Animal Models of Diabetic Complications Consortium
(U01 HL70524)

Annual Report
(2004)

Mouse Models of Diabetic Vascular Disease
Rockefeller/Columbia/NYU

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Submitted February 25, 2005

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

The overall goal is to create mouse model(s) in which diabetes worsens macro vascular 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 initially introduced by streptozotocin will be replaced by breeding in the traits Pdx1+/- and $Ins2^{Akita/+}$; insulin resistance will be bred in by the traits IRS1-/- and Akt2-/-; hyperglycemia and insulin resistance by Ob/Ob trait. Based on Dr. Goldberg's work we suspect that hyperlipidemia plus hyperglycemia and/or insulin resistance may not be sufficient to create a mouse model of diabetic macrovascular disease. Therefore, we also intend to breed in sensitizing traits such as the human aldose reductase transgene or SOD2+/-. To reduce the number of animal models we will reduce the hyperglycemia, insulin resistance, and sensitizer traits to one each. However, due to unforeseen technical difficulties it is too soon to specify which will be most useful and cannot at this point make the final selections.

Major achievements have been:

Project 1: Dr. Stoffel has demonstrated that Foxa-2 is an important insulin sensor of the liver and a key activator of glycolysis, β-oxidation and ketogenesis. He has shown that constitutive active expression of Foxa2 reverses hepatic insulin resistance and restores normoglycemia in all rodent models with type 2 diabetes studied (ob/ob, aP2-Srebp-1c, diet-induced obese mice). This model will allow the Stoffel group in collaboration with others in the AMDCC to study the role of hepatic insulin resistance in the development of diabetic complications.

Project 2: Dr. Goldberg has developed a model of diabetes-accelerated atherosclerosis, which involves the introduction of a human aldose reductase transgene. He has hypothesized that AR replacement, or some other sensitizer, is necessary for mice to replicate human diabetic pathophysiology. Further studies to optimize this model are planned.

Project 3: Dr. Breslow's experiments with the Pdx1+/- mouse model of hyperglycemia have called into question the simple notion that mere hyperglycemia promotes atherosclerosis susceptibility. In addition, he has made significant progress in establishing a platform model to test the effects of hyperglycemia, insulin resistance, and potential glucotoxicity sensitizers on atherosclerosis progression.

Project 4: Dr. Fisher has shown that 1. Lesion regression in mouse models can be accurately assessed non-invasively by MRI, 2. Hyperglycemia alone does not impair fatty streak lesion regression after normalization of plasma lipid values are normalized after transplantation, and 3. During regression macrophages can leave lesions through luminal route or adventitial routes (presumably via the lymphatics) after they acquire the characteristics of dendritic cells.

Project 5: Dr. Dansky has shown that the response to arterial injury in diabetic mice is highly dependent upon the model used. His studies suggest an important role of leptin in response to injury.

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.

3. Collaboration with other AMDCC groups:

As models are finalized, tissues are being shipped to other AMDCC core laboratories for evaluation of neuropathy, retinopathy, nephropathy, and uropathy. In addition, extra mice are being bred for shipment to AMDCC core laboratories for functional studies pertaining to neuropathy, uropathy and cardiomyopathy. 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).

5. Address previous EAC comments:

EAC general comments:

Focus efforts and fully phenotype best 3-4 models: The main strategy of the Rockefeller/Columbia/NYU group has been to create mice with diabetic dyslipidemia and then introduce hyperglycemia or insulin resistance or both and assesses 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 initially introduced by streptozotocin will be replaced by breeding in the traits Pdx1+/- or $Ins2^{Akita/+}$; insulin resistance will be bred in by the traits Akt2-/- or one of the models to be developed by Dr. Stoffel; hyperglycemia and insulin resistance by Ob/Ob trait. Based on Dr. Goldberg's work we suspect that hyperlipidemia plus hyperglycemia and/or insulin resistance may not be sufficient to create a mouse model of diabetic macrovascular disease. Therefore, we also intend to breed in sensitizing traits such as the human aldose reductase transgene or SOD2+/-. Some of this work has been delayed due to our finding that mice used to breed in the necessary traits were out bred and the need for backcrossing before starting the definitive experiments. However, our intention is to determine the best inducer of hyperglycemia, the best inducer of insulin resistance, and the best sensitizer. This will allow us to whittle the number of models down to 3 or 4 as requested (i.e. C57BL/6J LDLR-/- plus 1. high glucose plus sensitizer, 2. insulin resistance plus sensitizer, 3. Ob/Ob plus sensitizer.

Expand the phenotyping of the models: As our models are finalized, we will ship tissues to other AMDCC core laboratories for evaluation of neuropathy, retinopathy, nephropathy, and uropathy. In addition, extra mice will be bred and shipped to AMDCC core laboratories for functional studies pertaining to neuropathy, uropathy and cardiomyopathy. 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.

Other potential vascular complications should be evaluated: In fact the Rockefeller/Columbia/NYU group was favorably cited in this regard for the work going on at Columbia by Dr. Dansky involving arterial injury.

All standard assays *must* be published on the website: We have played a major role in posting methods on the web site. We are currently experimenting with a Movat's trichrome staining protocol and will shortly put it on the web site.

There is a problem of differentiating complications due to hyperglycemia, hyperlipidemia, hyperinsulinemia, and lack of control of insulin/glucagon regulation. This remains a major issue and we must think about how to categorize the models: Throughout our work we have made special efforts to distinguish complications due to hyperlipidemia, hyperglycemia, and insulin resistance and we will continue to do so.

EAC specific comments:

One finding was that mice made hyperglycemic did not show larger lesions, but this result may be compromised by use of the C57BL/6 strain. The lesions did increase for AR transgenics and this does suggest involvement of this enzyme. Why are cholesterol and triglyceride values elevated in the 0.5% cholesterol-fed HuAR transgenics versus wild-types? The main strategy of our group has been to use C57BL/6J LDLR-/- mice and then to optimize conditions to bring about diabetic macrovascular disease. There are many obvious reasons to have chosen this strain including the fact that it has been well studied for atherosclerosis and that many transgenic and knockout traits have been bred to this background. Our initial results with diabetic dyslipidemia and hyperglycemia led to the concept of the need to sensitize this strain and the work of Dr. Goldberg using the human aldose reductase transgene has borne this out. In Dr. Goldberg's data set presented in tables in his summary of progress indicate that the aldose reductase transgene does not affect cholesterol or triglyceride levels.

The aldose reductase-Tg animals are very interesting but it isn't clear as to why a targeted expression approach wasn't followed. It would be fruitful to express AR in cells that are specific to the complication in mind such as endothelial cells. Also, complexity of lesions would be important to quantify. If no major changes in complexity are observed, then different time points could be evaluated for plaque vulnerability. These are excellent points. The human aldose reductase transgenic mouse was available and the investment of time and resources to prove the "sensitizer" hypothesis much more reasonable than to start with targeted expression. However, Dr. Goldberg has plans to target transgene expression to ECs and macrophages to further refine the model. More extensive evaluations of lesions over time in different locations will be undertaken as the model is refined. This will include evaulation of lesions for features of vulnerable plaque.

While the Pdx+/- mice were disappointing, it may be interesting to compare them to STZ treatment of C57Bl/6J LDLR-/- mice for insight as to what differs in the response to hyperglycemia. Nevertheless, future studies with the Pdx+/- mice should be given low priority. It appears that STZ treatment must be carried out in the presence of a sensitizer (the human aldose reductase transgene) for hyperglycemia to increase diabetic macrovascular disease. In view of the disadvantages of the STZ model, we are trying to replace this part of the model with a genetic hyperglycemia model. The Pdx1+/- trait is one possibility; however, we are also checking the Akita trait. Assuming the STZ-huAR transgene interaction was due to hyperglycemia and not some other property of the STZ treatment, the genetic hyperglycemia models should work and we will choose the best one.

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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 □-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. Generation of mutant mice harboring a conditional constitutive active Foxa2 allele. In order to study the long-term effects of constitutive active Foxa2T156A mutation on insulin resistance and the development of atherosclerosis we generated a mutant allele in which we replaced exon 3, which contains amino acid residue T156, with an identical sequence except an alanine substitution at position 156. The locus was successfully targeted in ES cells and injected into blastocysts. Several high-chimeric animals were generated and transmitted the mutant allele. We are currently crossing this mutant allele into a c57BL/6 background as well as to ob/ob mice. This mutant mouse model will allow us to study atherosclerosis regression following activation of Foxa2t156A and improvement of insulin resistance and hyperglycemia.

2. Generation of a dominant-negative mutant Foxa2 allele. We have attempted to generate a dominant negative mutant alelel by deletion of the Foxa2 c-terminal transactivating domain. This

mutant can still bind to the consensus sites of promoters in Foxa2 target genes, however, it fails to activate these genes in vitro. We planned to generate a recombinant adenovirus expressing the dominant negative Foxa2 and use it to treat mice, thereby inducing hepatic steathosis and insulin resistance (without affecting insulin secretion). However, we were unable to generate this recombinant adenovirus. The reason for this is most likely a toxic effect of the mutant Foxa2 in LE293 cells. We have changed our strategy and decided to create a transgenic mouse in which the dominant negative Foxa2 is placed downstream of a liver-specific promoter (TTR) and a loxP-STOP-loxP cassette. Using this strategy will prevent expression of dnFoxa2 and allow the liver specific induction at specific timepoints following adenovirus-mediated Cre expression. Once these animals have been generated they will be characterized.

Plans for the coming year: We will create mouse models with conditional Foxa2T156A and dnFoxa2 alleles. These mice will be characterized for insulin sensitivity, glucose homeostasis, and lipid metabolism. These mice will also be crossed to hypercholestrolemic LDLR-/- mice to study effects on atherosclerosis progression and regression.

Most significant achievement: We have demonstrated that Foxa-2 is an insulin sensor of the liver and a key activator of glycolysis, β-oxidation and ketogenesis. Constitutive active expression of Foxa2 reverses hepatic insulin resistance and restores normoglycemia in all rodent models with type 2 diabetes studied (ob/ob, aP2-Srebp-1c, diet-induced obese mice). This model will allow us to study the role of hepatic insulin resistance in the development of diabetic complications.

Publications:
Wolfrum, C., Asilmaz, E., Luca, E., Friedman, J.M., Stoffel, M. Foxa2 regulates glucose and fatty acid metabolism in the liver during starvation and in diabetes. *Nature* **432**:1027-1032, 2004.

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. Initiially, 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: We have been studying a line of mice expressing a human AR (hAR) transgene driven by a mouse histocompatibility gene promoter. This transgenic mouse line was crossed on to the 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 aldose reductase expression and did not result in levels of the enzyme that were non physiological or pharmacological.

LDLR-/- mice with and without the hAR transgene and with and without STZ induced diabetes were fed the AIN76a (low fat) diet containing 0.15% cholesterol for 6 weeks and the animals evaluated metabolically and for aortic lesion area by the en face method. The transgenic and non transgenic mice had comparable cholesterol levels and comparable increases in cholesterol after STZ treatment. Although there was a trend to increased aortic lesion area on STZ alone, a significant increase was observed only in HuAR transgenic mice treated with STZ. The former is probably a lipid effect whereas the latter appears to be due to the interaction of the transgene and the hyperglycemia induced by STZ independent of an effect on cholesterol (see table 1A).

Table 1A: Blood glucose, lipids and lesion area in control and streptozotocin (STZ)-treated mice fed HCD for 6 weeks.

	Ldlr-/- (n=14)	**hAR-*Ldlr-/-*** (n=13)	**L*dlr-/-* STZ** (n=11)	**hAR-*Ldlr-/-* STZ** (n=16)
Glucose (mg/dl)	147 ± 6	148 ± 8	530 ± 32*	473 ± 22*
TC (mg/dl)	1569 ± 223	1358 ± 158	3033 ± 275*	3302 ± 269*
TG (mg/dl)	90 ± 4	93 ± 9	130 ± 10	149 ± 16
HDL (mg/dl)	58.33 ± 3.70	51.38 ± 4.54	57.56 ± 9.13	67.20 ± 5.23
Total lesion (%)	8.01 ± 0.62	10.71 ± 1.22	23.41 ± 2.18	37.54 ± 2.72#

Aortic arch (%)	14.88 ± 0.46	13.74 ± 0.24	39.11 ± 3.22	64.97 ± 2.99[#]

In a second study, LDLR+/- mice with and without the hAR transgene and with and without STZ induced diabetes were fed the Paigen diet (very high cholesterol plus cholic acid) for 12 weeks and the animals evaluated metabolically and for aortic lesion area by the en face method. Whereas in the previous experiment cholesterol levels were in the 1,500 to 3,000 mg/dl range, in this experiment cholesterol levels were much more reasonable averaging about 500 mg/dl. In addition, STZ treatment alone did not increase cholesterol levels as it in LDLR-/- mice fed the AIN76a 0.15% cholesterol diet. In this experiment, there was a trend to increased aortic arch but not total aortic lesion area on STZ alone, with a significant increase in both in HuAR transgenic mice treated with STZ. In this model there is a clear interaction of the transgene and the hyperglycemia induced by STZ on aortic lesion area in the absence of any significant lipid changes (see table 1B).

Table 1B: Blood glucose, lipid profile and lesion area in mice fed a very high cholesterol, cholic acid containing (CCA) diet for 12 weeks.

	***Ldlr*+/-** (n=8)	**hAR-*Ldlr*+/-** (n=8)	***Ldlr*+/- STZ** (n=16)	**hAR-*Ldlr*+/- STZ** (n=12)
Glucose (mg/dl)	127 ± 9.34	108 ± 6	436 ± 38*	420 ± 34*
TC (mg/dl)	483 ± 51	530 ± 33	543 ± 43	525 ± 54
TG (mg/dl)	47 ± 6	50 ± 5	49 ± 4	49 ± 6
VLDL-C (mg/dl)	269 ± 40	319 ± 27	318 ± 34	310 ± 38
LDL-C (mg/dl)	106 ± 10	138 ± 3	125 ± 13	147 ± 10
HDL-C (mg/dl)	77 ± 4	63 ± 7	70 ± 9	58 ± 11
Total lesion (%)	8.24 ± 1.04	7.92 ± 0.85	8.91 ± 0.95	16.1 ± 1.88[#]
Aortic arch (%)	3.31 ± 0.68	4.48 ± 1.19	15.03 ± 2.84	30.69 ± 4.23[#]

Data are expressed as Mean ± SEM. *P<0.05, relative to non-diabetic controls, [#]P<0.05 compared to non-hAR expressing diabetic mice. Lipid measurements were done in plasma samples from 6 h fasted mice. Glucose was measured in whole blood using a glucometer.

Plans for the coming year: Studies will be done to define the best genetic background (LDLR-/- vs. LDLR+/-), dietary regime (AIN76a varying the cholesterol content, Paigen diet, Western-type diet), and duration of dietary treatment for the HuAR transgenic mouse model. In collaboration with Dr. Breslow we will explore genetic models for inducing hyperglycemia rather than using STZ. Finally, we will attempt to define the size and morphology of lesions in the HuAR transgenic mouse model at multiple locations (aortic root, BCA and aortic surface).

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.

Publications:

Goldberg IJ, A Isaacs, E Sehayek, JL Breslow, L Huang. Effects of streptozotocin-induced diabetes in apolipoprotein AI deficient mice. Athero. 172:47-53, 2004.

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 chows 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 heterozygous KO **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 heterozygous KO showed a trend towards **increased** aortic root cross sectional lesion area but this was not statistically significant. The male Pdx-1 heterozygous KO 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. The role of combined hyperglycemia and insulin resistance in atherosclerosis progression was assessed by comparing aortic root cross sectional lesion area between C57BL/6JLDLR-/- and C57BL/6JLDLR-/-Ob/Ob 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. Although the atherosclerotic lesions in these mice are still being assessed, 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 females than males. The IPGTT results indicated 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.

3. We have chosen for our platform hyperlipidemia model the C57BL/6J LDLR-/- mouse. However, we are still working on the best model for hyperglycemia alone and insulin resistance alone. In addition, we are collaborating with Dr. Goldberg to study the effects of sensitizers in concert with genetic models of hyperglycemia on atherosclerosis susceptibility.
a. With regard to hyperglycemia, although the Pdx1+/- model is indeed hyperglycemic, the elevation seen in C57BL/6J LDLR-/- Pdx-1+/- compared to C57BL/6J LDLR-/- Pdx-1+/+ are less than 2 fold elevated. In the course of the last year, due to Dr. Dansky's work as well as the work of others in the AMDCC, we became aware that the Akita mouse displays consistently higher glucose levels than the Pdx1+/- mouse. Therefore, we obtained the Akita mice from Jackson Laboratory and these are now being bred to the C57BL/6J LDLR-/- background. These mice will be used to verify the results with Pdx1+/- mice as well as in studies with hyperglycemia dependent sensitizers such as the HuAR transgene.
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 have obtained the Akt2-/- mice from Jackson for breeding to the C57BL/6J LDLR-/- background. Unfortunately, when we genotyped the Jackson mice with ~100 microsatellite markers, we found the mice to be ~10% out bred. These mice are currently being back crossed to C57BL/6J LDLR-/- mice and we are checking markers at each generation of backcross. We expect fully backcrossed mice within ~3 generations, and then be in a position to test the role of this trait on atherosclerosis susceptibility.
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, to our surprise, upon genotyping these mice we found them to be ~20% out bred necessitating backcrossing as in the paragraph above before commencing atherosclerosis studies. While this is proceeding, we decided to obtain another potential sensitizer from Jackson, the SOD2+/- mouse. Again, to our surprise, these mice were also ~20% out bred and are now being backcrossed as well.

C. Plans for the coming year:
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 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 also study 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. After backcrossing to the C57BL/6J background, in collaboration with Dr. Goldberg, we will breed the HuAR transgenic LDLR-/- and/or the SOD2+/- LDLR-/- mice to Akita LDLR-/- mice to see if these sensitizers bring out glucotoxicity in genetic hyperglycemia as they do in the STZ model.
4. We will work with the Akt2-/- model and the new models being developed by Dr. Stoffel to determine the role of insulin resistance alone in atherosclerosis susceptibility.

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:

Choudhury RP, Trogan E, Rong JX, Elmalem VI, Dansky HM, Breslow JL, Witztum JL, Fallon JT, Fisher EA. HDL retards the progression of atherosclerosis and favorably remodels lesions without suppressing indices of inflammation or oxidation.*Arterio Thromb Vasc Biol.* 2004;**24**:1904-1909.

Teupser D, Pavlides S, Tan M, Gutierrez-Ramos J, Kolbeck R, Breslow JL. The major reduction of atherosclerosis in fractalkine (CX3CL-1) deficient mice is at the brachiocephalic artery rather than the aortic root. *Proc Natl Acad Sci USA.* 2004;**101**:17795-17800.

Project 4: "Assess effect of diabetes on atherosclerosis regression/remodeling

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 had a myocardial infarction yet). 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 and improving imaging by MRI, mechanistic studies with convenient non-invasive assessments could be applied to many mouse models of diabetic vascular disease.

Summary of accomplishments: There have been 3 major accomplishments. The first 2 are directly supported by this grant and the third is highly related. The first accomplishment to note is the demonstration that the regression process that occurs in the transplant model can be accurately assessed non-invasively by MRI. In a recent paper (Trogan et al, ATVB, 2004), we show with MRI of living mice that over the course of a month, lesions in the regressive environment decrease in apparent size, while those in the progressive environment continue to enlarge. When the animals were sacrificed, these apparent changes were confirmed by conventional histopathological analyses. In addition, new imaging methods were developed so that imaging could now be extended in living mice to the thoracic region. Previously, motion artifacts above the diaphragm prevented this. Furthermore, algorithms were applied to the acquired images to deduce plaque composition, which agreed well with the histopathological results. The second accomplishment is our completion of the study partially summarized last year, in which hyperglycemia did not impair the regression of a foam-cell rich lesion when plasma lipid values are normalized after transplantation. Interestingly, when the results were compared to those studied in Dr. Breslow's study, it appears that hyperglycemia, in the absence of lipid changes, does not adversely affect either the progression or regression of lesions in LDLR-/- mice. The third accomplishment is our publication of a study that shows that during regression of mouse atherosclerosis, cells thought to be macrophages can leave the lesions through either a luminal route or an adventitial route (presumably via the lymphatics) after they acquire the characteristics of dendrtitic cells (Llodra et al, PNAS 2004). Since the pioneering studies of Dr. Gerrity over 20 years ago that suggested that foam cells may transmigrate out of lesions into the vessel lumen, the role of macrophage emigration as a regulator of foam cell content has been actively discussed, but inadequately investigated. We show in this paper that there is simultaneous influx and efflux of foam cells, with influx predominating during progression and efflux during regression. It is exciting to speculate that in diabetes, advanced atherosclerosis progression may have as a component decreased foam cell emigration, but this remains to be proved.

Plans for the coming year: 1) In response to the EAC's suggestion that advanced lesions are studied, we have begun a study that is summarized on the Table (Jan- I do not know where you will put that table I sent in a separate e-mail- if not here, please reference it). The animal number will be increased to complete the groups and the lesions analyzed to determine the effects of hyperglycemia

on the regression of advanced (i.e., beyond the foam cell stage) lesions. 2) Also, in response to the EAC, we have started to develop a non-invasive model of lesion regression. In collaboration with Dr. Robert Raffai (UCSF), we will study the "apoE hypomorphic" mouse, which Dr. Raffai has recently shown to be useful for this purpose (Raffai, R., Loeb, S., Weisgraber, K. Apolipoproein E promotes the regression of atherosclerosis independently of lowering plasma cholesterol levels. ATVB 25:436, 2005). Basically, this is an apoE-/- model which has 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.

Most Significant Achievement: Several accomplishments are noted including: 1. the demonstration that the regression process that occurs in the transplant model can be accurately assessed non-invasively by MRI, 2. completion of the study showing that hyperglycemia alone does not impair the regression of a foam-cell rich lesion when plasma lipid values are normalized after transplantation, and 3. the demonstration that during regression macrophages can leave lesions through either a luminal route or an adventitial route (presumably via the lymphatics) after they acquire the characteristics of dendritic cells.

Publications:

Trogan E, Fayad ZA, Itskovich V, Aguinaldo JS, Mani V, Fallon JT, Chereshnev I, and Fisher EA. Serial studies of mouse atherosclerosis by in vivo magnetic resonance imaging detects lesion regression after correction of dyslipidemia. Arter. Thromb. Vasc. Biol. 24:1714-9, 2004.

Llorda J, Angeli V, Liu J, Trogan E, Fisher EA, and Randolph GJ. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. Proc. Natl. Acad. Sci. 101:11779-84, 2004.

Frias JC, Williams KJ, Fisher EA,[‡] and Fayad ZA[†] (†co-corrresponding authors). Recombinant HDL-like nanoparticles, a specific contrast agent for MRI of atherosclerotic plaques. Journal of the American Chemical Society 126:16316-7, 2004.

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

Responsible Investigator: Dr. Hayes Dansky

Rationale and Relevance: Diabetes is an independent risk factor for restenosis following surgical and mechanical revascularization. Approximately one third of all patients undergoing balloon angioplasty/ tent implantation require repeat revascularization procedures because of target vessel restenosis. 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 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:

Report Progress of each mouse strain used: We have been using a mouse model of vascular injury to evaluate the effect of type 1 and 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. Femoral artery endoluminal wire injury was performed in diabetic insulin2 (*ins2)akita* (model of type 1 diabetes) and leptin receptor db/db mutant mice (model of type 2 diabetes). Neointimal size in i*ns2akita* mouse arteries was unchanged when compared to non-diabetic wild type littermates. In contrast, neointimal formation in *lepr db/db* mice was surprisingly reduced by ~90% compared to nondiabetic *lepr*+/+$^{+}$ mice. In addition, four hours following arterial injury, medial smooth muscle cell death was diminished in *lepr db/db* arteries, suggesting that the initial response to arterial injury was altered in *lepr db/db* mice. It is unclear why diabetes did not accelerate the response to arterial injury. The differential response to arterial injury in *lepr* db/db mice suggests a potential role for leptin in the regulation of neointimal formation in response to arterial injury.

We have performed arterial injury studies in several additional models of diabetes. Bilateral femoral artery injury was performed in C57Bl/6 ob/ob mice. These mice have a mutation in the gene for leptin and have obesity and diabetes. We found no difference in the response to arterial injury in male ob/ob mice. These results are consistent with findings of another group who found no significant increase in neointimal formation in ob/ob mice after $FeCl_3$ induced carotid artery injury (Schafer et al. ATVB 2004:24: 112-17). We have also looked at a diet induced obesity/diabetes model. C57BL/6 wild type mice were fed a chow or high fat diet for a period of 9 weeks. High fat diet fed mice were obese, had impaired glucose tolerance and fasting hyperglycemia, and hyperinsulinemia. Arteries isolated 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. Despite alteration in endothelium dependent vasodilation, there was no increase in neointimal formation in response to

femoral artery injury. In fact, there was a trend toward a decrease in neointimal formation in the obese/diabetic mice. It is unclear why femoral artery injury in mouse models of diabetes results in a decrease in neointimal formation.

Plans for the coming year: We have 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. Preliminary data reveal intimal hyperplasia in the transplanted vein four weeks following transplantation. We plan on finishing these studies to determine the effect of type 1 and type 2 diabetes on vein graft stenosis. In addition, we are exploring whether diabetes increases neointimal formation using different methods of arterial injury.

Most significant achievement: Our studies using the femoral artery injury model have shown that the response to arterial injury in a diabetic mouse host is highly dependent upon the model used. Our studies suggest a potential role of leptin in this response.

Publications:

Stephenson K, Tunstead J, Tsai A, Gordon R, Henderson S, and Dansky HM. Neointimal formation after endovascular arterial injury is markedly attenuated in db/db mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2003;23(11):2027-33.

A manuscript describing the effect of type 2 diabetes on endothelial function and on the response to arterial injury has been recently submitted.