ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM (P01 DK57733)

UPDATE REPORT

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Project Number and Title: **CORE 1 – "**AMDCC RETINOPATHY CORE**"**

MARCH 2004 CORE REPORT- RETINOPATHY
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Rationale and relevance
Diabetes is the leading cause of blindness in the United States for patients age 20 to 74. This vision loss or impairment usually occurs as a result of proliferative diabetic retinopathy or macular edema. Proliferative diabetic retinopathy causes vision loss as a result of unregulated growth of abnormal blood vessels out of the retina, resulting in hemorrhages and formation of fibrovascular scars. Available evidence suggests that retinal ischemia, caused by increasing numbers of nonperfused capillaries, leads to the production and release of vasoproliferative factors which eventually stimulate retinal neovascularization. Macular edema does not cause total vision loss, but causes a loss of visual acuity.

Diabetic retinopathy is a progressive disorder, and several other lesions that are characteristic of the retinopathy have been found to predict the likelihood of vision loss in diabetic patients. These include the amount of capillary nonperfusion, the number of microaneurysms, and the extent and location of *intra-retinal* neovascularization. Histo-pathologic correlations have demonstrated that many nonperfused capillaries are "acellular"; acellular capillaries were functional capillaries that degenerated until all the remains is a basement membrane tube lacking endothelial cells or pericytes.

Essentially all species tested to date can develop the earliest stages of diabetic retinopathy (acellular capillaries, pericyte loss, capillary basement membrane thickening) if maintained diabetic long enough. However, diabetic animals have not been documented to develop three key features that are integral in the human disease:

1. Pre-retinal neovascularization
2. Retinal edema (only primates have a macula, so macular edema cannot be studied in nonprimates. Retinal edema is believed by some to occur by similar physiologic abnormalities, and to be a possible surrogate for macular edema)
3. Evidence of visual loss or impairment (other than due to cataract)

Mice have only been used rarely in studies of diabetic retinopathy to date, and there has been no systematic characterization of any mouse models of diabetic retinopathy. The development of retinal lesions in diabetic rats has been much better characterized than mouse, but there are a variety of significant metabolic differences between rats and mice which might influence the development of retinopathy. Whether rats or mice are a better model for the series of steps that lead to diabetic retinopathy in humans is not yet clear.

Phenotyping of Mice for the Presence of Diabetic Retinopathy
In man, the development of diabetic retinopathy is dependent on both the degree of glycemic control and the duration of diabetes. The degree of diabetes in mice is evaluated in 2 ways: tail blood glucose and glycated hemoglobin levels.

Electrophysiologic tests of retinal function currently are the only way available to gauge visual function in mice, and this can be measured at multiple timepoints while animals are alive. Retinal thickness might be measured noninvasively as a surrogate for retinal edema, as is being done currently

with patients. High-resolution optical coherence tomography can measure the retinal thickness noninvasively in humans, but this has not yet been reported using mice. Alternate methods to assess retinal edema are terminal procedures, and include lyophilization of the isolated retina to determine water content, or measuring vascular permeability as a surrogate. Assessment of neovascularization and retinal ischemia are very difficult noninvasively in the mouse. Fluorescein angiography is used to document neovascularization in patients, but the small size and dramatic curvature of the mouse eye make this very difficult to do reliably and reproducibly. Histologic assessment of pre-retinal neovascularization is a quantitative and relatively easy procedure, and gives positive identification of the number and location of the new vessels. Additionally, measurement of the number of acellular (nonperfused) retinal capillaries gives a quantitative surrogate parameter that should predict the progression to the advanced, neovascular stages of the retinopathy.

Thus, evaluation of electrophysiology and retinal thickness might be made at multiple time points while animals are alive, but assessment of changes in the retinal vasculature should be done when animals are killed after long durations of diabetes. Six months of diabetes is the first time that histologic evidence of the retinopathy (number of acellular capillaries) becomes significantly greater than normal in diabetic C57Bl/6 mice in our hands.

I propose to focus initially on the measurements that can be taken after animals are killed, and that animals not be killed before 6 months of diabetes. The most important contribution of a retinopathy core facility would be assessment of histological changes of the retinal vasculature for features of advanced diabetic retinopathy (intra-retinal microvascular abnormalities (IRMA) and pre-retinal neovascularization) or accelerated development of lesions characteristic of the "background" stages of the retinopathy (extensive formation of acellular capillaries, pericyte ghosts, microaneurysms). Studies of retinal electrophysiology and retinal edema will be valuable, but they likely will require that living animals be sent to a core laboratory for study, and those methods or interpretation of their results need to be better worked out first. Our studies to date indicated that apoptosis of capillary cells (as assessed by the TUNEL technique) is strongly associated with and precedes the development of retinopathy, and precedes the appearance of acellular capillaries and pericyte ghosts by a number of months, so I include it as option as well.

Vision loss also might be accounted for by loss of retinal neurons, such as ganglion cells. Nonvascular cells of the retina, including ganglion cells, have been found to undergo apoptosis in diabetic rats before appearance of accepted vascular markers of the retinopathy in the same animals. One manuscript recently failed to find this in diabetic mice, but the duration of diabetes was only a few months. We will evaluate if there is neuronal cell loss in the retinas of diabetic mice that we analyze.

Analysis of the trypsin digest preparation of the isolated retinal vascular is labor-intensive. For the calculation of initial budget that we received from this core facility, we planned to analyze retinas from 14 different models during this year. These measurements of retinopathy are expected to be completed at no cost to AMDCC members. Analyses will be conducted on a first come, first served basis.

Summary of Accomplishments

Mouse studies

C57Bl/6 mouse. We are just completing our initial study of the natural history of diabetic retinopathy in the C57Bl/6 mouse. The retinal vasculature was isolated using the trypsin digest method. At 6 months of diabetes, we detected a significant increase in the number of acellular capillaries and

TUNEL-positive (apoptotic) capillary cells in diabetic animals compared to age-matched nondiabetic mice. The microvascular lesions became progressively more numerous at 12 and 18 months of diabetes, but there was no evidence of pre-retinal neovascularization at any duration. Lesions of retinopathy were not significantly increased above normal after 4-5 months of diabetes, demonstrating that duration of diabetes is important, as it is in other species. There was no evidence of neurodegeneration (loss of retinal ganglion cells) at 6 months of diabetes, and there was a slight decrease in the b-wave of the electroretinogram at 3 months of diabetes. We will be analyzing the electroretinogram and looking for neurodegeneration at longer durations of diabetes in the near future.

Akita mouse. Retinal histopathology in the spontaneously diabetic Akita mice has been studied at 6 months of diabetes, and will be studied also at 12 months in the near future. Acellular capillaries and pericyte ghosts were statistically greater than normal at 6 months of diabetes (but just barely).

A/J mouse. This inbred mouse has been made diabetic, and will be killed to assess retinopathy initially at 6 months of diabetes. This strain is of interest because a colleague at my institution (J. Nadeau) has generated a group of mouse models in which every chromosome has been swapped (one at a time) between A/J and C57Bl/6 mice. We anticipate that this model may be valuable to begin to dissect the genetic basis of diabetic retinopathy, especially if the A/J develops retinal lesions significantly faster than the C57Bl/6.

iNOS knockout mouse. Animals have been made diabetic, and are about to be killed after 6 months of diabetes for assessment of susceptibility to develop retinal lesions.

bcl2 transgenic (endothelial specific) mouse. This model has been created by over-expressing *bcl2* under the preproendothelin promoter. Animals now have been killed, and retinopathy is being quantitated now after 8 months of diabetes.

Rat studies

Previously we learned that diabetic Lewis rats develop acellular capillaries and pericyte loss significantly faster than diabetic Sprague Dawley rats. Lewis rats had a significant increase in lesions at 8 months of diabetes (compared to their nondiabetic controls), whereas Sprague Dawley showed no increase at all at that duration. We have now cross bred these two strains, made the F2 generation (n=100) diabetic, and killed them after 8 months of diabetes. In the next year, we will do trypsin digests to assess pathology in these offspring. Having that data, personnel from our Genetics department will do gene scans on tissue saved from each animal to determine which genes are best associated with the accelerated (or resistance to) development of lesions of diabetic retinopathy.

Plans for the coming year

1. Discuss with AMDCC personnel regarding setting up the database to import the types of data you will generate, and to train personnel in my lab how to transfer data to the database immediately after we are done with analysis.

2. Establish detailed validation criteria for murine models of diabetic retinopathy to be added to AMDCC website. A draft of this will be prepared for the March AMDCC meeting.

3. Post procedure for how to isolate and send eyes to my lab on the website. Contact individual PIs about what models they are studying, and what ocular tissues might be available.

4. Evaluate retinopathy for other PIs.

Eyes already received:

decorin knockout (K. Sharma)

Investigators who have expressed interest in sending eyes within the coming year:

Feldman
Brosius
Danesghari
Gerrity

5. Finish evaluating retinopathy for animals in my lab.

Akita, A/J, iNOS knockout, *bcl2* transgenic (endothelial specific), IL-1 receptor knockout

6. Other mouse models that I am especially interested in getting from others:

db/db,aldose reductase transgenic, superoxide dismutase knockout, RAGE transgenic

7. Assess electroretinogram measurements in diabetic mice, and attempt to measure retinal thickness noninvasively by optical coherence tomography. Correlate this measurement with retinal thickness measured in fixed and embedded tissue sections.

8. Do trypsin digests to assess pathology in F2 generation of rats, and then collaborate on gene scans to determine which genes are best associated with the accelerated (or resistance to) development of lesions of diabetic retinopathy in rats.

Achievements within the last 6 months.

We have begun to make arrangements to assess retinopathy for several AMDCC PIs.

Procedure for other labs to collect eyes for assessment of retinal vascular disease

To evaluate retinopathy in animal models that other investigators develop, we will need to receive both eyes from at least 5 and preferably 10 experimental animals, and an equal number of age-matched normal controls. The controls are essential to include because many of the lesions characteristic of the retinopathy are found also in nondiabetic animals and humans (although quantitatively fewer). Duration of diabetes should be 6 months or longer.

Carefully remove the eyes as described below, and send immediately to us in 10% buffered formalin (pH 7.4). We would need to receive both eyes within about 4 days of death and fixation if apoptosis is to be assessed (if apoptosis is not to be assessed, the duration in formalin is not important). We will then log the samples in, isolate the retina from one eye, prepare trypsin digest quantitate the lesions (acellular capillaries, pericyte ghosts, microaneurysms, IRMA). On the other eye, we would dehydrate, embed in paraffin, cut cross-sections of retina, stain with conventional stains, and count pre-retinal capillaries (new vessels) and number of ganglion cells.

1. What we want:

Both eyes in formalin from at least 5 animals per experimental group. The longer the duration of diabetes, the better (C57bl/6 mice require at least 6 mos diabetes before we can detect retinal microvascular lesions). Include also age-matched wildtype nondiabetic controls and diabetic controls.

2. Eye collection

All tissue needs to be collected fresh (within 10-15 min of death) to get best results. Tissue post-mortem more than 1 hour does not work!

- Anesthetize animal (we have not yet found that any particular anesthetic is better or worse than others).
- Place thumb and pointer finger on either side of eye. By spreading thumb and pointer finger, stretch the skin around the eye.
- Using fine, sharp scissors, make 3-5 deep (5-10mm) cuts while forcing scissors into the eye socket. The eye now should be attached only by the optic nerve leading into the brain. Push the scissor back into the eye socket again to cut the nerve, so that there is some optic nerve still attached to the eye. **Do not** push on the skull to protrude the eye and then cut horizontal across the skull; this can damage the eye. Be careful not to push or compress the eye.
- Drop both eyes into 10% buffered formalin (pH 7.4) in a microfuge tube. Variances in pH seem to influence how well the tissue can be stained later, so check the pH (especially if the fixative is prepared in your lab). Label each tube with animal number. Also send a summary sheet listing all animals and experimental description with date of death; please put your name and email address on the sheet as well. Group identifications need not be on this sheet until analysis is completed. Parafilm each tube to minimize the likelihood of leaks, and put tubes in a zip-lock bag.
- We will need to receive both eyes within about 4 days of death and fixation if apoptosis is to be assessed (if apoptosis is not to be assessed, the duration in formalin is not important). Please give me a few days warning that tissue about when tissue will be shipped so that we can watch for it. Email tracking number once it is sent.