VALIDATION OF MODELS OF CARDIOVASCULAR DISEASE IN DIABETES

I. VASCULAR

A. Definition of human diabetic vascular disease

1. Accelerated atherosclerosis, peripheral vascular disease, and microvascular disease in the setting of 1) insulin resistance 2) dysmetabolic syndrome 3) impaired glucose tolerance (IGT) 4) type 2 diabetes mellitus or 5) type 1 diabetes mellitus
2. Acceleration of atherosclerosis is defined as an increase in extent of lesions (number of lesions/unit time) or progression of lesion histology (necrotic lipid cores with fibrous caps surrounded by a proteoglycan matrix or more unstable plaques). Diabetes lesions may also be characterized by increased inflammation (cells or cytokines), increased expression of certain genes (osteopontin or PAI-1), and increased calcification compared to lesions from nondiabetic humans.

B. Methods

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. 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. 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.
3. **Quantitation of Atherosclerotic Cross Sectional Lesion Area in the Aortic Root and Brachiocephalic Artery:**
   <u>*Preparation of Aortic Root and Brachiocephalic Artery for Lesion Quantitation:*</u>

   1. Prepare a 1 ml syringe by filling with 15µl 0.5 M EDTA ph 8.0 and then capping with a 23g needle. The syringe will be used for drawing blood from the heart.

2. Prepare a 10 ml syringe with 50-100 µl of heparin (1000 units per ml), then fill the syringe to 10 ml with PBS. Remove all the air from the syringe. Cap with a 30g needle. The syringe will be used to flush the heart.
3. Anesthetize the mouse.
4. Determine weight and length of the mouse.
5. Tape the mouse's arms to 3-4 layers of paper towels and place under a light source.
6. Cut the skin of the mouse from the abdomen to the top of the thorax.
7. Open the abdominal wall below the ribcage.
8. Lift the sternum with tweezers and cut the diaphragm. Then cut away the lower part of the ribcage to partially expose the heart
9. Draw blood from the heart by sticking the needle of the 1 ml syringe (see 1) into the apex of the left ventricle. Draw blood by slowly pulling the plunger, twisting the needle. Do not draw for over a minute to avoid blood clots. Transfer the blood into an Eppendorf tube. Mix the tube by inverting it a few times. Centrifuge the tube of blood and transfer the plasma into a fresh tube.
10. Make a small incision in the right atrium for drainage.
11. Stick the 10 ml syringe into the apex of the left ventricle and flush the blood from the mouse with 10 ml of PBS.
12. If the liver is to be saved take several sections and immediately freeze at –80 °C.
13. Dry the mouse by turning it over and pressing it against paper towels. Transfer to clean, dry paper towels and re-tape the arms.
14. Place the mouse under a dissecting microscope.
15. Remove the remaining ventral part of the ribcage, carefully cut the right clavicle leaving the brachiocephalc artery and its branches intact. Using microdissection scissors, cut out all of the fat around the ascending aorta and brachiocephalic artery.
16. Cut all the fat and tissue surrounding the heart, including the pulmonary artery, and veins.
17. Flush the heart again through the right ventricle, left atrium, and left ventricle using a 10 ml syringe containing a total of 3 ml of PBS to clean out residual blood.
18. Cut the brachiocephalic artery distal to where it branches into the right subclavian and right carotid and then cut it at its branching site from the aorta so you end up with a Y-shaped piece. Put it in a base mold (Fisher cat no 22-038217) and using a 10 ml syringe perfuse it with 1 ml of PBS to get it clean. It is important while handling the brachiocephalic artery to touch it only at its ends and not in the middle.
19. Cut the aorta proximal to the branching site of the brachiocephalic artery.
20. Take out the heart with the aorta. The atria must remain intact as they serve as landmarks when cutting the heart in the atherosclerosis assay.
21. Flush the heart-aorta preparation through the aorta with a 10 ml syringe containing 2 ml of PBS and place the heart in a 20 ml scintillation vial containing 10 ml of formaldehyde solution (10% v/v in aqueous phosphate buffer, Mallinckrodt).
22. Take the cleaned piece of the brachiocephalic artery and place it in a new base mold, fill it up to the first lip of the base mold with Tissue Tek O.C.T. compound. Move the brachiocephalic artery around carefully to make sure it fills with OCT.
23. Turn the Y-shaped piece of brachiocephaic artery piece so the prongs of the fork face the bottom of the base mold and freeze it in that position on a block of dry ice.

Embedding Hearts in Gelatin for Aortic Root Analysis:

1. Fix hearts in buffered formalin for 2 weeks at 4°C.

2. Place each heart in an embedding capsule; and place up to 40 capsules in a 1000 ml beaker. Wash under cold running tap water for 1 hour.
3. Make gelatin solutions containing 5%, 10% and 25% gelatin in H_20 and dissolve at 50°C in a water bath.
4. Place washed heart capsules in a shallow plastic container, float container in a 42°C water bath and cover heart capsules with 5% gelatin for 2 hours.
5. Pour back gelatin into a bottle (can be stored at 4°C for future use) and cover hearts with 10% gelatin for 2 hours.
6. Pour back gelatin into a bottle (can be stored at 4°C for future use) and cover hearts with 25% gelatin. Incubate in water bath over night.
8. Place container with heart capsules in refrigerator for 3 hours.
9. Remove heart capsules from solidified gelatin, discard gelatin.
10. Remove embedded hearts from capsules, trim gelatin around the heart to a cube and place trimmed hearts back into buffered formalin.
11. Store at 4°C for a minimum of 24 hours before sectioning the hearts.

Sectioning Hearts for Aortic Root Analysis:

1. Locate the left and right atria. Using a sharp razor-blade cut the bottom half of the heart off in a plane parallel to the atria. NOTE: It is essential that the gross cut is parallel to the atria so that a cross section of all three aortic valves is in the same geometric plane. Discard lower half of the heart.
2. Make a second cut above the top of the heart parallel to the first cut on the bottom.
3. Store the heart at 4°C in buffered formalin until ready to section.
4. Mount the trimmed heart on a sample pedestal in the cryostat using OCT compound, the aortic side of the block facing the pedestal.
5. Cover the rest of the gelatin block with OCT and let it freeze.
6. Set section thickness to 30µm and start sectioning until the part of the block that contains the heart tissue is visible. If a complete heart section is not visible, reposition the pedestal.
7. Once a complete heart section is visible, place it on a slide and to check the anatomic location of the section (initially you will see just a thick muscular wall). (note: to flatten the section for better viewing under the microscope dip the slide in water).
8. Cut approx. 20 of these 30µm sections and check under the microscope to see if the aortic sinus approaches. The presence of the left and right atria suggests that the sinus is close. The aortic sinus will usually appear rounded, the aortic wall won't be thick yet and the valves won't be distinct. Start saving the sections when the valve leaflets become clearly visible. (Reposition the pedestal if necessary to have all 3 leaflets of the valve in one plane)
9. Change thickness of sections to 12 µm and save every other section. Put 4 sections on one slide in this order, and label mouse and slide no:

1	2	mouse no.
3	4	slide no.

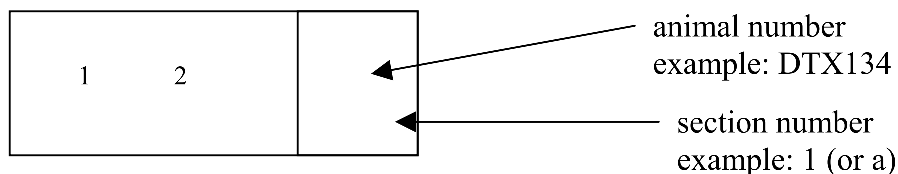
10. Cut and save sections until the aorta is rounded and all of the valve leaflets are gone. Usually, this is after 20 to 24 sections (5 to 6 slides).
11. Place slides in a covered glass dish with formalin vapor (put some formalin on bottom of dish) and store slides at 4°C for at least 12 hours prior to staining.

Staining Heart Sections for Aortic Root Analysis:

1. 1 min. H_2O
2. 1 min. H_2O
3. 30 sec. 60% isopropanol
4. 18 min filtered oil red-O (0.24% in 62.5% 2-propanol)
5. 30 sec. 60% isopropanol rinse
6. 1 min. H2O
7. 1 min. H2O
8. 2 min Harris Hematoxylin
9. 1 min Bluing Solution (H_2O with a few drops of ammonia)
10. 1 min. H_2O
11. 30 sec. Light Green
12. 1 dip H_2O
13. Keep in H_2O until ready to put cover slip on the slide
14. Fix cover slips with glycerol gelatin
15. Wash slides to remove excess glycerol gelatin

Sectioning Brachiocephalic Artery for Quantitation of Cross Sectional Lesional Area:

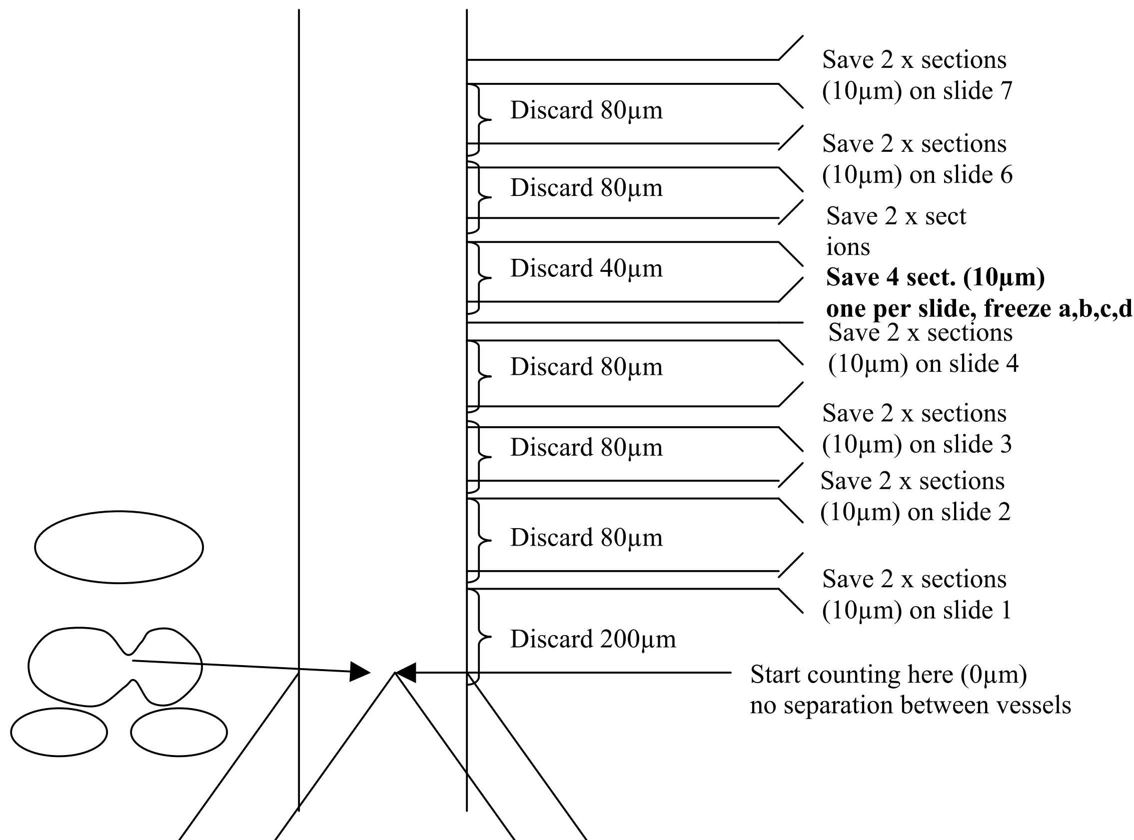
1. Set cutting temperature of microtome to –30°C. Mount specimens embedded in OCT on cutting pedestal, the opening of the Y shaped vessel should face towards the microtome cutting blade.
2. Set section thickness to 10µm. Start sectioning the right carotid and right subclavian arteries up to the point where they first combine into the brachiocephalic. A section of this point will not have the separation between the two vessels and will be considered the starting point for the following distances.
3. Once this point has been defined, cut 200µm discarding the sections.
4. Use Fisher Colorfrost plus slides to save sections
5. Save the following 2 sections (10µm each) on a the first slide (slide 1) in the following order:



6. Proceed sectioning and discard the following 80µm (8 sections)
7. Save the next 2 sections (10µm, each) → slide 2
8. Proceed sectioning and discard the following 80µm (8 sections)
9. Save the next 2 sections (10µm, each) → slide 3
10. Proceed sectioning and discard the following 80µm (8 sections)
11. Save the next 2 sections (10µm, each) → slide 4
12. Save 4 additional sections (10µm each), one per slide → slides a,b,c,d
    The purpose is to have consecutive sections available for immunostaining. These

sections are not relevant for quantification and can be discarded if immunostaining will not be done.

13. Proceed sectioning and discard the following 40µm (4 sections)
14. Save the next 2 sections (10µm, each) → slide 5
15. Proceed sectioning and discard the following 80µm (8 sections)
16. Save the next 2 sections (10µm, each) → slide 6
17. Proceed sectioning and discard the following 80µm (8 sections)
18. Save the next 2 sections (10µm, each) → slide 7



Staining Brachiocephalic Artery Sections for Lesion Quantitation:

1. 1 min. H_2O
2. 1 min. H_2O
3. 30 sec. 60% isopropanol
4. 18 min filtered oil red-O (0.24% in 62.5% 2-propanol)
5. 30 sec. 60% isopropanol rinse
6. 1 min. H2O
7. 1 min. H2O
8. 2 min Harris Hematoxylin
9. 1 min Bluing Solution (H_2O with a few drops of ammonia)
10. 1 min. H_2O
11. 30 sec. Light Green
12. 1 dip H_2O

13. Keep in H_2O until ready to put cover slip on the slide
14. Fix cover slips with glycerol gelatin
15. Wash slides to remove excess glycerol gelatin

Quantification of Aortic Root and Brachiocephalic Artery Lesion Area by Image Pro Software:

1. Use a microscope equipped with a video imaging system attached to a computer with Image Pro software
2. Quantify all 5 slides obtained from one aortic root and seven slides obtained from one brachiocephalic artery, preferentially use section number 1 on each slide. If section number 1 is not readable, go to the second section of the slide.
3. Measure only the area of oil red-O stained lesions on top of the internal elastic lamina, do not quantify any oil red-O stained material underneath the internal elastic lamina. In some cases, lesions may be sheared off the internal elastic lamina and are found in the lumen. Those lesions should also be counted.
4. The atherosclerotic lesion area is expressed as the mean of the lesion areas of one section per slide from the 5 aortic root slides or the 7 brachiocephalic artery slides.

4. Aortic Surface Lesion Area Quantitation by the En Face Method:

1. Mice are fasted prior to sacrifice.
2. The mice are weighed prior to euthanasia.
3. The mice are euthanized with an overdose of isoflurane (death is noted by the lack of respiration).
4. The abdominal cavity is opened using a midline incision and transverse cuts are made in the abdominal wall to improve drainage of the perfusate.
5. Blood is collected from the abdominal vena cava using a heparinized 1 ml syringe and a 1.5 inch 22 gauge needle. (The needle is bent away from the bevel to ease the insertion of the needle). The needle is pushed up through the vessel after collection of the blood and the intervening section of the vena cava scrapped with a scalpel to provide drainage.
6. Blood is placed into a chilled heparinized tube and the sample is centrifuged for 5 minutes at 10,000g.
7. The thoracic cavity is opened by cutting the ribs laterally to the sternum, with the sternum being retracted toward the head. The diaphragm is cut next to the ribs to improve drainage.
8. A 22-gauge needle attached to a gravity perfusion set up is inserted into the left ventricle and PBS containing 0.5 mM EDTA is started. The PBS flush is continued until the blood is removed (perfusate coming from the vena cava is clear).
9. The perfusion is now changed to a 4% paraformaldehyde, 7.5% sucrose, and 0.5 mM EDTA solution in PBS for 15 minutes.
10. The animal then flushed for a short time with PBS/EDTA solution to remove the paraformaldehyde from the lumen for the vessel.
11. The ribs, lungs, gastrointestinal and reproductive systems are removed, leaving the heart and kidneys *in situ*. The liver and spleen are weighed and along with a piece of the pancreas are saved in the fixative solution.

12. The mouse is taped to the dissection pad over the front and hind limbs allowing access to the body cavity.
13. The aorta is dissected out under the dissection microscope using mini-Vanna scissors and forceps. The aorta is dissected out from the heart until 3-5 mm after the iliac bifurcation. All the major arteries are branching off the aorta are left in place until later, excepting the gastric and hepatic arteries that were previously severed when the GI tract was removed. All adventitial tissue is removed by careful dissection.
14. The vessel is split *in situ* by making a small transverse cut partially through the iliac artery to allow scissors to be inserted into the lumen of the artery. The cut proceeds anteriorly until the kidneys are reached and then the other iliac artery is split. The cut is then continued anteriorly being angled at the aortic arch to the inside curve of the arch until the heart is reached. The heart is now removed and weighed, being saved with the other organs. The right and common carotid arteries are then severed close to the aorta and the scissors are placed into the right carotid towards the common carotid to begin a second cut, this cut allows the arch to lay flat the pinned. This second cut through the carotids and the innominant (brachiocephalic) artery until the aorta is split. At this point the aortic arch will resemble a Y. The renal arteries are now severed close to the aorta and the kidneys are removed from their capsules, weighed and saved. The iliac arteries are severed 3 mm past the bifurcation and the aorta removed.
15. The arteries can now be stored in PBS in the refrigerator or pinned and imaged.
16. The aortae are pinned onto a standard black wax dissection pan using 0.15 mm-black anodized pins (Fine Science Tools).
17. The pan is filled with PBS to prevent the vessels dehydrating and the vessels are pinned flat.
18. Sufficient pins will be used in order to make the vessel lay flat, typically requiring more pins in the arch than the thoracic and abdominal portions of the vessel. A slight tension is placed on the vessel when pinning to allow the vessel to lay flat. Additionally, at this point any remaining adventitia must be removed from the vessels using forceps and scissors.
19. The pan containing the pinned vessels is stained using the following procedure. The PBS is drained and replaced by 70% ethanol for 5 minutes. The 70% ethanol is then drained and the Sudan IV solution is placed into the pan for 15 minutes. The Sudan IV is then drained and the vessels are destained using 80% ethanol for 3 minutes. After 3 minutes the 80% ethanol is drained and the stained vessels are rinsed under running water to remove all ethanol. The drained pan is then filled with filtered PBS (this removes particles that could interfere with the images) until the liquid level covers the pins completely.
20. A marker bar containing three 15mm segments with separate colors is pinned next to the vessel prior to obtaining the image to provide a delineation of the vessel into segments. The aortic arch thoracic segments are proximal to the diaphragm (above the take off of the hepatic artery). The abdominal aorta is distal to the diaphragm.
21. Images of the vessels are then obtained and the extent of atherosclerosis is determined using ImagePro software.

5. Histology: Lesion Complexity Index

This latter defines the ratio of fatty streaks to complex lesions. Each of the slides is reviewed for the following lesion features: fibrous caps, cholesterol clefts, necrosis. The following quantification is applied for lesion area and complexity indiex:

Animals are fasted from 6 am to 12 noon and then the following studies are performed. Upon sacrifice, blood is removed from the inferior vena cava into EDTA (final concentration; 0.05M) and plasma/red blood cells are stored for analysis. The aorta is perfused gently with PBS in a retrograde manner and the heart is removed and stored in buffered formalin (10%). Cryostat sections are prepared and embedded in gelatin (25%). Serial sections, 10 µm thick, are cut from the level of the aortic valve leaflets up to =480 µm above the leaflets in the aortic sinus; every other section is retrieved and placed onto gelatin-coated slides (5%); four sections are placed onto each slide for a total of five slides. Sections are then stained/counterstained with oil red O and hematoxylin/light green. Quantitation of atherosclerotic lesion area is performed on one section from each slide (beginning at the site where three distinct valves first appear) using a Zeiss microscope and image analysis system (Media Cybernetics); mean lesion area from slides two through five is reported. On each of the same slides as above, the total number of lesions is counted. The complexity index is calculated from the ratio of fatty streak (FS)/total lesion number or complex [C] lesion (defined by presence of cholesterol clefts, necrosis or fibrous cap formation)/total lesion number. The sum of the ratio of FS/total and C/total is one.

6. **Lipids:**

Simple analyses include total cholesterol (TC), high-density lipoprotein cholesterol (HDLC), triglyceredes and free fatty acids, measured by standard technology. HDLC vs. non-HDLC is measured using ultracentrifucation. Particle size is measured by FPLC analyses.

Cholesterol specifics are as follows:

Reagent: Cholesterol HP, 12 x 48ml (Roche 704036), ready to use

Controls: Wako Control Serum I, 10 x 5 ml (Wako 410-00101), low control

Standard: NERL Diagnostics aqueous cholesterol (NERL 1305) (set of 5 standards at 50, 100, 200, 300, 400mg/dl)

1. Use standards at 0, 50, 100, 200 mg/dl. Prepare standard at 0mg/dl using PBS. Do not use the standards at 300 and 400 mg provided in the original set, because they exceed the linear range of the assay. According to the supplier, standards should be stored at 15-30°C (i.e. room temperature) (This is very important!).
2. Prepare a work sheet with 8 rows and 12 columns to correspond to the wells of a 96 well plate. Enter places for standards, controls, and unknown samples in duplicate.
3. If values greater than 200mg/dl are expected, dilute the unknown samples by transferring 10 µl of the unknown to a new tube containing an amounts of PBS appropriate for the expected cholesterol concentration (ie 90 µl PBS, as a 1:10 dilution may be appropriate for apoE deficient mice fed high fat/cholesterol diets)
4. Vortex and spin the standards, controls and unknown samples.
5. According to the places indicated on the work sheet add 10 µl of the standards, controls and unknown samples to the wells of a clear 96 well flat-bottom micro-titer plate.
6. Add 250 µl of reagent to each of the wells to be tested using a repeater pipette.
7. Place the plate in a humidified chamber consisting of a covered Tupper Ware box with wet paper towels on the bottom. Place the Tupper Ware box on an orbital shaker at room temperature for 2 minutes and then in a 37 °C incubator for 30 minutes.
8. Program the location of the standards, controls, and unknown samples on the work sheet into an absorption plate reader (such as Spectra Max 250 made by Molecular Devices).

9. Set the wavelength to 505 nm.
10. Read the plate.
11. Save the results and print the information. The plate reader will calculate the concentration of cholesterol in the unknown samples. If the samples have been diluted, multiply the results by the dilution factor (10 in this case).

Quality control:

12. Check linearity of the standard curve.
13. Check if the concentration of the control agrees with the expected values.
14. Check the duplicates and if they differ by more than 10% re-assay the sample.
15. Samples that exceed the highest standard (200mg/dl) should be re-assayed after dilution (see 3 above).

7. **Non-invasive Blood Pressures** are obtained from the mice using the BP-2000 Blood Pressure Analysis System from Visitech Systems, Inc. (Apex, NC).
8. **Inflammatory Markers**
   Circulation: Mouse plasma adiponectin and leptin concentrations are measured with RIA kits obtained from Linco Research (St. Charles, MO). IL-6 and TNFα concentrations are determined with ELISA kits obtained from BD Pharmingen (San Jose, CA). Plasma PAI-1 levels are measured with an ELISA obtained from Molecular Innovations (Southfield, MI). sICAM-1 concentrations are measured with an ELISA available from Amersham Biosciences (Piscataway, NV) and sVCAM-1 levels are determined with an ELISA obtained from R & D Systems (Minneapolis, MN).
   Tissue Expression: Real-time RT-PCR can be used to quantitative expression of inflammatory genes in blood vessels. Genes include: Osteopontin, PAI-1, I-CAM, V-CAM, TNF-α, Egr-1, tissue factor, CD-68 (macrophage accumulation).
9. **Immunohistochemistry** Characterization of Cellular Components in the Blood Vessel Wall/Atherosclerotic Plaque

1. Mononuclear phagocytes:
Vendor: Serotec
Recommended primary antibody: RAT anti-mouse CD68 MCA1957
Recommended secondary antibody per Serotec: Goat anti-rat IgG (mouse absorbed) - STAR72

2. Smooth muscle cells:
Vendor: Sigma Aldrich
Recommended primary antibody: monoclonal anti-actin, alpha smooth muscle antibody, developed in mouse
Isotype: IgG2a
Product Number: A2547

Recommended secondary antibody: Per consultation with Sigma Aldrich on available products for recognition of the IgG2a isotype

3. Endothelial cells:
Vendor: Pharmingen
Recommended primary antibody: Rat (LEW/Cr1BR)IgG2a,k Anti-CD31

Clone: MEC13.3
Product Number: 550274

Recommended secondary antibody: Per consultation with Pharmingen on available products for recognition of epitopes per the specific rat strain

4. CD4 T cells:
Vendor: Pharmingen
Recommended primary antibody: Rat (Lou/WS1)IgG2a,k Anti-CD4
Clone: H129.19
Product number: 550278

Recommended secondary antibody: Per consultation with Pharmingen on available products for recognition of epitopes per the specific rat strain

10. As a general guideline for AMDCC investigators atherosclerotic lesion analysis should take place at one of 4 different time points depending on the goal of the analysis and the model used:

5. Early lesion evaluation in apo E knockout mice: wean mice at 4 weeks of age onto experimental diet, feed diet for 12 weeks, sacrifice mice and evaluate lesions at 16 weeks of age
6. Early lesion evaluation in LDL receptor knockout mice: wean mice at 4 weeks of age onto experimental diet high in fat and/or cholesterol, feed diet for 16 weeks, sacrifice mice and evaluate lesions at 20 weeks of age
7. Early lesion development in mature LDL receptor knockout mice: wean mice at 4 weeks of age onto chow or low fat low cholesterol diet, feed diet for 8 weeks, switch mice to experimental diet high in fat and/or cholesterol and feed diet to mice for 16 weeks, sacrifice mice and evaluate lesions at 28 weeks of age
8. Advanced lesion evaluation in either apo E or LDL receptor knockout mice: wean mice at 4 weeks of age onto experimental diet high in fat and/or cholesterol, feed diet for 40 weeks, sacrifice mice and evaluate lesions at 44 weeks of age

C. Validation of Animal Models

Primary Screening

1. Presence of insulin resistance and/or diabetes.
   Fasting glucose > 150 mg/dl for diabetes
   Fasting insulin > 0.5 ng/ml for hyperinsulimia
   Confirm with intraperitoneal glucose tolerance test
2. Advanced lesions compared to insulin sensitive, euglycemic control
3. No other significant pathologies present

Secondary Screening

1. Abnormal lipid profile: ↑TG; ↓HDLC
2. ↑ Non-HDLC
3. ↑ inflammatory markers and adipocytokines
4. ↑ Body fat
5. Presence of hypertension
6. Presence of atherosclerotic plaques in smaller and larger vessels: ↑ inflammation,

↑ calcification in lesions

7. Gene profiling to compare with human studies which are ongoing

D. Acceptable model:

Acceptable Animal Models of Vascular Disease in Diabetes

1. Hyperglycemic (type 1 or type 2 diabetes) or insulin resistant animals that develop accelerated atherosclerotic lesions compared to normoglycemic, insulin sensitive controls. Acceleration of atherosclerosis is defined as a 25 percent faster lesion progression.
2. Lesions acceleration must not be solely due to elevated circulating cholesterol levels (could be due to an exacerbation of an insulin resistant or diabetic pattern).
3. Model shows increased size of early lesions, or progression to fibrous plaques, or plaque rupture or occlusion compared to nondiabetic controls. As a secondary criteria, models should show signs of increased inflammation and/or thrombotic tendencies of lesions compared to normal controls.

II. DIABETIC CARDIOMYOPATHY

A. Definition of Human Disease:

In the broadest sense, diabetic cardiomyopathy can be defined as the presence of cardiac contractile dysfunction in the setting of diabetes and in the absence of underlying coronary artery disease and hypertension. In reality, hypertension and coronary artery disease often co-exist and will accelerate the development of cardiac dysfunction. Left ventricular hypertrophy (LVH) and decreased fractional shortening was associated with type 2 diabetes independently of hypertension (Strong Heart Study). Therefore LVH should be included in the definition of the diabetic cardiomyopathy (at least in individuals with type 2 diabetes). The most commonly described feature of diabetic cardiomyopathy is diastolic dysfunction as evidenced by impaired diastolic filling (reduced E/A ratios by echocardiography) or decreased end diastolic volume. Impaired systolic function was described in type2 diabetics (Strong Heart Study) and is more likely to occur in the presence of hypertension. Small numbers of diabetic patients with significant congestive heart failure (in the absence of hypertension and coronary artery disease) have been described and their syndrome attributed to diabetic cardiomyopathy. Histologic changes are not pathognomonic for diabetes and share many features with changes described in patients with hypertensive heart disease or ischemic cardiomyopathy. The findings include cardiomyocyte hypertrophy, replacement and interstitial fibrosis and variable amounts of patchy small vessel disease (intimal and subintimal proliferation, arteriolar thickening and perivascular fibrosis). In individuals with advanced CHF, increased apoptosis of myocytes, endothelial cells and cardiac fibroblasts have been observed.

Definition of Diabetic Cardiomyopathy in mouse models. The presence of LV dysfunction (decreased ejection fraction and/or evidence of diastolic dysfunction such as decreased E/A ratios – relative to age and strained matched controls) in association with interstitial or replacement fibrosis (need not be extensive) in the setting of hyperglycemia and/or insulin resistance. In models of type 2 diabetes the presence of LV hypertrophy (>20% increase in cardiac mass adjusted for tibia length) and histological evidence of myocyte hypertrophy should also be included. (Given the high heart rate of the mouse, it is technically challenging to measure E/A ratios. Thus data from echocardiography and invasive catheterization may need to be combined to confidently diagnose diastolic dysfunction in the mouse heart).

B. Screening:

Metabolic screening (serum insulin, glucose and glucose tolerance tests) will be performed as described above for vascular disease. Echocardiography will be performed to non-invasively determine LV mass, and indices of systolic and diastolic function. Hearts will be excised for the determination of heart weight adjusted for tibia length, and then preserved in buffered formaldehyde for histological analysis (Hematoxylin -Eosin and Masson's Trichrome).

C. Model Validation:

Mice that meet the initial screening criteria (statistically significant reduction in LV function - >10%, increased heart weight tibia length - > 20% for type 2 diabetes models, and histological changes) will undergo additional analysis in order to validate/ verify the existence of cardiomyopathy. (1) Invasive assessment of LV function (by direct trans-carotid LV catheterization). This analysis will give additional information about LV contractile and relaxation parameters, and LV end diastolic pressure. (2) Determination of cardiac contractile function, and pressure volume relationships in Langendorrf perfused hearts (to determine passive elastic properties of the LV, which will be impaired in the face of interstitial fibrosis). (3) Cardiac metabolic parameters in isolated perfused hearts to determine if the characteristic metabolic disturbances of diabetes–decreased glucose oxidation and altered fatty acid utilization are present. (4) Determination of expression levels of genes whose expression is altered in the diabetic heart such as – GLUT4, GLUT1, SERCA-2, α-MHC (decreased), ß-MHC and UCP3 (increased). (5) Evaluation of the cardiac responses to stress. Potential paradigms include: pressure overload hypertrophy induced by aortic banding, ischemia/reperfusion injury in response to coronary artery ligation and catecholamine induced injury achieved by minipump administration of isoproterenol (5day duration). (6) More extensive histological analysis to examine for apoptosis – using immunohistological techniques such as TUNEL assays, terminal deoxynucleotidyl transferase (TDT) or in situ ligation of hairpin probes, and to examine for evidence of lipotoxicity – oil red staining and /or electron microscopy. (7) Transcriptional profiling to obtain expression data to be compared with human data when this becomes available.

D. Methods:

(a) ***Cardiac morphology and histology***. Hearts are briefly perfused with 10% buffered formaldehyde and then are paraffin embedded, sectioned and stained with Hematoxylin & Eosin (general morphology) and with Masson's Trichrome to quantify fibrosis. The degree of fibrosis is quantified using image analysis protocols that quantify the relative abundance of blue staining (representing fibrosis). For immunohistochemistry and oil red O lipid staining, hearts are initially perfused with Krebs Buffer, then rapidly frozen to –80ºC prior to cryosectioning. Heart weights are adjusted to tibia length given differences in body weights between diabetic and control animals. After initial dissection, tissue is removed from the tibia by boiling briefly in 0.1N NaOH. Lengths are measured from the condyles to the tip of the medial malleolus using micrometer Vermer calipers.

(b) ***Mouse echocardiography.*** Transthoracic echocardiography will be performed in awake mice. This protocol maintains heart rates at >600 bpm. Heart rates are generally maintained at > 400/minute with this regimen. The chest hair is removed with a topical depilatory agent. Limb leads are attached for ECG gating and the animals are imaged in the supine position with a 13 MHz linear probe attached to a state-of-the-art echocardiograph (Vivid FiVe ™, Cardiovascular Ultrasound System, General Electric). 2-dimensional images and 2-D guided M-mode images are taken in both short and long axis projections. High 2-D frame rates (up to 600 frames/s) allow adequate temporal resolution to make accurate measurements. LV cavity size and wall thickness are measured in at

least 3 beats from each projection and averaged. LV mass is calculated according to a standard cube formula (100). LV systolic function is assessed by calculating fractional shortening from M-mode tracings and fractional area change or ejection fraction from 2-D images.

(c) ***Measurement of in vivo cardiac contractile performance using a micro-manometer tipped catheter:*** Indices of left ventricular contractile performance are determined in mice by percutaneously introducing a 1.4 Fr. Millar catheter into the carotid artery and gently advancing this into the left ventricular cavity. Pressure waves are recorded captured and analyzed using pClamp 8.2 software. These studies are performed under avertin anesthesia (avertin, 0.2ml/10g body weight); heart rates are generally maintained at > 400/minute with this regimen.

(d) ***Isolated perfused heart analysis:*** These methods have previously been published by members of the University of Utah group (*J. Clin Investigation* 2002; 109: 629-639, *J. Clin Investigation* 1999; 104: 1703-1714).

(e) Ischemia/reperfusion and aortic banding methods have been extensively published.
Following aortic banding, 2 cohorts of mice (10 per genotype) will be followed. In the acute cohort, serial echocardiography will be performed twice per week for 2 weeks after which the mice will be sacrificed. In the chronic cohort, serial echocardiography will be performed weekly for 6 weeks in order to determine if there will be differences in the transition from compensated hypertrophy to failure. Infarct size and post-reperfusion apoptosis will be determined as described (Circulation 2000.101:660-7, Am J Physiol. 1999. 277:H763-9).

E. Acceptable model:

16. Insulin resistant or diabetic animal that develops cardiac hypertrophy, diastolic and/or systolic dysfunction and histological changes of increased interstitial or replacement fibrosis and myocyte hypertrophy compared to insulin sensitive or non-diabetic control. Given the possibility that STZ in high doses may have direct cardiotoxicity, for changes in type 1 models to be deemed specific, they should be present in independent models of islet cell failure such as the NOD or the Akita mouse. Moreover, these changes (if acute) should be reversed or ameliorated by treatment of the diabetes by insulin. It is recognized that chronic changes might not necessarily be reversible.
17. Correction of hyperglycemia, in general, attenuates cardiac changes in type 1 diabetes models.