Animal Models of Diabetic Complications Consortium
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Annual Report
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Project Title: Creating glucose responsive cardiovascular complications in the mouse

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Part A: Principal Investigator's Summary

This Project has led to a continued intellectual and experimental collaboration between the two PIs and their laboratories. This has included meetings approximately every 6 weeks and weekly phone communications. Thus, both major projects are proceeding and being critiqued by the PIs and an outside reviewer, Dr. Jan Breslow (Rockefeller University).

The Project includes methods to produce and evaluate mouse models of two major cardiovascular complications of diabetes: atherosclerosis and heart failure. Studies were performed to evaluate the effects of high fat versus high cholesterol diets on insulin actions and atherosclerosis in LDL receptor knockout (*Ldlr-/-*) mice. In addition, overexpression of aldose reductase, an atherosclerosis-exacerbating gene, was studied in this context.

Atherosclerosis and its pathophysiology is being evaluated in two ways. Dr. Fisher's laboratory has developed methods to study atherosclerosis regression in mice. As proposed in the application, the surgical artery transplant techniques are being complemented with methods to reverse hyperlipidemia in mice. Using adenoviral gene transfer, the Fisher laboratory is establishing techniques and the chronology of vascular change that occurs when LDL receptors are expressing in hypercholesterolemia *Ldlr-/-* mice that have established atherosclerosis.

To study the effects of the expression of aldose reductase, the investigators have made several constructs to create new transgenic mice. These include a tet-on-off system to express aldose reductase in specific tissues in a chronological manner.

To evaluate the effects of aldose reductase on heart function, a new line of transgenic mice in which aldose reductase is expressed in cardiomyocytes via the myosin heavy chain (MHC) promoter was created. These mice are currently being bred and will be phenotyped in the coming year.

1. Project Accomplishments (2008)

Aim 1 creation of new mouse models of diabetic cardiovascular disease:
Production of an Inducible hAR mouse: We completed the construction and testing of the two plasmids to make a transgenic mouse in which AR expression can be induced by adding tetraclycine to the drinking water ("tet-on" system). The separate plasmids are called rtTA (which produces the transcription factor that activates the second plasmid) and TRE/hAR (which will express human AR when activated). Before sending the plasmids to Jackson Lab, cells were co-transfected with both plasmids, or as a control with only the TRE/hAR plasmid (to test for "leaky" expression), or no plasmid. All cells were incubated with tetraclycine. The no and single plasmid cells had barely detectable enzymatic activities for hAR. In contrast, the double plasmid cells had ~ 10X as much hAR mRNA, protein, and enzymatic activity than the other two groups of cells. With these encouraging results, the plasmids were sent to Jackson Labs to make transgenic mice on the *Ldlr-/-* background.

MHC-hAR mice: The second proposed mouse model was created. An MHC promoter was placed 5' to the hAR cDNA, injected into mice and two lines of transgenic animals were produced. These mice have a >20 fold increased in hAR expression and this increase is localized to the heart. The two lines are being characterized for hAR activity, effects on cardiac function as the mice age, and influence of diabetes. Initial characterization shows that MHC-hAR mice have increased heart failure markers, ANF and BNP, compared to littermate controls. Studies of the effects of STZ-induced diabetes on this background are in progress. If, as expected, the animals develop cardiac pathology, they will be sent to the MGHC for breeding.

Aim 2 to study the development of vascular lesions in diabetic mice
Non-surgical model of regression: We have tried 2 approaches. In the first, we treated *Ldlr-/-* mice with a helper virus-dependent adenovirus containing an expression cassette for the LDL receptor. This virus was given to us by Dr. Larry Chan, Baylor College of Medicine and had been previously shown to lower LDL cholesterol (LDL-C) levels to near normal after ~10 days after administration to the mice. The lowering was sustained for months as reported by Dr. Chan's laboratory. We confirmed the LDL-C lowering, finding that by 5 weeks after adenovirus injection the LDL-C went from ~1200 (on a high cholesterol diet) to 150 mg/dL (n=10). Associated with this was a reduction in macrophage content at the aortic root of over 50%. Thus, this approach appears to be suitable to using STZ to induce hyperglycemia concurrent with virus injection to determine whether glucose levels in the 400-500 mg/dL range impair the loss of macrophages. We used another variation of the *Ldlr-/-* mouse, the "Reversa mouse" to test another non-surgical approach. In this mouse, a "genetic switch" is thrown, which reduces VLDL secretion and LDL-C levels to normal over the course of 7-10 days. At the time the switch was thrown, half of the mice were treated with STZ. The resulting hyperglycemia was associated with ~3X more macrophages in aortic root plaques than in the normoglycemic mice, suggesting the loss of the benefits of reversing the hyperlipidemia. By applying laser capture microdissection and gene expression analyses, we have also found that hyperglycemia increased macrophage expression of a number of inflammatory factors, such as VCAM, ICAM, MCP-1, as well as of an ER stress responder (CHOP). Overall, then, the

feasibility of non-surgical models to test some of the hypotheses proposed in the grant application has been clearly established.

Surgical models of regression: Some aspects of the originally proposed studies are better suited to the surgical (transplantation) model. In particular, the use of the Akita mouse to provide a hyperglycemic plasma environment avoids toxicity of STZ, but is more conveniently suited to be a recipient of an aortic segment containing a plaque from a *Ldlr-/-* mouse. To get all of the genotypes proposed in the application (combinations of Akita, *Ldlr-/-*, hAR), extensive breeding still needs to be completed. The appropriate matings and selection by genotyping are ongoing.

HAR and atherosclerosis progression: Ongoing atherosclerosis progression studies have assessed the effects of diet, Akita, and aging on atherosclerosis progression in the hAR-*Ldlr-/-* background. Young *Ldlr-/-* mice ± hAR were begun on a high fat diet to create insulin resistance or a high cholesterol-only diet. The mice developed hypercholesterolemia, however, the levels of hyperglycemia and insulin resistance were modest. In this setting neither high fat/insulin resistance nor the introduction of hAR altered atherosclerosis progression. In a second study, hAR/Ldlr-/- was crossed onto the Akita background. The presence of Akita led to a 2 fold increase in plasma cholesterol levels which averaged ~1500 mg/dl. After 20 weeks, these mice have extensive lesions, the lesions were greater in the diabetic more hyperlipidemic mice, but hAR did not alter lesion size in these mice with very extensive atherosclerosis.

A study is underway to test whether high fat insulin resistance is greater in older mice. As was reported to the AMDCC group by Dr. Hsueh, we found that feeding a high fat diet to mice that were greater than 6 months old led to much greater plasma cholesterol levels and more insulin resistance. We are testing whether hAR will alter lesion size in this model.

Finally, in an effort to understand how diabetes alters plasma lipids, we studied the effects of STZ-diabetes on parameters that affect the metabolism of plasma lipoproteins. Diabetic mice ate approximately 60% more diet than did non-diabetic controls on the same 0.15% cholesterol diet. However adjusting the diets by lowering the cholesterol to 0.075% thereby reducing the cholesterol intake of the STZ-treated mice, did not reduce plasma cholesterol to non-diabetic mouse levels. Kinetic studies showed that STZ-treatment did not increase lipoprotein production. Rather initial decay, associated with lipoprotein trapping, was reduced.

2. Collaborations:

Within the AMDCC: The NYC Project has developed a on-going collaboration with Dr. Abel to assist with the evaluation of diabetic cardiomyopathic mice. Animals with lipid-induced cardiomyopathy have been sent to the University of Utah and glucose and fatty acid oxidation in isolated perfused hearts have been studied. When additional data are obtained on the MHC-hAR mice, these animals will also be studied as part of this collaboration.

We are in discussion with both the AECOM and U of Colorado groups to design studies to test the roles of lipid uptake into kidney proximal tubules.

With Jax: Vectors to produce the inducible hAR mice have been sent to Jackson Laboratories.

With the MMPCs: Although we have had no formal use of the MMPCs, along with Dr. Abel studies are planned with the UTSW laboratory to develop methods to simultaneously assess the contributions of glucose and fatty acids to TCA cycle intermediates using labeled isolated perfused hearts.

Outside the AMDCC: An ongoing collaboration has continued with Dr. Breslow, Rockefeller U. Dr. Breslow attends the joint data presentation meeting with Drs. Goldberg and Fisher. In addition, his laboratory created the Akita/Ldlr-/- mice ± hAR and studies to assess the effects of diabetes on atherosclerosis and regression in the model are continuing.

Dr. Fisher has established a collaboration with Dr. L. Chan (Baylor) to utilize helper dependent adenoviral infection to reverse hypercholesterolemia and atherosclerosis in Ldlr-/- mice.

3. Address previous EAC comments

The constitutive hAR Tg mice should be reposited with JAX by Spring 2008.
This will be done.

Results were presented that the hAR-/-/LDLR-/- mouse made diabetic by STZ has no difference in atherosclerosis but has an effect on survival. It would be interesting to follow-up on this observation to determine what the mechanisms of death is - potentially thrombosis? Cardiac arrhythmia?
The mice develop several vascular events including bowel infarction and hemoplegia presumably due to spinal artery occlusions.

What effect does the fructose diet have on the development of IR and DM in the hAR-/- mice?
This experiment has not been done. Fructose feeding increases cholesterol in mice models. It is possible that fructose will reproduce the abnormalities seen with hAR and diabetes. In no other models does fructose feeding lead to diabetes or insulin resistance.

Dr. Fisher presented data to indicate that cultured macrophage hypoxia may be one of the mediating factors in the accelerated atherosclerosis. However, one question is whether they will be able to differentiate an effect of hypoxia vs. oxidant stress (We believe the proposal was made that oxidant stress induces hypoxic genes). In addition

to monitoring hypoxia *in situ*, expression of hypoxia-inducible genes other than VEGF would be helpful.
Both hypoxia and aldose reductase increase oxidant stress. Because plaques have hypoxic areas, we hypothesize that diabetic hAR-expressing foam cells will have even greater ROS. We intend, as suggested by the EAC, to monitor the separate effects of each of these factors and their combination. In addition to VEGF, our read-outs will be other known factors upregulated by hypoxia, such as GLUT1, HIF-1alpha, TNFα, PAI-1, and PGK. In addition, we will assess ROS by measurement of glutathione and its regulating enzymes, peroxynitrate, TBARs (MDA in tissues) and DCF fluorescence (as an indicator of H_2O_2 production by NADPH oxidase). These assays will be performed in both tissue and in cell culture models of foam cells (with and without hAR expression) under normoxic and hypoxic conditions.

4. Publications:

Hsueh, W, Abel ED, Breslow JL, Maeda N, Davis RC, Fisher EA, Dansky H, McClain DA, McIndoe R, Wassef M K, Rabadan-Diehl C, Goldberg IJ. *Circ Res* 100, 1415-1427, 2007

Dansky, HM, IJ Goldberg. Effects of diabetes on murine lipoproteins and vascular disease. Curr Drug Targets 8:1196-202, 2007

Goldberg IJ, Y Hu, J Wei, LA Huggins, MG. Rackmill, H Hamai, BN Reid, WS Blaner L-S Huang. Decreased lipoprotein clearance is responsible for increased cholesterol in streptozotocin treated LDL receptor knockout mice, Diabetes, accepted