita mice and generated Mpv17-/-:Akita mice. These mice also exhibited significant resistance to the development of diabetes as shown by their much slower increase of blood glucose level over time, compared to control mice.

Together, our preliminary studies under Aim 2 of this grant demonstrate the surprising and novel result that Mpv17 deficiency protects mice against type 1 diabetes in two models, STZ (autoimmune diabetes model) and Akita (ER stress diabetes model). Further studies will thus provide opportunity to reveal novel mechanisms or pathways in Type 1 diabetogenesis.

2. Collaboration:

See also Part A

3. Publications:

Seel also Part A

Project 2: "Role of Cd36 in tubulo-interstitial of DN"

Responsible Investigator: Susztak, Katalin

1. Detailed Project Accomplishments:

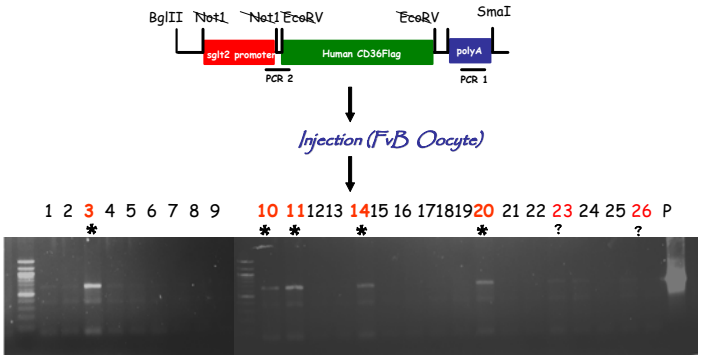

Figure 1. Schematic representation of the targeting construct (above). Results of the tail genotyping PCR (bellow). 7 transgenic founders are shown in red.

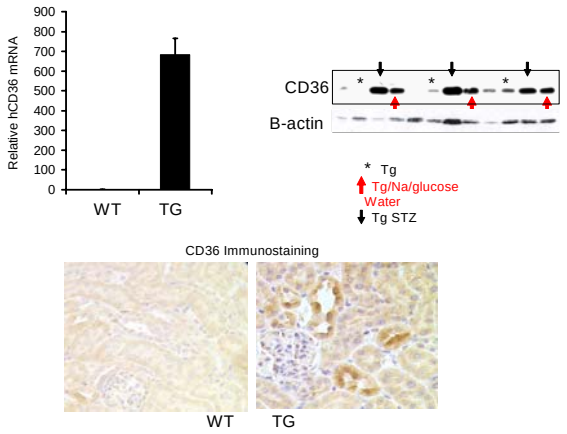

Figure2. Expression of the CD36 transgene in the sglt2CD36 transgenic mice. A. QRT-PCR analysis of human CD36 mRNA in wild type and in transgenic mice. B. Western Blot analysis of CD36 expression in wild type transgenic, NaCl and glucose treated transgenic and STZ induced diabetic mice. C. Immunostaing (for CD36) of kidney sections of wild type and diabetic transgenic animals.

Human CD36 cDNA was cloned under the sglt2 (sodium/glucose) cotransporter promoter. The targeting vector was injected to FvB oocytes. By tail genotyping PCR, 8 founder lines were identified, 4 males and 3 females. All four male founders were infertile, 1 female founder died and the remaining 2 female founder transmitted the transgene to its progeny. Transgenic pups were born with expected frequency. Transgene expression was analyzed at the mRNA level by QRT-PCR and on protein level by Western blots and immunocytochemistry. Transgenic animals had ~200 fold increase in the transgene mRNA levels expression compared to wild type mice (Fig2). Western blots failed to show an increase of the transgene expression at baseline. Animals were challenged with a high sodium and high glucose diet or were made diabetic to enhance the transgene expression (as the sglt2 promoter is known to be sensitive to these measures). Following enhanced salt and glucose load transgenic animals had approximately 3-4 fold higher level of CD36 levels at the whole kidney level. Immunohistochemistry analysis also identified the Cd36 transgene

in the renal tubules (Fig2). Thereby we concluded that we have successfully created transgenic animals with CD36 overexpression in the renal tubules.

In order to evaluate the effect of increased CD36 expression on the development and progression of diabetic nephropathy. 4 weeks old male animals (n=5) were made diabetic via low dose streptozotocin injection (50 mg/kg ipx5 daily). At 12 weeks of age albuminuria was highly increased in CD36 transgenic diabetic animals compared to wild type (FvB) diabetic animals (1720 μg/mg creat vs. 60 μg/mg creat, respectively). Histological analysis (PAS staining) showed dilated tubules with tubular epithelial degeneration and increased interstitial infiltration. Tubular apoptosis rate (quantified by TUNEL stain) in the kidney cortex was also significantly increased (0.373% vs. 1.5%) in transgenic diabetic animals compared to wild type diabetic animals. Oil Red-O staining confirmed the enhanced tubular lipid accumulation in transgenic diabetic animals.

Thereby we concluded that increased proximal tubular CD36 expression plays an important role in diabetic tubular degeneration and renal disease progression.

2. Collaboration:

See also Part A

3. Publications:

See also Part A