Animal Models of Diabetic Complications Consortium
(U01 HL70524)

Update Report
(September 2001-January 2004)

Mouse Models of Diabetic Vascular Disease
Rockefeller/Columbia/NYU/Mount Sinai Group

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Part A:

Principal Investigator's Summary

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.

Major achievements have been:

Project 1: Dr. Stoffel's group continues to develop novel models of diabetes. In particular, they have demonstrated that Foxa-2 is the major liver insulin sensor and a key activator of glycolysis, β-oxidation and ketogenesis and that constitutive active expression of Foxa2 reverses hepatic insulin resistance and restores normoglycemia in rodent models of type 2 diabetes (ob/ob, aP2-Srebp-1c, diet-induced obese mice). Based on this information it will be possible to create a mouse model of hepatic insulin resistance and study how this affects diabetic complications.

Project 2: Dr. Goldberg has shown that even hypercholesterolemic mice in the setting of low HDL do not develop accelerated atherosclerosis when made diabetic. He has interpreted this to mean that there is a gene present in humans, but missing in mice, that mediates the toxic effects of diabetes/hyperglycemia. In preliminary experiments he has shown that the introduction of a human aldose reductase transgene on to the LDLR-/- background do develop accelerated atherosclerosis when made diabetic. This is a major breakthrough and may provide the consortium with a clue to the ideal mouse model for diabetic complications.

Project 3: Dr. Breslow's group has provided basic data about semi synthetic diets that can achieve hypercholesterolemia and atherosclerosis in LDLR-/- mice without toxicity from very high cholesterol and cholic acid components and without the weight gain induced by high fat feeding. They also showed that the aortic root and brachiocephalic arteries are the best for assessing lesion progression and have called into question the wide use of the whole aorta en face method. Finally, they showed that hyperglycemia by itself does not exacerbate atherosclerosis and may even ameliorate it.

Project 4: Dr. Fisher's group has shown that hyperglycemia per se does not impede the regression of the macrophage foam cell-rich lesions of atherosclerosis. They have also shown that during regression there is up regulation of cholesterol efflux genes in lesional macrophages but no up regulation of PPARγ. In addition to pioneering in showing the feasibility of using Laser Capture Microscopy for macrophage gene expression in the artery wall, they have recently shown that proteomic analysis of lesional macrophages is also feasible.

Project 5: Dr. Dansky has shown using the femoral artery injury model that there is markedly decreased response to injury in db/db mice but not akita mice. This demonstrates a previously unappreciated role for leptin in neointimal formation and suggests new mechanistic and therapeutic approaches to vascular disease in diabetics.

Interrelationships of projects:

Project 1 has provided projects 3 to 5 with mouse models of hyperglycemia and or insulin resistance to study for atherosclerosis susceptibility and has continued to develop new mouse models for future studies. Project 2 has interacted with project 3 to study the effect of hypercholesterolemia in the presence of low HDL on atherosclerosis in STX diabetic mice. Projects 3, 4 and 5 are studying the effects of hyperglycemia in the absence of insulin resistance utilizing Pdx-1+/- mice on atherosclerosis progression, regression and injury/remodeling. Project 2 is collaborating with projects 3 and 4 to study the effects of the human aldose reductase transgene on atherosclerosis progression and regression in the genetic Pdx-1+/- model of hyperglycemia. Each project has interacted with the Mouse Core to obtain and breed mice.

Collaborations: The Rockefeller/Columbia/NYU/Mount Sinai investigators have closely collaborated with each other as specified in the paragraph above. The group leaders of the 5 projects and their colleagues meet every 2 months to view results, plan experiments, and discuss future collaborations. Each group has used the Mouse Core to obtain animals.

Pertinent non-AMDCC Collaborations: Mike Brownlee of Albert Einstein College of Medicine has been very helpful in planning experiments related to what might be missing in the mouse that could bring out diabetic complications.

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Part B:

Update by Project Leaders

Responsible Investigator: Dr. Markus Stoffel, MD, PhD

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

A. 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.

B. Summary of Accomplishments:

We have shown that in normal mice, plasma insulin inhibits the forkhead transcription factor Foxa2 by nuclear exclusion and that in the fasted state (low insulin) Foxa2 activates transcriptional programs of β-oxidation, ketogenesis and glycolysis. In insulin resistant/hyperinsulinemic 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. Our results indicate that activation of Foxa2 is responsible for a significant proportion of the response to starvation. Furthermore, chronic hyperinsulinemia in insulin resistant syndromes results in the cytoplasmic localization and inactivation of Foxa2 and, through a viscous cycle, leads to insulin resistance, increased lipid accumulation and glucose production in the liver. Our studies establish a novel mouse animal model with reversible hyperglycemia and hepatic insulin resistance that can be used to study the effects of hyperinsulinemia and hyperglycemia on atherosclerosis progression.

C. Plans for the coming year:

We will characterize a Foxa2T156A knock-in mouse and mouse model with a conditional Foxa2T156A allele. 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 atherosclerosis progression.

D. 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:

Richter, S., Shih, D.Q., Pearson, E., Wolfrum, C., Fajans, S.S., Hattersley, A., Stoffel, M. (2003): Regulation of Apolipoprotein M Gene Expression by MODY3 Gene Hepatocyte Nuclear Factor–1□: Haploinsufficiency is associated with reduced serum apoM levels. *Diabetes* 52:2989-2995.

Wolfrum, C., Besser, D., Luca, E., Stoffel, M. (2003): Insulin regulates the transcriptional activity of forkhead transcription factor Hnf-3□/Foxa-2 by Akt-mediated phosphorylation. *Proc Natl Acad Sci U S A* 100: 11624-11629.

Wolfrum, C., Asilmaz, E., Luca, E., Friedman, J.M., Stoffel, M. (2004) Foxa2 regulates glucose and fatty acid metabolism in the liver during starvation and in diabetes. (submitted)

Responsible Investigator: Dr. Ira Goldberg, M.D.

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

A. Rationale and Relevance:

The etiology of the increased atherosclerosis in patients with diabetes is unknown. Our studies are an effort to determine this relationship. Our data suggest that in the mouse, elevated cholesterol is a more potent vascular toxin than hyperglycemia. These data complement those obtained by other studies in mice and results found in several other species (pigs, rabbits). These data are similar to those in human studies that suggest that lipid abnormalities and hypertension have a greater impact on macovascular pathology than does hyperglycemia.

Unquestionable patients with diabetes develop more vascular disease. Thus, there may be a genetic factor in humans, but not mice, that permits the expression of the deleterious effects of hyperglycemia on the vasculature. By using genetically altered mice it may be possible to uncover this factor and then direct interventions specifically to diabetic macrovascular disease. Our goal is to uncover factors that determine the increased risk of atherosclerosis in diabetic patients. This might allow therapies directed to this target and might identify genetic factors that predispose, or protect, individuals from diabetic vascular complications.

B. Summary of Accomplishments:

Our studies initially focused on genetic alterations in lipoprotein metabolism that might have obviated the presumed atherogenic effects of diabetes. We recreated a human-like diabetic dyslipidemia using three genetic modifications: transgenic expression of human apoB, transgenic expression of cholesteryl ester transfer protein, and heterozygous knockout of lipoprotein lipase. The mice developed increased LDL and VLDL. HDL decreased but not to human levels when the mice were placed on a high fat diet. Only diabetic mice that showed a marked increase in plasma lipids developed more atherosclerosis. There was no evidence for an effect of diabetes, produced with streptozotocin (STZ), exclusive of the lipid abnormalities.

We next created diabetes in mice that were deficient in both apoAI and LDL receptors. The hypothesis was that the high HDL in the mouse was protective from the effects of hyperglycemia. In addition, to avoid the increased glucose found in mice fed high fat diets, the animals were given a chow-cholesterol diet (see Project 3 for a discussion of the use of diets in these models). This study showed again that diabetes per se did not accelerate atherosclerosis, i.e. elimination of the high HDL in the mouse did not bring out the untoward vascular effects of diabetes.

C. Plans for the coming year:

We have initiated studies using a new line of transgenic mice that were created. A transgene for aldose reductase (AR) was crossed onto a LDL receptor knockout background. The effects of STZ-induced diabetes in both homozygous and heterozygous LDL receptor knockout mice will be assessed.

D. Most significant achievement:

We have shown that the mice used do not develop increased atherosclerosis in the setting of diabetes. This suggests that either there is a gene that is present in humans, but

missing in mice, that mediates the toxic effects of diabetes/hyperglycemia, or the mice express a protective factor other than HDL.

Publications

Kako Y, M Massé, LS Huang, AR Tall, IJ Goldberg Lipoprotein lipase deficiency and CETP in streptozotocin treated apoB-expressing mice. J Lipid R. 43:872-877, 2002

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

Responsible Investigator: Dr. Jan L. Breslow, M.D.

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

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:

A study was designed to test the effects of low-fat, semi synthetic diets containing increasing amounts of cholesterol in C57BL/6J and FVB/N LDLR-/- mice on lesion development at the aortic root, brachiocephalic artery and whole aorta (en face measurement). Animals were sacrificed at 20 weeks of age having been on the diet for 16 weeks. The low fat semi synthetic diet containing 0.00% or 0.02% cholesterol was sufficient to induce hypercholesterolemia and atherosclerosis in C57BL/6J mice a the aortic root and brachiocephalic artery, but did not produce significant lesions in the aorta measurable by the en face method. Raising dietary cholesterol to 0.15%, 0.3% or 0.5% more than doubled plasma cholesterol levels, increased lesion area at the aortic root and brachiocephalic artery and also resulted in significant en face lesions. The FVB/N mice had comparable cholesterol levels, but were atherosclerosis resistant and had many fold smaller lesions.

A study is underway to test the effect of hyperglycemia in the absence of insulin resistance on atherosclerotic lesion development. Hyperglycemia is induced by breeding the heterozygous Pdx-1 knockout trait on to the LDLR-/- background (all animals C57BL/6J). Animals were fed the 0.02% cholesterol semi synthetic diet from weaning at 4 weeks of age to sacrifice at 20 weeks of age. In females hyperglycemia actually decreased aortic root lesion area and had no significant effect on total cholesterol, HDL and non-HDL cholesterol or triglycerides. The study in male mice is still underway.

Finally, C57BL/6J IRS-1 heterozygous knockout mice were gotten from The Joslin Clinic in order to generate insulin resistant LDLR-/-IRS-1-/- mice. The IRS-1+/- mice were bred on to the LDLR-/- background and when intercrossed only 2/63 mice were IRS-1-/-. This precludes using this as a model for insulin resistance. Excessive loss of IRS1-/- mice has not been previously reported, however, all of these studies were with out breds.

C. Plans for the coming year:

We plan to complete the LDLR-/-Pdx-1+/- study in male mice. We will carefully examine lesion morphology in these mice. In collaboration with Dr. Goldberg we will breed the HuARTg to the LDLR-/-Pdx-1+/- mice to see if this will bring out glucotoxicity. Similarly, we will also breed the MnSOD+/- trait to the LDLR-/-Pdx-1+/- mice to test the theory of whether glucose induced ROS promote atherosclerosis. We will also work with Dr. Stoffel to develop another model of insulin resistance in the absence of hyperglycemia to test its effect on lesions.

D. Most significant achievement:

We have established a semi synthetic diet that achieves hypercholesterolemia and atherosclerosis in LDLR-/- mice without toxicity from very high cholesterol and cholic acid components and without the weight gain induced by high fat feeding. We also showed that the aortic root and brachiocephalic arteries are the best for assessing lesion progression and call into question the wide use of the whole aorta en face method. Finally, we showed that hyperglycemia by itself does not exacerbate atherosclerosis but may even ameliorate it.

Publications:

Teupser D, Persky AD, Breslow JL. Induction of atherosclerosis by low fat semi synthetic diets in LDL-receptor deficient C57BL/6J and FVB/NJ mice: Comparison of lesions at the aortic root, brachiocephalic artery and whole aorta (en face measurement). *Arterio Thromb Vasc Biol.* 2003;**23**:1907-1913.

Responsible Investigator: Dr. Edward Fisher, M.D. Ph.D.

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

A. Rationale and Relevance:

Patients with diabetes have increased risk of coronary artery disease. Their plaque burden is significant and it is likely that efforts to halt progression will be incompletely effective in reducing risk. Greater risk reduction will require, therefore, regression of plaques. Knowledge about the factors that either impede or promote regression in the diabetic state is currently lacking. By using a novel transplantation approach, coupled with mouse models that can separate the effects of hyperglycemia from insulin resistance, we can apply conventional and advanced methods of analyses to implicate factors relevant to the regression of atherosclerosis in diabetes.

B. Summary of Accomplishments:

We have completed one study in which we followed the same protocol as that used in Project 3, except that after feeding the LDLR-/- mice the semi-purified cholesterol-containing diet until 20 weeks of age, aortic arch segments containing lesions were transplanted into the abdominal aorta of either wild type mice or PDX1+/- mice (which are normolipidemic, insulin deficient, and hyperglycemic). Four weeks later, the recipient mice were sacrificed and lesional analysis of the grafts was performed. The results were compared to pre-transplant lesions taken from mice treated in parallel to the donor mice. All mice in the study were on the C57BL6 background.

At four weeks, compared to pre-transplant values, in both types of recipients, macrophage foam cell content was close to nil, with significant reductions in lesional areas. There was the suggestion that the regression process was more effective in the PDX1+/- recipients, however, in that the lesional area in the PDX1+/- recipients was ~20% of the pre-transplant mean, whereas it was ~30% in the wild type recipients. Our findings along with those using Pdx-1+/- female LDLR-/- in Project 3, suggest that hyperglycemia per se is not adverse in terms of atherosclerosis progression or regression.

In the original proposal, we planned on applying laser capture microdissection/Taq Man PCR techniques to study changes in macrophage gene expression in regressing (our project) or progressing (Projects 2 and 3) lesions. We have examined lesions in the wild type recipients and have found that within 3 days of placing a complex plaque in a wild type environment, factors associated with cholesterol efflux, such as LXRa and ABCA1, were upregulated by 3-5 fold, whereas PPARγ, whose expression we found low in pre-transplant lesions, is not. These preliminary results raise the issue of whether PPARγ agonists are benefical in diabetic atherosclerosis because of direct or indirect effects.

In addition to gene expression studies, we have now established proteomics as an additional approach. By harvesting approximately 2000 lesional macrophages (far fewer than what is needed for gene expression studies), we have obtained from the lysate material the mass spectra of the proteomic profile using a Ciphergen SELDI-TOF instrument (through a collaborator at the USDA, Dr. Earl Harrison). By taking samples from lesions in the early stages of the regression process, it will now be possible to detect

differences in the proteomic profiles in lesions placed in different metabolic environments.

C. Plans for the coming year:

We are analyzing gene expression in the lesions of the aldose reductase models in collaboration with Dr. Goldberg (Project 2). We are repeating the PDX1+/- regression study with more advanced lesions (animals fed to up to 30 weeks of the diet) and for the analysis, we will incorporate gene expression and proteomic assays. We will also start regression studies in insulin resistant models. A colony of Akt2 -/- mice on the C57BL6 background, kindly provided by Dr. Morris Birnbaum, U. of Pennsylvania, is being expanded for this purpose. Advanced lesions will be allowed to develop in the LDLR-/- mice, and transplantation into wild type or insulin resistant mice performed.

D. Most significant achievements:

1) Hyperglycemia per se does not impede the regression of a macrophage foam cell-rich lesion; 2) Regression is associated with up regulation in lesional macrophages of cholesterol efflux factors, but not PPARγ; 3) Proteomic analysis of lesional macrophages is quite feasible.

Publications:

No full papers yet. Manuscripts combining our results with those from Projects 2 and 3 are planned. An oral presentation was made at the AHA meeting, Nov. 2003, on the regression studies in general.

Responsible Investigator: Dr. Hayes Dansky, M.D.

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

A. 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/stent 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; however, restenosis in diabetic 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.

B. Summary of Accomplishments:

We used 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 humans. Femoral artery injury response includes endothelial denudation, platelet adherence, smooth muscle migration/proliferation and matrix deposition. 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. Increased HDL in leprdb/db diabetic mice may have played a role in the marked reduction in neointimal size in lepr db/db mice. In addition, 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.

C. Plans for the coming year:

We plan to perform additional arterial injury studies using other models of type 1 and type 2 diabetes. Other models include: C57BL/6 wild type mice fed a high fat diet to induce obesity/diabetes, IRS1-/- mice, and pdx1+/- mice. 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. Preliminary data reveals intimal hyperplasia in the transplanted vein four weeks following transplantation. We

propose to transplant wild type isogenic veins into db/db and ins2akita hosts to evaluate the effect of diabetes on vein graft stenosis.

D. Most significant achievement:

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

E. 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.

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Part C:

Update by Core Leader

Responsible Investigator: Jan L. Breslow, M.D.

Core 1: "Mouse Core"

Rationale and relevance:

The mouse core is necessary to acquire and breed mice with the necessary genotypes to test the effects of hyperglycemia, insulin resistance or the combination on atherosclerotic lesion development.

Summary of Accomplishments:

Animals have been bred and distributed to the investigators in the RU/Columbia/NYU/Mount Sinai group.

Plans for coming year:

Continue as above.