Animal Models of Diabetic Complications Consortium
(U01 HL70524)

Annual Report
(2005)

Mouse Models of Diabetic Vascular Disease
Rockefeller/Columbia/NYU

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Submitted February 2006

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

The overall goal is to create mouse model in which diabetes worsens macrovascular disease. Our main strategy is to create mice with diabetic dyslipidemia and then introduce hyperglycemia or insulin resistance or both and assess effects on atherosclerosis progression and regression and arterial response to injury. Our main animal model is the C57BL/6J LDLR-/- mouse fed the AIN76a diet containing 0.02% cholesterol. Hyperglycemia, induced by streptozotocin treatment in earlier studies, will be achieved by breeding in the Pdx1+/- and $Ins2^{Akita/+}$traits; insulin resistance by breeding in the IRS1-/- and Akt2-/- traits; hyperglycemia plus insulin resistance by breeding in the Ob/Ob trait. As part of our group's efforts, Dr. Goldberg showed that streptozotocin induced hyperglycemia in the C57BL/6J LDLR-/- mouse failed to accelerate atherosclerosis. However, if the mice were sensitized by the presence of the Human Aldose Reductase Transgene (HuARTg), hyperglycemia could accelerate atherosclerosis. Therefore, it appears that our basic model requires the presence of a sensitizer and two are being compared, the HuARTg and SOD2+/- traits.

Experimental results to date have eliminated some of the possibilities enumerated above. The $Ins2^{Akita/+}$ trait resulted in more consistent and higher glucose levels than the Pdx1+/- trait, and in future experiments hyperglycemia will be produced solely with the $Ins2^{Akita/+}$ trait. We found that homozygosity for the IRS1 knockout trait was lethal on the pure bred C57BL/6J background, and have abandoned this model of relatively pure insulin resistance. We also found that the C57BL/6J LDLR-/- Ob/Ob mice are insulin resistant but males were not hyperglycemic and females only mildly so. Thus this model can be used to study relatively pure insulin resistance and if necessary hyperglycemia can be added by breeding in the $Ins2^{Akita/+}$ trait. We also acquired Akt2 knockout mice as another model of relatively pure insulin resistance. However, the mice we were able to obtain were out bred. We have succeeded in backcrossing the AKT2 knockout trait on to the C57BL/6J background, but will not have the time to test its effect on atherosclerosis by breeding it to the LDLR-/- background. We will archive these mice for others who might want to pursue this line of research. We are still experimenting to determine which sensitizer, HuARTg or SOD2+/-, is better. Ultimately we will focus our studies on C57BL/6J LDLR-/- mice made hyperglycemic with the $Ins2^{Akita/+}$ trait and/or insulin resistant with the ObOb trait, and sensitized with either the HuARTg or SOD2+/- trait.

Major achievements have been:

Project 1: Dr. Stoffel has demonstrated that Pgc1b is a potent and specific cofactor of Foxa-2 and that the concerted actions of these factors regulate β-oxidation, ketogenesis and apoM synthesis. They also regulate VLDL secretion by stimulating the expression of MTP and DGAT2. This pathway is inhibited by insulin signaling (via PI3kinase and AKT2) and it offers an explanation for the long sought mechanism by which insulin inhibits VLDL secretion.

Project 2: Dr. Goldberg has succeeded in developing a model of diabetes-accelerated atherosclerosis. This occurs when a human transgene for AR is introduced in the presence of hyperglycemia. He has hypothesized that AR replacement is necessary for mice to replicate human

pathophysiology. This model will allow testing of methods to reduce disease by pharmacological and dietary approaches.

Project 3: Dr. Breslow has called into question the simple notion that mere hyperglycemia promotes atherosclerosis susceptibility. He has also made significant progress in establishing a platform model to test the effects of hyperglycemia, insulin resistance, and potential glucotoxicity sensitizers on atherosclerosis progression. His next set of studies should reveal the best model(s) for studying diabetic macrovascular disease.

Project 4: Dr. Fisher has shown that regression of atherosclerosis in a mouse model requires the function of the dendritic cell-related factor CCR7 and that foam cells can be depleted from even advanced plaques by reversing hyperlipidemia and that this process is independent of mild hyperglycemia.

Project 5: Dr. Dansky has used a variety of mouse models of vascular disease (i.e. femoral artery injury model, vein graft model, examination of isolated vascular rings) and has demonstrated that the effect of diabetes on vascular disease is highly dependent on the vascular model and the method of diabetes induction. These studies suggest that a specific set of diabetes associated metabolic abnormalities underlie each vascular phenotype.

2. Collaboration within your group:

The Rockefeller/Columbia/NYU AMDCC group meets bimonthly to present progress, exchange ideas and to promote collaborations. Dr. Goldberg's hypothesis that the mouse requires a sensitizer to develop diabetic macrovascular disease has resulted in close collaboration with Dr. Breslow. The 2 groups will further refine the model to examine whether or not genetic forms of hyperglycemia can substitute for STZ induced diabetes. In addition, the groups will explore another sensitizer, the SOD2+/- trait. Drs. Breslow and Fisher are collaborating closely to adapt as many of the atherosclerosis progression models for study of regression by Dr. Fisher. Drs. Stoffel, Breslow, Fisher and Dansky will adapt the new models of insulin resistance developed by Dr. Stoffel for studies of atherosclerosis progression and regression as well as arterial response to injury. Dr. Stoffel has been the principal consultant to the entire group on mouse models of diabetes. In addition, Drs. Goldberg and Breslow have provided expertise on mouse models of hyperlipidemia. Drs. Goldberg and Fisher are collaborating on studies of atherosclerosis regression and jointly applying for continued AMDCC funding.

3. Collaboration with other AMDCC groups:

As models are finalized, tissues are being shipped to other AMDCC core laboratories for evaluation of retinopathy, nephropathy, uropathy, cardiomyopathy and neuropathy,. In addition, extra mice are being bred for shipment to AMDCC core laboratories for functional studies pertaining to uropathy, cardiomyopathy and neuropathy. For example, tissues from the C57BL6J LDLR-/- Ob/Ob study have been shipped and additional mice are now being bred for shipment and functional studies.

4. Pertinent non-AMDCC Collaboration:

Drs. Goldberg and Breslow have been collaborating with Dr. Mike Brownlee of Albert Einstein College of Medicine to determine the best sensitizer traits to breed on to the C57BL/6J LDLR-/- model to promote diabetic macrovascular disease. In addition, Dr. Fisher has been collaborating with Dr. Robert Raffai of UCSF to develop a non-transplant model of lesion regression. They are using the "apoE hypomorphic" mouse developed by Dr. Raffai (Raffai, R., Loeb, S., Weisgraber, K. Apolipoprotein E promotes the regression of atherosclerosis independently of lowering plasma cholesterol levels. ATVB 25:436, 2005). Dr. Breslow is collaborating with the Laboratories of Mike Rosenfeld in Seattle and Renu Virmani in Gaithersberg Maryland to improve the staining and immunohistochemistry of lesions to better assess qualitative differences in lesions between models.

5. Address previous EAC comments:

Phenotyping existing models: There are 3 major ongoing experiments as shown in the Feldman tables: All animals are C57BL/6J LDLR-/- fed AIN 76a 0.02% cholesterol. Exp #1 +/+ vs. Ob/Ob at 20 and 32 weeks, kidneys and eyes have been shipped and extra animals are being bred for shipment for functional bladder, heart and nerve conduction studies. Exp #2 wild-type vs. $Ins2^{Akita/+}$ vs. HuARTg vs. $Ins2^{Akita/+}$ HuARTg. Exp #3 wild type vs. $Ins2^{Akita/+}$ vs. SOD2+/- vs. $Ins2^{Akita/+}$ SOD2+/-. Eyes and kidneys will be harvested from mice in Experiments #2 and #3 and shipped for evaluation and extra mice will be sent for functional studies of the bladder, heart and nerve conduction.
A paper by the CV working group is in preparation.
Validation criteria for diabetic retinopathy and uropathy will be established by experts in those areas. We have a necropsy protocol for our laboratory and will work to make uniform one for the consortium.
All of our experiments have analyzed male and female mice separately.
In the C57BL/6J model chow does not raise cholesterol levels sufficiently to induce the development of atherosclerotic lesions. We have chosen to use the AIN76a semi-synthetic diet with 0.02% cholesterol to raise cholesterol levels and induce lesions. Other diets can be used as well including low fat (chow)-high cholesterol and high fat-high cholesterol.
The data we have uploaded is compatible with other data bases.
We are now trying to further up-grade the quality of our staining and immunohistochemistry of lesions by collaborating with the Laboratories of Mike Rosenfeld in Seattle and Renu Virmani in Gaithersberg Maryland. This is an ongoing process.
Kidneys are currently harvested, frozen and shipped to the Nephropathy core lab.
We are not working with the C57BL/6 tg GLUT, db/db mice.

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Part B:

Update by Project Leaders

Project 1: "Creation of New Mouse Models of Diabetes"

Responsible Investigator: Dr. Markus Stoffel

Rationale and Relevance: In recent years, research has identified specific effects of hyperglycemia and insulin resistance on the vasculature of the diabetic patient. Atherosclerosis is known to develop earlier in the diabetic patient and is more aggressive due to the metabolic effects of hyperglycemia and insulin resistance. The results of many large, randomized, prospective trials have provided practice changes in the management of the patient with diabetes. Trials such as the Framingham Study identified risk factors associated with atherosclerosis. Additional studies, such as the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study, provided information about risk factors for diabetes and contributed to treatment recommendations for the person with type 1 or type 2 diabetes. In spite of these advances the molecular etiology of the increased atherosclerosis susceptibility in patients with diabetes remains poorly understood. The goal of our study is determine factors/genes that promote vascular disease by generating genetic mouse models in which insulin resistance and hyperglycemia can be modified. These studies will ultimately lead to a molecular understanding of the role of insulin resistance/hyperglycemia in atherosclerosis development in type 2 diabetes and may facilitate rational designs for preventive/ therapeutic clinical trials in humans.

Summary of Accomplishments: We have followed up on our observation that Foxa2 is inactivated by nuclear exclusion following activation by the insulin/PI3kinase/Akt pathway *in vitro*. In previous studies we have shown that in normal mice, plasma insulin inhibits the forkhead transcription factor Foxa2 by nuclear exclusion and that in the fasted state Foxa2 activates transcriptional programs of β-oxidation, ketogenesis and glycolysis. In diabetic animals models of type 2 diabetes (e.g. ob/ob, aP2-Srebp-1c or diet-induced obese mice), Foxa2 is inactive and permanently located in the cytoplasm of hepatocytes. In these animals, adenoviral expression of Foxa2T156A, a nuclear, constitutive active Foxa2 that cannot be inhibited by insulin, decreases hepatic triglyceride content, increases hepatic insulin sensitivity, reduces glucose production, normalizes plasma glucose levels and significantly lowers plasma insulin concentrations. These changes are associated with increased expression of genes encoding enzymes of fatty acid oxidation, ketogenesis and glycolysis. These results indicated that activation of Foxa2 is responsible for a significant proportion of the response to starvation and that chronic hyperinsulinemia in insulin resistant syndromes leads to insulin resistance, increased lipid accumulation and glucose production in the liver. During the last year we have followed up the characterization of the above animal models. The pertinent findings are summarized below:

1. Insulin regulates VLDL synthesis/secretion through Foxa2/Pgc1β

Activation of Foxa2 in the liver leads to increased β-oxidation and secretion of fatty acids in the form of triacylglycerols (TAGs), a process impaired in type 2 diabetes. Using a genetic and biochemical approach, we have demonstrate that Foxa2 is coactivated by PPARgamma coactivator beta (Pgc-1β). Adenoviral expression of Foxa2 and Pgc-1β in livers of ob/ob mice results in decreased hepatic TAG content and increased plasma TAG concentrations. In addition, the concerted action of Foxa2/Pgc-1β activates genes in mitochondrial β-oxidation and enhances fatty acid metabolism. Furthermore, Foxa2/Pgc-1β induce the expression of microsomal triglyceride transfer protein (MTP), thereby increasing VLDL secretion. This process is inhibited by insulin through a Foxa2-dependent

mechanism. These data demonstrate that Foxa2/Pgc-1β regulate hepatic lipid homeostasis by affecting the clearance rate of fatty acids through oxidation and/or secretion of lipids in response to insulin.

2. Foxa2 regulates apoM synthesis, secretion, plasma preβ-HDL levels and reverse cholesterol efflux.

The metabolic syndrome is characterized by constellation of insulin resistance, hypertension, low plasma HDL and increased TAGs levels. The molecular mechanisms by which insulin resistance causes low plasma HDL levels are poorly understood. We have shown that Foxa2 is a potent activator of apoM expression, a major deteminant of HDL and preβ-HDL levels. Insulin-resistant mice (ob/ob, aP2-Srebp-1c), which have permanently inactivated Foxa2 due to nuclear exclusion, were treated with Ad-Foxa2T156A, a constitutive active form of Foxa2. Expression of this constitutively active form of Foxa2 in these models led to a 6-fold induction of apoM expression, a 2-3-fold increase in preβ-HDL and a 50% increase in plasma HDL levels. Furthermore, HDL particles of ob/ob mice treated with Ad-Foxa2T156A were significantly more potent acceptors of ^{14}C-cholesterol in reverse cholesterol assays using cholesterol-laden macrophages compared to HDL from ob/ob mice treated with Ad-GFP. We also showed that the transcriptional activation of apoM by Foxa2 is mediated by a highly conserved HNF3 binding element in the apoM promoter. Together, these data demonstrate that Foxa2 is a major regulator of HDL levels through the regulation of apoM expression.

Plans for the coming months: We will continue charcterizing mouse models with conditional Foxa2T156A and dominant negative Foxa2 alleles. These alleles have now been back-crossed on to a C57/B6 background (7th generation) as well as on to an Ob/Ob mutant background. These mice will be characterized for insulin sensitivity, glucose homeostasis, and lipid metabolism.

Most significant achievement: We have demonstrated that Pgc1β is a potent and specific cofactor of Foxa-2 and that the concerted actions of these factors regulate β-oxidation, ketogenesis and apoM synthesis. They also regulate VLDL secretion by stimulating the expression of MTP and DGAT2. This pathway is inhibited by insulin signaling (via PI3kinase and AKT2) and it offers an explanation for the long sought mechanism by which insulin inhibits VLDL secretion.

Publications: Wolfrum, C and Stoffel, M. Coactivation of Foxa2 through Pgc-1β promotes liver fatty acid oxidation and triglyceride/VLDL secretion. *Cell Metabolism* **3**:99-110, 2006.

Project 2: "Creation of New Mouse Models of Diabetic Dyslipidemia"

Responsible Investigator: Dr. Ira Goldberg

Rationale and Relevance: The overall hypothesis of this project is that mice do not develop diabetic macrovascular due to a genetic deficiency that prevents the full vasculopathic impact of diabetes. Initially, we hypothesized that this might be due to the failure of mice to develp the diabetic dyslipidemia found in most patients with type 2 diabetes. Although we performed several experiments to produce a diabetic dyslipoproteinemia-like profile, we were unable to show that this altered atherosclerosis. We subsequently have developed data showing that transgenic expression of aldose reductase (AR) leads to greater atherosclerosis in diabetic mice.

Summary of Accomplishments:

A. Effects of aldose reductase (AR) expression: We completed a study using a line of mice expressing a human AR (hAR) transgene driven by a mouse histocompatibility gene promoter. This transgenic mouse line was crossed onto both the LDLR-/- and LDLR+/- backgrounds and the mice made diabetic with STZ. We determined that the hAR transgene was expressed in mouse heart and macrophages at levels between non-transgenic mice and comparable human tissues. We concluded from this that the transgene was restorative of AR expression and did not result in non-physiological levels of the enzyme.

The expression of hAR increased atherosclerosis in diabetic mice with total and partial deficiency of the LDL receptor. hAR did not alter disease in non-diabetic mice. Studies were initiated to assess the reasons for the increased disease. We reported that macrophages from hAR expressing mice had increased expression of several inflammatory genes. In addition, gene expression and protein levels of two scavenger receptors, SR-A and CD36, were increased as was the uptake of the modified lipoprotein acetyl LDL). Moreover, we studied homogenates of aortas from control and diabetic hAR/LDLR-/- mice and found a reduction in glutathione and glutathionine regenerating enzymes in the diabetic hAR expressing vessels.

B. Effects of high fat versus only cholesterol on insulin resistance and diabetes. In an effort to create a model of diet induced insulin resistance and diabetes, we assessed the effects of a high fat versus a high cholesterol only diet on glucose, lipids, and atherosclerosis in LDLR-/- mice. The diet chosen contained butter fat and allowed us to compare data with those obtained using a different source of fat (see Project 5). Although the high fat diet led to increased weight and a rise in insulin levels, differences in plasma glucose levels were not significant. In addition, there were no changes in the extent of atherosclerotic lesions.

Plans for the coming months:

A. **Effects of AR inhibition on atherosclerosis development:** We have begun to determine if an AR inhibitor, zapolrestat (Pfizer), will reduce atherosclerosis in STZ-treated hAR/LDLR-/- mice. Initially we will use STZ diabetic mice of genotypes hAR/LDLR-/- and LDLR-/- with and without zapolrestat treatment. The zapolrestat was obtained and has been compounded into the diets. The studies will follow animals for 6 weeks and plasma lipids, glucose, and extent of atherosclerosis will be determined.

B. Physiology of hyperlipidemia in STZ-treated LDLR-/- mice: We and others have noted that STZ-treatment lead to greater concentrations of cholesterol in the LDLR-/- background. Similarly, Dr. Breslow has observed that the combination of LDLR-/- and ob/ob leads to a marked increase in plasma lipids. We will determine why this occurs by the comparing the following in LDLR-/- ± STZ-induced diabetes: food intake, lipoprotein profiles, apoB protein using Triton, expression of lipoprotein receptors – LRP, SR-B1, perlecan – by real time PCR and Western blot analysis.

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Most significant achievement: We have succeeded in developing a model of diabetes-accelerated atherosclerosis. This occurs when a human transgene for AR is introduced. We hypothesize that AR replacement is necessary for mice to replicate human pathophysiology. This model will allow testing of methods to reduce disease by pharmacologic and dietary approaches.

Publications:

Goldberg IJ. Why does diabetes increase atherosclerosis? I don't know. J Clin Invest, 114:613-5, 2004.

Vikramadithyan RK, Hu Y, Noh H-L, Liang C-P, Hallam K, Tall AR, Ramasamy A, **Goldberg IJ**. Human Aldose Reductase Expression Accelerates Diabetic Atherosclerosis in Transgenic Mice. J Clin Invest, 115 2434-2443, 2005

Project 3: "Assess the effect of diabetes on atherosclerosis progression"

Responsible Investigator: Dr. Jan L. Breslow

A. Rationale and Relevance: The aim of this project is to assess the impact of Type II Diabetes on the progression of atherosclerotic lesions using mouse models. The mouse is normally quite resistant to atherosclerosis because of low plasma cholesterol levels. Therefore, we selected as our main experimental model the LDLR-/- mouse, which has elevated levels of LDL. On a chow diet this mouse only has cholesterol levels of ~200 mg/d and does not develop significant lesions. It was necessary to develop a diet protocol that would allow these mice to develop lesions, yet not by itself cause excessive weight gain or insulin resistance. It was also necessary to assess lesion development at different sites to make sure we were observing the most relevant phenotype. Having settled these issues, experiments are now being conducted to assess the effects of hyperglycemia, insulin resistance or the combination on lesion development. Appropriate models will be developed in which diabetes worsens lesions without greatly exacerbating other risk factors, such as lipoprotein levels.

B. Summary of Accomplishments:

1. The role of genetically induced hyperglycemia in atherosclerosis progression was assessed by breeding the Pdx1+/- trait on to the LDLR-/- background and comparing aortic root cross sectional lesion area between C57BL/6J LDLR-/- Pdx-1+/+ and C57BL/6J LDLR-/- Pdx-1+/- mice weaned at 4 wks, then fed semi-synthetic AIN76a diet containing 0.02% cholesterol for 16 weeks, and sacrificed at 20 weeks of age. In females hyperglycemia induced by the Pdx-1+/- trait **decreased** aortic root cross sectional lesion area but had no significant effect on plasma lipid or lipoproteins. In males hyperglycemia induced by the Pdx-1+/- trait showed a trend towards **increased** aortic root cross sectional lesion area, but this was not statistically significant. The male C57BL/6J LDLR-/- Pdx-1+/- mice had lower cholesterol and non-HDL cholesterol (of marginal significance) and lower triglycerides (significant). The results in females contrast with those in males. In females hyperglycemia appears to decrease lesions and in males, despite lower levels of atherogenic lipoproteins, there is a trend for hyperglycemia to increase lesions.
2. In an attempt to assess the effects of combined hyperglycemia and insulin resistance on atherosclerosis progression C57BL/6JLDLR-/- and C57BL/6JLDLR-/-Ob/Ob mice were compared. These mice were weaned at 4 weeks, fed a semi-synthetic AIN76a diet containing 0.02% cholesterol for 16 weeks, and sacrificed at 20 weeks of age. In males and females the Ob/Ob knockout caused a significant increase in lesion area in both the aortic root and brachiocephalic artery. Interestingly, this increase was more marked in males. The lesions in males wild-type for the Ob/Ob gene were smaller than the lesions observed in the females, but when the Ob/Ob knockout was introduced, the males developed larger lesions than the females. There were gender specific effects of Ob/Ob mutation on the C57BL/6J LDLR-/- background with regard to insulin and glucose metabolism. Fasting insulin concentrations were ~ 2 times higher in Ob/Ob females than in Ob/Ob males. The IPGTT revealed that Ob/Ob females had significantly elevated glucose levels compared to control, whereas in males there was an overall trend to higher glucose levels but significance was not achieved except minimally at the 30 minute time point. The combination of higher insulin and glucose levels suggests females were more insulin resistant than males. This contrasts somewhat with the overall effects as well as gender effects of the Ob/Ob mutation with regard to lipid and lipoprotein levels. Cholesterol, non HDL-cholesterol and triglycerides were all very significantly

elevated in Ob/Ob mice of both genders; whereas this was true for HDL cholesterol in Ob/Ob females, the elevation seen in Ob/Ob males was only marginally significant. Lesions were so impressive in the C57BL/6J LDLR-/- Ob/Ob mice at 20 weeks that we decided to extend the study to see if they progressed to unstable plaques. In this study, C57BL/6J LDLR-/- and C57BL/6J LDLR-/- Ob/Ob mice were weaned at 4 weeks, fed the same semi-synthetic AIN76a diet containing 0.02% cholesterol for 28 weeks, and sacrificed at 32 weeks of age. This study is still underway, but preliminary results indicate a two-fold increase in lesion area at both the aortic root and brachiocephalic artery compared to the 20 week time point and the plaques are currently being examined for signs of lesion instability.

3. We have chosen for our platform hyperlipidemia model the C57BL/6J LDLR-/- mouse and at this point have selected the $Ins2^{Akita/+}$ trait to bring out hyperglycemia alone, the Ob/Ob trait to bring out mainly insulin resistance, and the combination of $Ins2^{Akita/+}$ and Ob/Ob for hyperglycemia and insulin resistance. In addition, we are collaborating with Dr. Goldberg to study the effects of sensitizers in concert with genetic models of hyperglycemia on atherosclerosis susceptibility. The experimental details are as follows:

a. In a comparison of C57BL/6J LDLR-/- Pdx-1+/- compared to C57BL/6J LDLR-/- Pdx-1+/+ we found that the former only had less than a ~2-fold increase in glucose. Upon examination of another hyperglycemia model, $Ins2^{Akita/+}$, we found it had consistently higher glucose levels than the Pdx1+/- mouse. Unfortunately, a genome scan of the $Ins2^{Akita/+}$ mice from Jackson Laboratory were found to be outbred and we have now bred them to the C57BL/6J LDLR-/- background. Studies with these mice are now underway to verify that hyperglycemia alone has minimal effects on atherosclerosis as we found for the Pdx1+/- mice. We are also using the $Ins2^{Akita/+}$ trait in combination with the HuARTg and SOD2+/- sensitizer traits to assess the combined effects on atherosclerosis.

b. With regard to insulin resistance, as previously noted our first choice was the IRS1-/- model. Although IRS1+/- mice could be brother-sister mated to produce IRS1-/- mice on an out bred background, when the IRS1+/- trait was bred to the C57BL/6J background, a necessary requirement for model stability as well as for atherosclerosis studies, no C57BL/6J IRS1-/- mice were obtained. As an alternate model, we obtained the Akt2-/- mice from Jackson for breeding to the C57BL/6J LDLR-/- background. Unfortunately, when we genotyped these mice we found them to be ~10% out bred. After 3 generations of backcross they are now inbred on the C57BL/6J LDLR-/- background. We will not have time to assess these mice for atherosclerosis, however, we will offer them to the Mutant Mouse Regional Resources Centers (MMRRC) for cryopreservation so they can be made available to the scientific community.

c. With regard to sensitizers, we obtained the HuAR transgenic mice from the AMDCC members at the University of Michigan with the intention of immediately breeding them on to the C57BL/6J LDLR-/- background. However, these mice were found to be ~20% out bred necessitating backcrossing before commencing atherosclerosis studies. Another potential sensitizer, the SOD2+/- mouse was obtained from Jackson and also found to be ~20% out bred. Both the HuARTg and the SOD2+/- traits have now been bred onto the inbred C57BL/6J LDLR-/- background and are now being crossed with C57BL/6J LDLR-/- $Ins2^{Akita/+}$ mice to compare the sensitizers for their abilities to promote diabetes accelerated atherosclerosis.

C. Plans for the coming months:

1. We plan to complete the LDLR-/-Pdx-1+/- study by characterizing the lesions morphologically and immunohistochemically. We will then prepare a manuscript jointly with Dr. Fisher describing the effect of this form of genetic hyperglycemia on atherosclerotic lesion progression and regression.

We also plan to confirm the Pdx1+/- findings in the $Ins2^{Akita/+}$ model to verify the effect (or lack thereof) of hyperglycemia alone on atherosclerosis susceptibility.
2. We plan to complete the Ob/Ob study by both quantitative and qualitative analysis of aortic root and BCA lesions. In addition to the 20 week time point, we will complete the study of the mice at 32 weeks with the hope that the advanced lesions at this stage will show signs of plaque vulnerability. We will continue to distribute tissues to other AMDCC core laboratories for assessment of neuropathy, retinopathy, nephropathy, and uropathy. Extra female mice will be bred for shipment to AMDCC core laboratories for functional studies pertaining to neuropathy, uropathy and cardiomyopathy. We recognize that the C57BL6J LDLR-/- Ob/Ob model has been reported by others, but our study is novel with regard to the qualitative analysis of lesions, especially as they progress, and for all of the ancillary studies done by the other AMDCC core laboratories.
3. Now that we have fully backcrossed the HuAR transgenic, SOD2, and $Ins2^{Akita/+}$ mice onto the C57BL/6J LDLR-/- background, we will breed the HuAR transgenic LDLR-/- and the SOD2+/- LDLR-/- mice to $Ins2^{Akita/+}$ LDLR-/- mice to see if these sensitizers bring out glucotoxicity in genetic hyperglycemia as they do in the STZ model.
4. The Akt2+/- mice fully backcrossed onto the C57BL/6J LDLR-/- background will be submitted to the MMRRC for cryopreservation so that they are available to other investigators.

D. Most significant achievement: We have called into question the simple notion that mere hyperglycemia promotes atherosclerosis susceptibility. We have made significant progress in establishing a platform model to test the effects of hyperglycemia, insulin resistance, and potential glucotoxicity sensitizers on atherosclerosis progression. Our next set of studies should reveal the best model(s) for studying diabetic macrovascular disease.

Publications:
Teupser D, Tan M, Persky AD, Breslow JL. Atherosclerosis quantitative trait loci are sex- and lineage-dependent in an intercross of C57BL/6J and FVB/N low-density lipoprotein receptor -/- mice. *Proc Natl Acad Sci USA*. 2006;**103**:123-128.

Project 4: "Assess effect of diabetes on atherosclerosis regression

Responsible Investigator: Dr. Edward Fisher

Rationale and relevance: The atherosclerosis disease burden in the diabetic population is high, making factors that retard further progression as well as those promoting regression important in reducing CAD risk. We have developed a model of regression in which the hyperlipidemia of the LDLR-/- or apoE-/- mouse can be rapidly normalized. This would simulate the aggressive lipid management now recommended for diabetics (who are now classified as "coronary heart disease risk equivalents", even if they have not yet had a myocardial infarction). The separate effects of hyperglycemia or insulin resistance on the capacity of normolipidemia to regress lesions could then be studied. In addition, by developing molecular methods to analyze the gene expression in plaque cells, mechanistic studies of changes in foam cells could be applied to many mouse models of diabetic vascular disease.

Summary of accomplishments: There have been 2 major accomplishments related to the funded studies: 1) Last year, we noted our publication (Llodra et al., PNAS 2004) in which we showed that during regression of mouse atherosclerosis, foam cells migrate from the lesions. Because dendritic cells (DCs) are monocyte derived cells that travel from tissue to lymph nodes, we hypothesized that macrophage foam cells acquired DC-like properties in a regression environment. To test this, we focused on the chemokine receptor CCR7, which is known to be required for DC migration. First, using laser capture microdissection of foam cells from lesions, in the isolated RNA we found induction of CCR7 gene expression only during regression. Immunostaining confirmed this induction at the protein level. Notably, the administration during regression of antibodies that block CCR7 function substantially impeded regression. These novel results therefore establish that the assumption of a DC-like state is part of the regression process in our mouse model. These and related findings are published in Trogan et al., PNAS early edition, 2006; 2) The Advisory Committee requested us to study the effects of hyperglycemia on plaques more advanced than in our first round of studies, which were predominately foam cell-rich. Thus, we fed LDLR-/- mice the western diet for 32 weeks (twice as long as in the first study, then performed transplants into either wild type (WT mice) or hyperglycemic PDX1+/- mice. As before, 4 weeks after transplantation, lesions were analyzed. In contrast to the first study, in which there were significant decreases in lesion size AND foam cell content independent of hyperglycemia, with more advanced lesions, foam cell content again decreased in both types of transplant recipients, but lesion size was not significantly reduced. Upon microscopic inspection of the lesions, the reason for this was obvious- as expected, as the plaques advanced, foam cells made up less of the intima than before, so the quantitative impact of their loss on lesion area became blunted. Nonetheless, the results of this second study are important in that foam cells in advanced plaques, though they may be fewer in number, are thought to be highly destabilizing in human lesions. In addition, many in the field have questioned whether advanced lesions can be favorably modified. Our results show in a mouse model that foam cells in advanced lesions can indeed be depleted, and that the hyperglycemia of the PDX1+/- mouse was not an interfering factor in this process.

Plans for the coming months: 1) We will investigate the factors that regulate the induction of CCR7 in regression- these studies are proposed in a pending R01, which was favorably reviewed and is awaiting final word from the NIH. 2) Also, in response to the Advisory Committee, we are

developing a non-invasive model of lesion regression. We proposed starting this last year, but there was some delay in obtaining the mice from our collaborator, Dr. Robert Raffai (UCSF). To review the strategy, we will study the "apoE hypomorphic" mouse. Basically, this is an apoE-/- model with an apoE transgene with a "floxed" neomycin gene in an intron. The neo cassette suppresses the expression of the transgene, but when it is excised by Cre-recombinase, the expression of apoE increases, thereby lowering plasma lipid levels. Cre-recombinase, in turn, is conditionally activated by injections of pIpC, meaning that lesion formation at any stage can be allowed to develop before apoE expression is turned on. Besides the non-surgical inducible advantage of this model, its other major advantage is that it involves only one modified locus. Thus, if the effects of modifying genes on regression are approached by breeding the mouse model of interest onto an atherosclerosis background, the combination genotype will be relatively easy to reconstitute in a few generations. Another application would be to study the effects of hyperlipidemia and its amelioration on diabetes-related pathology, such as kidney disease, retinopathy, neuropathy, etc.

To investigate the effects of hyperglycemia on regression in this model, we are expanding our colony and after feeding the mice Western diet for 32 weeks (to develop advanced lesions), we will invoke normolipidemia with or without STZ injection (following the AMDCC protocol for STZ induction of hyperglycemia). One month after establishment of normolipidemia (the same time period used in the regression study), we will analyzed the lesions of STZ-treated and saline injected normolipidemic mice. The results will be compared between the groups as well as against the results in a control group sacrificed at the time of normolipidemia induction. While the completion of these studies will likely fall beyond the official end of funding for this project, we are committed to completing at least one study.

Most Significant Achievements: 1) Regression of atherosclerosis in a mouse model requires the function of the dendritic cell-related factor CCR7; 2) Foam cells can be depleted from even advanced plaques by reversing hyperlipidemia and that this process is independent of mild hyperglycemia.

Related Publications:

Eugene Trogan, Jonathan E. Feig, Snjezana Dogan, George H. Rothblat, Véronique Angeli, Frank Tacke, Gwendalyn Randolph, and Edward A. Fisher. Gene Expression Changes in Foam Cells and the Role of the Dendritic Cell Migration Factor CCR7 During Atherosclerosis Regression in ApoE-deficient Mice, PNAS, early edition, 2006.

Eugene Trogan and Edward A. Fisher. Laser capture microdissection for analysis of macrophage gene expression from atherosclerotic lesions. Methods Mol Biol. 2005;293:221-31.

Project 5: "Assess the effect of diabetes on arterial injury"

Responsible Investigator: Dr. Hayes Dansky

Rationale and Relevance: Diabetes is an independent risk factor for atherosclerotic vascular disease and for restenosis following surgical and mechanical revascularization. The presence of diabetes significantly increases the risk for restenosis after PTCA/stent implantation. Recent clinical trials with rapamycin coated stents have reduced the incidence of restenosis even further; however, restenosis in diabetics patients still remains approximately two fold higher compared to patients without diabetes. In patients requiring surgical revascularization, the presence of diabetes markedly increases the risk of vein graft stenosis/occlusion after coronary artery bypass grafting. The mechanism(s) by which diabetes promotes restenosis are poorly understood. Our goal is to create mouse models of increased restenosis to study the mechanism(s) by which metabolic abnormalities affect vascular remodeling and restenosis.

Summary of Accomplishments: We have been using a mouse model of vascular injury to evaluate the effect of type 2 diabetes on restenosis. This model involves endoluminal wire injury of the femoral artery. Previous papers have documented that this model of arterial injury recapitulates many aspects of neointimal formation in response to angioplasty/stent implantation in humans. Our expectation was that diabetes would accelerate the response to arterial injury and result in an increase in neointimal size. We fed high fat diets to C57BL/6 wild type mice to induce obesity and type 2 diabetes. Femoral artery endoluminal wire injury was performed to determine whether diabetes would increase neointimal size in high fat diet fed diabetic mice compared to nondiabetic chow fed C57BL/6 mice. Diabetes had no significant effect on neointimal size. In fact, there was a trend toward a decrease in neointimal size in the setting of diabetes. While these effects may contradict clinical data in humans, our lab and others have demonstrated dramatic decreases in neointimal formation in diabetic mice.

We have also investigated the mechanism of endothelial dysfunction in high fat diet induced type 2 diabetic C57BL/6 mice. High fat diet fed mice were obese, had impaired glucose tolerance and fasting hyperglycemia, and hyperinsulinemia. Isolated arteries from high fat diet fed obese/diabetic mice had impaired endothelium dependent vasodilation and enhanced vasoconstriction to adrenergic agents. No difference in insulin mediated Akt and endothelial nitric oxide sythase (eNOS) phosphorylation were noted in the arterial wall, but eNOS dimer formation was absent in diabetic mice. Increased staining for tyrosine nitrosylation indicated that enhanced reactive oxygen species were generated in the arterial wall, resulting in the generation of peroxynitrite, disruption of eNOS dimers, and reduced NO dependent arterial vasodilation. These studies demonstrate that the diabetes associated factors involved in the increase response to arterial injury are distinct from the factors which impair endothelial vasodilation.

In collaboration with the Department of Cardiothoracic surgery at the Mount Sinai School of Medicine, we have also developed a model of vein graft stenosis in the mouse. This model involves the transplantation of the inferior vena cava into the abdominal aorta of the mouse. In a publication that is in press, we have demonstrated that vein graft stenosis increases in type 2 diabetic db/db mice. Increases in extracellular matrix deposition were partly responsible for the increase in neointimal size in diabetic mice.

Plans for the coming months: In collaboration with Dr. Ira Goldberg, we have compared atherosclerosis in LDLR-/- mice fed low and high fat diets. Preliminary data reveals that high fat diet induced obesity/diabetes does not increase atherosclerosis in LDLR-/- mice. We plan on finishing these studies over the next few months.

Most significant achievement: Our studies using a variety of mouse models of vascular disease (femoral artery injury model, vein graft model, examination of isolated vascular rings) have demonstrated that the effect of diabetes on vascular disease is highly dependent on the vascular model and the method of diabetes induction. These studies suggest that a specific set of diabetes associated metabolic abnormalities underlie each vascular phenotype.

Publications:

Molnar J, Yu S, Mzhavia N, Pau C, Chereshnev I, Dansky HM. Diabetes induces endothelial dysfunction but does not increase neointimal formation in high-fat diet fed C57BL/6J mice. Circ Res. 2005 Jun 10;96(11):1178-84.

Salzberg S, Filsoufi F, Anyanwu A, von Harbou K, Karlof E, Carpentier A, Dansky HM, Adams DH. Neointimal formation is increased in the setting of type 2 diabetes mellitus in a murine model of vein grafting. Circulation supplement (in press)