



VALIDATION OF MOUSE MODELS OF DIABETIC NEPHROPATHY

I. Definition of human diabetic nephropathy

1. In man diabetic nephropathy is a CLINICAL SYNDROME characterized by
 - i. Progressive renal insufficiency in the setting of hyperglycemia
 1. This is typically preceded by a period of glomerular hyperfiltration
 - ii. Albuminuria
 1. This is preceded by the development of microalbuminuria
 - iii. Characteristic pathologic changes including
 1. Glomerular basement membrane thickening on EM—with absence of immune deposits
 2. Mesangial matrix expansion and sclerosis ± nodular mesangial sclerosis (i.e. Kimmelstiel Wilson lesions)
 3. Tubulointerstitial fibrosis
 4. Arteriolar Hyalinosis

II. Areas where current mouse models fall short

1. Renal failure
 - i. At present no mouse model of diabetes develops renal failure
 1. Problems with serum creatinine determination inaccuracy
 2. No routine measure for determining GFR without sacrificing mice
2. Albuminuria
 - i. no benchmarks for the amount of albuminuria reflective of glomerular disease
 - ii. only modest increases in proteinuria detected in present models (at most ten fold whereas renal disease in man is associated with a 100-1000 fold increase in albuminuria
 - iii. Pathological changes are not robust: absence of wide-spread mesangial sclerosis, kimmelstiel Wilson nodules, arteriolar hyalinosis or tubulointerstitial fibrosis.

III. Diabetic models

1. Fasting measurements are determined after food has been removed for 6 hours (e.g. 7 am to 1 pm).
2. Insulin resistance and diabetes is determined by intraperitoneal glucose tolerance test, or euglycemic clamp. All are performed under awake, conscious conditions.

IV. RENAL PHENOTYPING

Glomerular filtration rate: No techniques for performing serial determination of GFR in mice are firmly established.

Four methods for determining glomerular filtration rate are being evaluated. Three methods are based on administration of the exogenous marker, inulin. The third approach explores the measurement of the endogenous marker of GFR, creatinine. The results using these approaches are summarized below.

- 1) **Anesthetized inulin clearance** values will be used as a comparator for values obtained in conscious mice since the most of the data in the literature has been

based on these determinations. (*Method: Animals will be anesthetized with 0.04 mg/g pentobarbital, and a polyethylene catheter (PE-90) inserted into the trachea to facilitate spontaneous ventilation. The left carotid artery and left jugular vein are cannulated with polyethylene catheters (PE-10) for intravenous infusions, to monitor mean arterial pressure, and to allow intermittent sampling of arterial blood. After surgery, normal saline (2% of body wt) is infused intravenously over 20 min to replace surgical losses. A priming dose of carboxyl-¹⁴C-inulin is given, followed by infusion of carboxyl-¹⁴C-inulin in normal saline at a rate of 25 μ l/min/100 g body wt. The bladder is cannulated via a supra-pubic incision with a PE-50 catheter to facilitate collection of urine. After 30 min of equilibration, renal function is measured during at least two consecutive 30 min clearance periods. Carboxyl-¹⁴C-inulin in plasma and urine is measured in a liquid scintillation counter (Nuclear Chicago-TM Analytical Inc., Elk Grove, IL) and clearances are calculated using standard formula (**Inulin clearance = (Urine_{Inulin} X Urine flow rate)/Plasma_{Inulin}(1).***)

- 2) **Creatinine Clearance** is the most widely utilized approach to estimate GFR in mice. This is now recognized to be inaccurate due to a significant artifact contributing to more than 80% of the apparent serum creatinine concentration of ~1 mg/dl as measured by a picric acid based method (2, 3). When authentic serum creatinine is measured by HPLC values of ~0.1 mg/dl are obtained. It remains to be determined whether GFR as measured by creatinine clearance corresponds to values obtained by the Gold-Standard e.g. –inulin clearance.
- 3) **Steady state inulin clearance:** Osmotic minipump techniques have been used to measure GFR in rats (4, 5) and the consortium has adapted this approach to mice. Short term and serial determination of GFR in conscious mice can be determined by intraperitoneal implantation of an FITC inulin filled osmotic minipump. The pump that has been tested, has a minimum lifetime that delivers a fixed amount of inulin over time. After steady state 3.5 \pm 0.5 μ l/min/gBW on normal diet and 5.5 \pm 0.5 on HS diet. These relatively values were obtained in female C57BL/6 mice and higher values have been obtained in other strains and male mice.
- 4) **Non-steady state inulin clearance:** Serial measurements of GFR can be made over a period of months rather than weeks using a non-steady state bolus approach to determine the decay rate of FITC inulin in plasma. Plasma is sampled at regular intervals over the course of 60-75 minutes and the decay rate fit to a bi-exponential decay rate. The first component reflects re-distribution of inulin from the plasma volume into the extracellular space, while the second phase If FITC inulin decay reflects renal clearance of inulin. GFR measured using this approach was 6.5 \pm 0.5 μ l/min/gBW or ~230 μ L/min/mouse and unchanged in sequential measurements made two weeks apart.

Proteinuria (albuminuria)

At present the degree and extent of albuminuria in mouse models of diabetic nephropathy is not dramatic. Akita mice (*Ins2*) and low dose streptozotocin models of diabetic nephropathy develop only 30-80 μ g/ albumin excretion per day (Breyer consortium observation). In the most robust diabetic mouse model (C57BLKS db/db mice) 24 hour protein excretion is only ~300 μ g/24 hours(6).

In contrast, much more significantly elevated twenty four hour protein excretion rates have been reported in several murine models of glomerular disease disease. Alport's disease mice generated by disruption of the Col IV/ α 5 gene (reference) exhibit roughly 2000 μ g/24 hours (7). Mice with genetic disruption of CD2AP exhibit even more robust albuminuria up to 10,000 μ g/24 hours/day (8). Thus the consortium views the achievement of a 24 hour albumin excretion rate of 3000 μ g to be a primary goal to improve current models of diabetic nephropathy (i.e. 100x above

normal). A secondary goal would be to identify a model where glomerular albumin excretion is increased by at least 10-fold in an inbred mouse strain (e.g. 129 or C57BL/6).

Urine albumin:Creatinine ratio's will also be standardized. In normal mice and most models of diabetes, Uprotein: U Creat is usually 0.1 to 0.2 ($\mu\text{g}/\mu\text{g}$). In mice with overt albuminuria this ratio is usually greater than 10 and always greater than 1. Correlation between this ratio and twenty four hour albumin excretion will be performed by consortia members.

These assays will be performed using a standardized approach. The consortium members agree upon using the **EXOCEL ALBUWELL M ELISA kit**. (<http://www.exocell.com/albuwellm.html>) . Units will be reported as μg albumin/mg Creatinine. If a 24 hour urine collection is made then μg Albumin/24 hours/mouse should also be reported.

PATHOLOGY. The renal pathology subcommittee has established criteria for diabetic nephropathy in mice (Based on conference call discussion November 6, 2002, with Drs. Killen, Meyer, Steffes, Striker and Fogo) as outlined below.

Pathologic definition of diabetic nephropathy in man:

In **early/mild** cases typical of human diabetic nephropathy changes include mild glomerular basement membrane thickening, mild mesangial expansion, with little if any interstitial fibrosis. There is accompanying mild to moderate vascular changes, typified by afferent and efferent arteriolar hyalinosis.

In more **moderately** advanced diabetic nephropathy, there is further expansion of the mesangial matrix, which may culminate in small to medium sized Kimmelstiel-Wilson nodules. There may even be focal segmental sclerotic lesions. Glomerular basement membrane thickening is generally more advanced, and there is mild to moderate accompanying interstitial fibrosis and moderate vascular changes as described above.

In **advanced human diabetic nephropathy**, there is extensive glomerular basement membrane thickening, often up to 4 to 5 times normal, with markedly expanded mesangial matrix, which may manifest as Kimmelstiel-Wilson nodules. Of note, Kimmelstiel-Wilson nodules do not occur in all cases of even advanced human diabetic nephropathy. There may be associated sclerosis, i.e. obliteration of capillary lumens, often with adhesion, and associated hyalinosis in glomeruli. There is associated moderate to severe tubulointerstitial fibrosis, and moderate to severe vascular changes as described above. In diabetic nephropathy due to type 2 diabetes, glomerular basement membrane thickening may not be as dramatic or as uniform as in diabetic nephropathy due to type 1 diabetes.

The criteria for established diabetic nephropathy in a mice: **Screening** should be performed by examining light microscopic (LM), PAS-stained 2-3 micron sections from paraffin embedded tissue that can be non-perfused. The three initial LM criteria are (all five criteria below required):

1) **Mesangial matrix expansion** (estimated by light microscopic screening, verified by scoring according to images of mesangial matrix expansion with greater than 50% increase in mesangial volume compared to normal controls, using NIH Image or similar program). This lesion should be present in the majority of glomeruli.

2) **Arteriolar hyaline**. Any degree of hyalinosis is accepted as indicative of a component of diabetic nephropathy.

3) **Tubular interstitial damage**, evident by fibrosis. In addition there may be tubular basement membrane thickening, as discerned by light microscopy. Any degree of fibrosis is acceptable as indicative of a component of diabetic nephropathy.

If all of the above three lesions are present, then further screening by EM should be done, to assess the following:

4) **GBM thickening**, verified by EM morphometry, representing more than 25% increase over age-matched control.

5) **Absence of electron microscopic dense material by EM.**

Additional considerations are as follows:

If such electron dense material is present, then further studies by immunofluorescence should be done. If IgG or IgA are present in a dominant or co-dominant distribution, supportive of immune complexes, the lesion is not pure diabetic nephropathy. IgM staining alone is not taken as definitive evidence of immune complex disease. Negative IF in the setting of EM densities would likely indicate that the electron dense material represents only sclerosis or hyalinosis.

In addition to the above criteria, there should be an absence of inflammation, endocapillary proliferation with neutrophils, or extracapillary proliferation.

Screening should be performed in animals that meet metabolic screening profiles and have albuminuria as delineated by other criteria. Appropriate controls that do not have those findings should be included for the overall screen.

Note: Additional lesions of segmental sclerosis or nodular sclerosis may exist. Nodular sclerosis, the so-called Kimmelstiel-Wilson nodule, would be considered an attractive bonus, but not a required feature of diabetic nephropathy in mice. If segmental sclerosis is present in the absence of mesangial matrix expansion and GBM thickening as outlined above, the lesion is deemed to not represent diabetic nephropathy, but rather segmental sclerosis due to other etiologies.

Note: For initial screening, perfusion fixation is not needed. This is based on the extensive experience with human material where perfusion fixation for obvious reasons is not done. This is also based on the consortium's goal, that we are screening for robust, reproducible lesions, and not for subtle early defects. Further studies to look at pathogenesis and mechanisms and measure specific components of the glomerulus would very likely be enhanced by directed, detailed perfusion fixation-based studies. Mice that do not meet all screening criteria may still be of interest for some further mechanistic studies. We anticipate that an ideal diabetic nephropathy model would show progression over time, and achieve more marked mesangial matrix expansion than the initial screening criteria cut-off. *Nevertheless, once a model meets the criteria for these features of diabetic nephropathy, it will undoubtedly be desirable to quantify the glomerular morphology in perfused fixed kidneys.*

Note: Images of examples of mesangial matrix expansion on PAS-stained sections, illustrating LM appearance of 25%, 50%, 100% or greater mesangial matrix expansion as verified by computer-assisted morphometry will be generated by Liliane Striker, and posted on the Website for use in LM scoring. LM scores of 0-4+ can then be assigned for screening purposes, based on the LM standards posted.

DATA ENTRY BY CONSORTIA MEMBERS TO THE AMDCC WEB-SITE (WWW.AMDCC.ORG).

V. **Acceptable Animal Models of RENAL Disease in Diabetes**

- A. Greater than 50% decline in GFR over the lifetime of the animal
- B. 100 fold increase in albuminuria > controls for that strain at the same age and gender.

- C. Pathology
 - a. Mesangial Sclerosis
 - b. Any degree of arteriolar hyalinosis
 - c. GBM thickening by >50% baseline
 - d. Tubulo-interstitial fibrosis

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PROTOCOLS:

Mouse Strain: C57BL/6J (i.e. from the Jackson Labs) will be used as the index strain. This strain is prone to atherosclerosis but resistant to nephropathy. Other strains including C57BLKS/J, ROP/os and 129/Svj may be nephropathy prone but this needs to be validated.

Systematic screening for nephropathy susceptibility will need to be determined for each mouse strain.

Diet. The group has mostly used chow diet, but some were using a semi-synthetic diet. (AIN76A; with added cholesterol and fat). Although Jan Breslow is currently evaluating the AIN76A diet, the mouse metabolic phenotyping centers (MMPC's) have decided against synthetic diets because of the **prohibitive costs**. Based on discussion between several MMPCs taking into account they have elected to utilize a common diet source from Harland Teklad

The MMPCs also decided that this would be irradiated chow. The product code is #7012 (also listed as #7912), Teklad LM-485 Mouse/Rat Sterilizable Diet (<http://www.teklad.com/standard/index.htm>). The MMPC at the University of Cincinnati will perform for some initial analyses on the fatty acid compositions of the chow, and the information will then be shared with all four MMPCs.

In the cases of feeding studies, it was agreed that a semi-purified diet should be used instead of the chow.

Blood sampling. Mice have a diurnal pattern, high metabolic rate, and eat at night. Overnight fasting introduces an overly prolonged stress, and causes insulin resistance.

It was recommended 4 and 6 hr fasting precedes serum measurements. (At Vanderbilt food is removed at 7am and the study performed at 1 PM (6 hrs).

Husbandry We need to determine whether the AMDCC should adopt the protocols utilized by the Mouse Metabolic Phenotyping Centers as much as possible. These include issues of light/dark cycles, sentinel animal health status, bedding type, frequency of bedding changes.

The most recent version of the MMPC protocols is attached to this email.

MODELS of Diabetes:

Streptozotocin (STZ) induced diabetes:

A low dose STZ protocol in mice is being evaluated (by Kumar Sharma from the Einstein/Jefferson/Minnesota group). STZ is made up fresh in 0.1 M Na Citrate buffer, pH 4.5. Daily STZ injections (50 mg/kg i.p.) are initiated at 7-8 wks of age and are administered for five consecutive days. This model relies in part, on a secondary autoimmune insulinitis following injury of the pancreatic β -cell by STZ.

Preliminary results In C57BL6, show diabetes develops in about 50% of mice 3 wks after the first round of injections with blood glucose levels typically 400-600 mg/dl. This schedule is repeated 7 wks after the first round of injections in those mice not developing diabetes. Insulin is not required. Mortality was less than 10% in diabetic mice. In 129/Svj (new designation by Jackson labs 129 P3/J), 80% of mice developed hyperglycemia by 3 weeks after first round of STZ injections. Due to severe hyperglycemia 7 weeks after the first round (400-600 mg/dl) the second round of STZ injections were not required. Body weights were essentially no difference between non-diabetic and diabetic mice with either strain.

Genetic Models of diabetes:

Several genetic models of diabetes are being examined been proposed including:

AKITA
C57BL6/J *Lep^{db}/Lep^{db}*,

C57BLKS/J *Lep^{db}/Lep^{db}*,
 TgPax1-HNF6, and
 NOD mice.

Glucose tolerance test: Glucose is measured by glucose analyzer and insulin by the Linco assay. For intraperitoneal glucose tolerance, mice are injected IP with a glucose solution at 1 mg glucose/1 g of mouse body weight. Blood samples are collected prior to the administration of the glucose for time zero and at 15, 30, 60 and 120 minutes post-glucose administration. Blood is collected from the retro-orbital sinus in lightly anesthetized mice (isoflurane inhalant) using sterile, heparinized microcapillary tubes, or from a warmed tail vein. The collected blood is immediately placed on ice, the plasma is separated by centrifugation as quickly as possible and the samples frozen.

Glucose "Clamp" for measurement of whole body insulin-stimulated glucose disposal

Version: 1

Edited by: Donald A. McClain

Summary

Reagents and Materials

Protocol

Summary: This is the standard protocol for measuring insulin action in terms of total body glucose disposal. It is the "gold standard" for quantifying insulin resistance/sensitivity. The euglycemic clamp involves two separate procedures with a recovery period of 4-6 days to allow healing and minimize stress. The first procedure involves the placement of a catheter into the right internal jugular vein of the mouse for infusion of insulin and glucose. The mice are anesthetized and the catheter placed. The exposed end of the catheter burrowed under the skin and exteriorized in the scapular region. After the recovery period, the mice are subjected to the clamp procedure after a fast of 6 hours. The mice are infused with insulin (18 mU/kg/min) and infusion of a 25% glucose solution is begun until an euglycemic state of ~ 6 mM is reached. Blood samples are collected at -40, -20, -10, 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 minutes from the tail vein for determination of plasma glucose and insulin. The glucose infusion rate is determined as a measure of insulin resistance. This procedure can be modified to include tracers.

Reagents and Materials:

Reagent/Material	Quantity Required	Vendor	Stock Number
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(See below)

Protocol:

1. Jugular Vein Catheterization:

a. Anesthetize mouse by injection of 12.5mg/ml Avertin (0.15ml/10 grams body weight, ip). Recipe for Avertin: dissolve 0.625g of 2,2,2-Tribromoethanol (Aldrich Chemical Company, Inc. T4,840-2) in 1.25ml tertiary amyl alcohol, bring solution up to 50ml with sterile water, store at 4C in the dark.

b. Give a prophylactic injection of 15mg/ml Ampicillin (Sigma A-0797, 0.15 ml/g b.w.ip).

c. Wait 10 minutes and check pedal reflex by extending hind limb and firmly pinching the hind paw. If the mouse attempts to withdraw limb, give an additional 50ul of Avertin. Periodically (about every 10 min) check for pedal reflex throughout surgery.

d. After anesthesia has been achieved shave area over right external jugular vein and nape of neck with electric razor. Clean shaved areas with Betadine solution and wipe off excess with 70% isopropyl alcohol pad.

e. Protect from dry eye with a small amount of ophthalmic lubricant (Lacri-Lube NP, Allergan No. 4240) in each eye.

f. Place the mouse on a pre-warmed surgical surface (Braintree Scientific Inc., Deltaphase operating board model 39 OP with Isothermal pad model 39 DP) covered with sterile drape. Wear sterile gloves and use aseptic technique throughout the surgery.

g. Make a small incision through the skin on shaved area of neck. Feed blunt end scissors (surgical scissor 10cm, Fine Science Tools, FST 14078-10) subcutaneously toward the right shoulder to create a path to exteriorize catheter later on.

h. Place the mouse on its back with head oriented towards surgeon. Loosely tape down chin and all four limbs in a spread-eagled fashion. With the aid of a dissecting microscope (Zeiss Semi 2000), make a horizontal incision (~1 cm) above the jugular vein anterior to the clavicle centered in the shaved area. Using small curved forceps (serrated fine forceps 10cm, FST 11052-10) to create a subcutaneous pocket on anterior side of incision. Gently tease fat tissue away from the incision site until the jugular vein can be visualized.

i. Clean a 1 cm section of the jugular vein anterior to the pectoral muscle of fat and other tissue by blunt dissection. Isolate the jugular vein by placing small curved forceps under vein. Feed three pieces (approximately 3cm each) of 6-0 braided silk suture material under the vein. Tie with suture tying forceps (FST 00272-13) the anterior ligature to occlude vein. Use two small curved hemostats (FST 13003-10) and attach to each end of the anterior ligature. Adjust hemostats to place light tension on vein. Make a tie to the posterior ligature but leave loose. The center ligature is left loose with no tie.

j. Prepare catheter by attaching a 45 cm length of Micro-Renathane tubing (Braintree Scientific, Inc., MRE 025) to a blunt ended 25-gauge needle. Attach needle to a 1cc syringe filled with a saline heparin mix (0.9%, 20U/ml). Fill catheter with saline heparin mix making sure there are no air bubbles present. The end of the catheter should be blunt. Using micro scissors (8 cm, FST 15002-08) make a small incision in the vein between the anterior and posterior ligatures making sure not to cut through vessel. Using catheter forceps (vessel cannulation forcep, FST 00574-11) to hold the catheter, feed the blunt end into the lumen of the vessel. Feed the catheter into the vessel until it just disappears past the pectoral muscle. Check for occlusions by aspirating the syringe catheter assembly. Blood from the vein should flow freely in and out of the catheter.

k. Secure the catheter by first tying off the posterior ligature. Next, tie the middle ligature around the vessel and catheter. Tie ends of posterior and middle ligature together to firmly secure catheter. Remove hemostats and tie off anterior ligature around catheter.

l. Trim off any excess thread. To exteriorize the catheter, use hemostats to break off needle from hub. Remove tape from chin and limbs. Gently turn mouse on its stomach. Feed blunt forceps subcutaneous through the hole in the nape of the neck to the jugular incision site. Grasp end of needle with forceps and pull catheter through leaving slack in the pocket created in the ventral incision site.

m. Return mouse to its back and place a small amount of 2.5% Lidocaine ointment (Xylocaine) on the surgical site. Using forceps, gather the skin together. Gently remove any moisture with gauze. Holding the skin, place surgical glue (Nexaband S/C 909004, J.A.Webster, Inc.) along incision. Return mouse to stomach. Place a small amount of Xylocaine on neck incision.

n. To attach mouse to the INSTECH Solomon tether system (Plymouth Meeting, PA) feed the catheter through the tether (CIH62) being careful not to put tension on the catheter itself. Place the mouse in the harness (CIH62) attached to the tether. Adjust the fit of the harness. Cut off any excess catheter to leave a small loop to connect to the end of the swivel (375/25).

o. Place the mouse in the Instech designed animal enclosure (STANK) and attach the swivel to the arm (SMCLA). Connect the line from a multi infusion pump (Harvard Apparatus, PHD 2000) to the swivel. The pump will infuse saline (0.9%) at a rate of 40ul/hr during the recovery period (48 hrs). The mouse should have free access to food and water throughout this period. Attach the lid to cage.

2. Clamp procedure:

a. After 48 h of surgical recovery the mouse is fasted overnight and transferred to a standard mouse housing cage with a tether arm attached to hold the tether system. A dual infusion pump (Harvard Apparatus, Pump 33) will be used to infuse 50% dextrose and insulin to

the mouse. A constant flow rate of insulin and a variable rate of 50% dextrose will be infused to maintain a constant blood glucose level.

b. A saline insulin mix (0.9%, 20mU/ml) is loaded in a Becton Dickinson (BD) 5 ml syringe (309603) and placed in the syringe position #2. Preset diameter and flow rate settings to 12.06mm and 2.5ul/hr respectively. The 50% dextrose is loaded in a 10 ml BD syringe (309604) and the diameter preset to 14.5mm with a flow rate of 13.2ul/hr/gbw. Attach blunt ended 25g needles to both syringes and run 25g tubing from the syringes to a Y connector. From the connector run enough 25g tubing to reach the Instech swivel. Start pump at ~100x the programmed flow rate for 10 minutes to void air in the lines and equilibrate the insulin 50% dextrose mix. Turn off the pump and set insulin and 50% dextrose flows to infusion rates, connect tubing to swivel.

c. Clip a small portion of the mouse tail and measure blood glucose level with a glucometer (Glucometer Elite XL) before starting pump.

d. Start pump and timer. Measure blood glucose after the first 15 minutes and at 10 minute intervals for a total of 60 minutes. Adjust the 50% dextrose flow rate during this period to stabilize blood glucose levels to 100-150 mg/dl (6.0-9.0 mmol).

e. Glucose disposal rate calculation= $(\text{flow rate}(\text{ul/hr})/60/\text{kgb.w.})/2$ This assumes glucose production rate (hepatic and renal gluconeogenesis and glycogenolysis) will be zero which is effectively the case at these levels of hyperinsulinemia. However, at lower insulin infusion rates (e.g. when generating an insulin dose-response curve for a particular action of insulin) the glucose production rate will need to be determined by isotope dilution (see the literature for protocols.) Glucose uptake into specific tissues can also be measured by the infusion of [¹⁴C]-2-deoxy-D-glucose at the end of the procedure (see literature).

INULIN CLEARANCE

SUMMARY OF METHODOLOGY :The study is conducted on mice. Under temporary anesthesia, Alzet micro-osmotic pumps (Model 1007D) were filled with 3% FITC-inulin solution. These pumps release their contents at a rate of 0.5 μ l/hr for inulin for at least seven days. Two mini-pumps were implanted in mouse peritoneal cavity through a midline incision (approximate 0.5 cm). After the mice regained consciousness, they were placed in metabolic cages. 48 hours later of surgery, collect mouse urine for 24 hours (day 3) and collect 100 μ l of blood from sphenous vein as described previously. Repeat the urine and blood collection on day 5 and day 7 after surgery. The concentration of FITC-inulin is detected by fluorometer and GFR is calculated based on the amount of inulin excreted through kidney in 24 hours and the concentration of inulin in plasma.

To confirm that fluorescence intensity represents inulin concentration, GFR calculated by the fluorescence will be compared to that calculated by the concentration of inulin measured by an HPLC technique.

Materials: the study was conducted on C57BL/6 mice, aged 6-8 weeks. The FITC-inulin is the product of Sigma Pharmaceuticals. Micro-osmotic pump is available in Alza Corporation and the model 1007D, with a release rate of 0.5 μ l/hr for 7 days, was used in this study.

Surgery: The mice were anesthetized with a solution of Ketamine (0.05mg/ g BW) and Rompun (0.005mg/ g BW). The micro-osmotic pumps were filled with approximately 100 μ l of a 3 % FITC-inulin solution. Two pumps were inserted into peritoneal cavity of mouse through an approximate 0.6 cm length of abdominal midline incision. After the mice fully weak up, they were placed into metabolic cages. Varying concentrations of sugar water were provided in order to speed the recovery period.

Blood and urine collection: After the recovery period, urine was collected from the metabolic cage after a 24-hour interval. Blood was collected at the end of the 24 hours, using a method adapted from Hem et al. Briefly, the conscious mice were restrained inside a 50-mL centrifuge tube with airholes drilled in the tip. The inner thigh was closely shaven and wiped with 70%

ethanol, revealing the saphenous vein. A small incision was made with a scalpel, and at most 0.1 ml blood was collected with a heparinized capillary tube (Fisher Scientific). This yielded, on average, a 40 µl plasma sample after centrifugation (4,000 RPM, 10 min).

Measurement of inulin in plasma and urine: After collection, the blood samples were centrifuged as described above and the urine samples were centrifuged as well (3,000 RPM, 5 min) in order to separate any impurities (food pellets) that might have collected in the urine collection tube. The plasma samples were then mixed with HEPES (pH 7.4): 40µl plasma + 10µl HEPES. The urine samples were diluted 1:5 (200 µl urine in 800 µl HEPES) and 1:10 in HEPES. The processed samples were then loaded onto a 96-well plate (COSTAR 3595), 50µl of sample/well. The fluorescence was measured with Fluoroscan Ascent FL (Labsystems), at excitation 485 nm, and emission 530 nm.

In order to ascertain the concentration of FITC-inulin in the samples, a standard curve was obtained both for urine and plasma. For each point on the urine standard curve, 200 µl of normal mouse urine was diluted into 300 µl HEPES. Then, 500 µl of FITC-inulin solution of varying known concentrations (dissolved in HEPES) was added to each tube. Five points were included on the urine standard curve, with inulin concentrations ranging from 0.05 mg/ml to 0.0008 mg/ml. For each point on the plasma standard curve, 160 µl of normal mouse plasma were mixed with 40µl of an inulin solution (made in HEPES) of known concentration. Four points were included on the plasma standard curve, with inulin concentrations ranging from 0.005 mg/ml to 0.0006 mg/ml.

Calculation of GFR: GFR is calculated by the amount of inulin excreted in urine divided by the concentration of plasma inulin and expressed in ml/min/g body weight.

MICROABLUMINURIA:

Manufacturer/Trade: Exocell, Inc. / Albuwell M

Catalogue Numbers: 1011 Strip Plate

Methodology: Competitive ELISA

Summary of procedure: Albuwell M is an indirect competitive ELISA designed to monitor kidney function in the mouse by measurement of urinary albumin. To complete the assay, sample and rabbit anti-murine albumin antibody are added to albumin coated wells. The antibody interacts and binds with the albumin immobilized to the stationary phase or with albumin in the fluid phase, hence the notion of competitive binding.

A subsequent reaction with anti-rabbit -HRP conjugate labels the probe with enzyme. After washing, only the antibody-conjugate bound to the stationary phase remains in the well, and this is detected using a chromogenic reaction. Color intensity is inversely proportional to the logarithm of albumin in the fluid phase. The assay may be completed in less than 2.5 hours.

Specimen Required: Urine, 10ul

Assay Range: 0.3-10 ug/ml

Precision: Intraassay and interassay precision for samples within the useful range of the assay have a C.V.<10% of the mean.

Creatinine Companion:

Exocell, Inc. / The Creatinine Companion

Catalogue Numbers: 1012 Strip Plate Methodology: Chemical Analysis Summary of procedure: The Creatinine Companion is a chemical assay designed to monitor urinary creatinine in a convenient microplate format. It is intended as a "companion" for Exocell urinary albumin assays.

The assay is based upon the Jaffe' reaction of alkaline picrate with creatinine. Standards or samples are added to a microplate, and alkalinepicrate reagent is subsequently added. Absorbance at 500 nm is determined after a 10 minute incubation on the benchtop. Subsequently, an acid solution is added, and absorbance is again determined after a 5 minute incubation. The difference between these absorbance values is directly proportional to the

creatinine concentration, and a standard curve is generated from the response to the standards. Unknown samples are evaluated by comparing response to the standard curve. The assay may be completed in less than 30 minutes.

The assay serves as a normalization procedure for other analytes in urine samples, particularly for urinary albumin (1,2). It allows for a measure of kidney function based on a spot urine sample rather than the usual 24 hour pool.

Specimen Required: Urine, 10 ul

Assay Range: 1.0-10 ug/ml

Precision: Intra- and interassay precision of samples within the useful range have a C.V.<10% of the mean.

References: 1. Bennet, P. H. et. al. Screening and management of microalbuminuria in patients with diabetes mellitus. Am. J. Kidney Dis. 25:107, 1995. 2. Warram, J. H. et. al. Effect of duration of type 1 diabetes on the prevalence of stages of diabetic nephropathy defined by urinary albumin/creatinine ratio. J. Am. Soc. Nephrol. 7:930, 1996.

Pathology

Kidneys will be fixed by immersion in 4% paraformaldehyde. They will be evaluated periodic acid shiff (PAS) and Electron microscopy, Except for specialized quantitative morphometric studies, tissues will not routinely be performed on perfuse- fixed kidneys.

PAS Staining protocol (Per Dr. Agnes Fogo/Vanderbilt University)

Supplies:

<u>Sigma</u>	1 (800) 325-5832
Periodic acid	#P-7875
Ammonia hydroxide, 28 – 30%	#A-6899
Hydrochloric acid	#H-7020
<u>Surgipath</u>	1 (800) 225-3035
Schiff's reagent	#03800
Harris hematoxylin	#01562
Xylene	#03665
Ethanol	#03686
95% Ethanol	#03682
Micromount (coverslip mounting media)	#01732

Preparation and storage of reagents:

0.5% percent periodic acid:

0.5 gram periodic acid + 100 ml distilled H₂O

Store in corrosive acid safety cabinet at room temperature

Schiff's Reagent:

Use as is and store at 4° C

Harris hematoxylin:

Filter before use and store at room temperature

1% acid alcohol for differentiation:

1 ml of hydrochloric acid + 99 ml 70% alcohol (30 ml distilled H₂O + 70 ml ethanol)

Store in corrosive acid safety cabinet at room temperature

Ammonia water for bluing:

3 ml of 28% ammonia hydroxide + 1000 ml distilled H₂O

Store in corrosive alkaline safety cabinet at room temperature

Xylene, ethanol, 95% ethanol and Micromount:
Store at room temperature in flammable safety cabinet

Staining Procedure

Cut 2-3 μm paraffin sections on glass slides, dried overnight at room temperature

- 1) Deparaffinize by immersion in xylene, 3 changes over a 10 minute period.
- 2) Hydrate by immersion in 2 changes of ethanol, 2 changes of 95% ethanol for 3 – 5 minutes each change, and then rinse in running tap water 3 minutes (place staining jar with slides in it under a gentle flow of water).
- 3) Rinse in 3 changes of distilled H_2O for 1 minute each
- 4) Oxidize by immersion in 0.5% periodic acid for 10 minutes
- 5) Rinse in 3 changes of distilled H_2O for 1 minute each
- 6) Immerse in Schiff's reagent for 20 minutes
- 7) Rinse in running tap water for 10 minutes
- 8) Immerse in Harris hematoxylin for 3 minutes
- 9) Rinse in running tap water for 1 minute
- 10) Differentiate in 1% acid alcohol by dipping very quickly for 3 dips (takes about 1.5 seconds total!)
- 11) Immediately rinse in running tap water for 1 minute
- 12) "Blue" in ammonia water by immersion for 30 seconds
- 13) Rinse in running water for 3 minutes
- 14) Dehydrate by immersing slides in 2 changes of 95% ethanol, 2 changes of ethanol, and 3 changes of xylene for 3 – 5 minute each change
- 15) Place 1 drop of Micromount on each slide on tissue section and place coverslip on top

Electron microscopy,

Cut tissue IMMEDIATELY after harvesting in about 1mm cube pieces with very sharp razor so tissue is not crushed, and place in 2% glutaraldehyde.

Tissue can be kept in fridge for a couple of weeks in glut before processing further, or can then be switched to a special buffer.

It is crucial to NOT let the EM tissue sit around while you get all the other samples etc at sacrifice- the tissue will dry out, there will be changes and artifact from suboptimal fixation.

Additional pathological characterization of renal disease will be carried out should the model exhibit characteristics consistent with diabetic nephropathy. Standardized protocols have yet to be agreed upon and will require additional evaluation:

Masson Trichrome

Jones Methamine silver stain

Immunofluorescence (IgG, IgA, IgM, C3):