

**Animal Models of Diabetic Complications Consortium
(U01 HL087946)**

**Annual Report
(2009)**

**“Dislipidemia, Lipoic Acid and Diabetic Vascular Complications in
Humanized Mice”**

**The University of North Carolina at Chapel Hill
Department of Pathology and Laboratory Medicine**

**Principal Investigator
Nobuyo Maeda**

**Address: 710 Brinkhous-Bullitt Building, CB7527, Chapel Hill NC 27599-7525
Phone: 919-966-6914
E-mail: nobuyo@med.unc.edu**

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

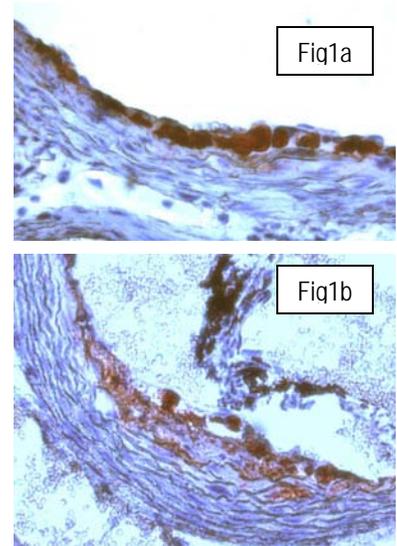
Hypothesis

1. Mice humanized lipoprotein metabolism system will develop a more human-like diabetic dyslipidemia and cardiovascular problems.
2. Genetically determined differences in the levels of endogenous antioxidants affect the development of cardiovascular complications of diabetes.

Recent Progress and Major Accomplishments

1-1. Akita mice expressing human apoE4 and human LDLR develop atherosclerosis without severe hypercholesterolemia. Non-diabetic E3 and E4 mice with 2 fold normal expression of the human LDLR (3h and 4h mice, respectively) have similar plasma fasting glucose and triglyceride levels to wild type mice. Their cholesterol levels are reduced because of reduced HDL-cholesterol to one third when on a normal chow (low-fat diet), and neither of these strains develops atherosclerosis. However, when challenged with a high-fat diet, 4h mice, but not 3h mice, accumulate cholesterol-enriched remnant particles and develop significant atherosclerosis.

To examine the effects of diabetes on atherosclerosis development, we generated mice that are homozygous for apoE3 or apoE4, heterozygous for human LDLR and mouse LDLR, with an Akita mutation (3hAkita and 4hAkita). At 2 months of age, both 3hAkita and 4hAkita males already had fasting glucose levels above 400 mg/dl, reflective of diabetes. However, there were no significant differences between 3hAkita and 4hAkita mice in regards to glucose, cholesterol or triglyceride levels. At 4 months of age, the diabetic 3hAkita and 4hAkita mice still had similar fasting glucose and plasma triglyceride levels. However, the diabetic 4hAkita males showed significantly higher plasma cholesterols than diabetic mice with 3hAkita males (110 ± 30 mg/dl vs 60 ± 5 mg/dl, $P < 0.02$). We also noticed that the life span of 4hAkita males appear to be significantly shortened compared to 3hAkita mice. Only two 4hAkita mice survived to 6 months of age to date (out of 10 mice), while all 3h Akita survived to this age. Importantly, when aortic root sections from the two surviving 6 mo old 4hAkita males were examined, both of them had clear atherosclerotic alterations. As shown in the Figure 1, right, the vessel wall is thickened, elastic layers are discontinuous, and lipid-containing macrophages (red staining) are present in the subendothelial areas (Fig1a), as well as deeper in the smooth muscle layers (Fig1b). Intimal area of aorta in Fig1b also contains cuboidal cells without lipid, suggesting the smooth muscle cells migration and proliferation. Thus 4hAkita mice demonstrate an initiation of atherosclerosis due to diabetes in the absence of severe diabetes-induced hyperlipidemia (cholesterol levels < 125 mg/dl).



The vast majority of mouse models of diabetic atherosclerosis currently used are models that are lacking in critical proteins of lipoprotein metabolism, such as apoE or the LDLR, or wild type mice fed an atherogenic diet containing 1.25% cholesterol and 0.5% sodium cholate. In these models, an increase in

atherosclerosis during diabetes is almost always associated with a severe diabetes-induced hyperlipidemia. This opens a question whether accelerated plaque development in diabetes is simply due to diabetes-induced dyslipidemia or there are contributions of the lipid-independent effects of diabetes. Since 4hAkita mice have all essential genes and protein components of lipid and glucose metabolism. We are excited with a potential of 4hAkita mice as a new model of diabetic atherosclerosis that is reflective of human physiology.

1-2. Diabetic dyslipidemia in LDLR^{-/-} mice expressing human apoE3 or apoE4. We reported last year that the LDLR^{-/-} males expressing human apoE4 isoform (4KO) develop significantly larger atherosclerosis than those expressing human apoE3 isoform (3KO) when they were made diabetic with STZ injection at 2 months of age. While average fasted glucose levels were lower in diabetic 4KO mice than in diabetic 3KO mice (326 ± 19 mg vs 441 ± 31 mg), plasma cholesterol and triglycerides were both higher in 4KO than in 3KO. Fast performance liquid chromatography (FPLC) showed that, after one month of diabetes, LDL (IDL) cholesterol levels rose dramatically in 4KO mice (320%), which was more increase than in 3KO mice (200%). The ratio of LDL/HDL in 4KO mice, a well-established risk factor for atherosclerosis in humans, was almost three times that in 3KO mice.

Sizes of VLDL particles isolated from diabetic 3KO and 4KO mice were not different as analyzed by electron microscopy. Relative apoE content in VLDL (% protein) was higher in non-diabetic 4KO mice than in 3KO mice and diabetes increased the levels by about 30% in both groups. Similarly, apoCIII content in VLDL from 4KO mice was 150% that from 3KO mice, and increased by diabetes by about 50% in both groups. Higher apoE and apoCIII contents are both inhibitory to lipolysis of lipoproteins. However, in vitro lipolysis of VLDLs isolated from diabetic 3KO and 4KO mice, as assayed using bovine lipoprotein lipase and heparinase-treated plasma from wild type mice as a source of lipase, did not reveal significant differences.

Modified lipoprotein particles, such as oxidized or glycated LDL, in the circulation are more likely to be taken up by macrophage and form foam cells in the atherosclerotic plaques, and apoE4 is less efficient than other isoforms for protecting lipoproteins from both oxidation and glycation in vitro. We therefore measured glycation in lipoproteins by using antibody against carboxymethyl lysines and by determination of free amine contents. Neither detected a significant difference between lipoprotein fractions isolated from diabetic 3KO and 4KO mice (21.5% and 18.7% glycation in 3KO VLDL and 4KO VLDL respectively and 13.3% and 11.9% respectively in LDL). If any, glycation tended to be less in lipoproteins containing apoE4. There was no significant difference in the amount of LDL oxidation as measured by TBARS assay. These data suggest that although increased in amount, plasma lipoprotein particles per se are not the major cause of accelerated atherosclerosis in diabetic 4KO mice.

1-3. Hepatic energy storage and gene expression in diabetic 3KO and 4KO mice. The harmful effects of apoE4 during diabetes may be attributable to changes in hepatic energy storage. The livers of diabetic 4KO mice had more fat stored in the form of triglycerides, and significantly less glucose stored in the form of glycogen than the livers of 3KO mice. Although these mice lack the LDLR, the activity of other apoE receptors, such as LDLR related protein (LRP), heparan sulfate proteoglycans (HSPGs) and the VLDL receptor (VLDLR), could account for an increase in lipoprotein/triglyceride uptake in hyperglycemic state. mRNA levels for *Ndst1* and LRP were not affected by diabetes nor differed between diabetic 3KO and 4KO livers. In contrast, diabetes increased the expression of the gene for VLDLR by 10 fold ($P < 0.0001$), and tended higher (2X) in diabetic 4KO mice than in 3KO mice ($P = 0.1$). Furthermore, genes related to insulin

sensitivity tended to be lower in diabetic 4KO mice. For example, adiponectin receptor 2 was significantly lower (36%) compared to 3KO mice (100%, $P < 0.02$). Potential explanations for these differences in energy storage include (1) isoform dependent differences in hepatic uptake of free fatty acid, glucose, and/or whole lipoproteins, (2) VLDL and/or glucose secretion rates, and (3) a shift in glucose versus lipid as the preferred source of energy. Experiments addressing these issues are in progress.

2-1. Enhanced atherosclerosis in mice with a reduced lipoic acid production. A 50% reduction in lipoic acid synthase (*Lias*) gene expression accelerates the development of atherosclerosis as the average size of atherosclerotic plaques in the aortic sinus of the 6 months old *Lias*^{+/-}*Apoe*^{-/-} male mice ($114 \pm 8 \times 10^3 \mu\text{m}^2$, $n=25$) was about 1.5X that in *Lias*^{+/+}*Apoe*^{-/-} male mice ($77 \pm 3 \times 10^3 \mu\text{m}^2$, $n=25$, $P < 0.01$). However, the difference was not present in females. RNA analyses of aorta showed that the expression of *Sod2* and *Mcp1* genes in *Lias*^{+/-}*Apoe*^{-/-} mice was significantly lower than in *Lias*^{+/+}*Apoe*^{-/-} mice (38%, $P < 0.02$ and 55%, $P < 0.05$ respectively), while the expression of *IL6* trended higher (240%, $P = 0.1$). This suggests that the reduced endogenous antioxidant enzymes are likely contributing to the enhanced inflammatory response and atherosclerotic plaque development. Expression of catalase and other genes will be analyzed as more specimens become available.

Since a large increase of oxidative stress is the hallmark of diabetes, we next tested whether a 50% reduction of *Lias* expression will enhance the diabetes-induced atherosclerosis. Both *Lias*^{+/-}*Apoe*^{-/-} and *Lias*^{+/+}*Apoe*^{-/-} males were treated with STZ at 2 months of age and hyperglycemia was monitored for 5 months. While their body weight was the same before the treatment with STZ, we observed that the body weight of diabetic *Lias*^{+/-}*Apoe*^{-/-} mice was significantly lower than the diabetic *Lias*^{+/+}*Apoe*^{-/-} mice ($P < 0.05$). Plasma levels of glucose, cholesterol and triglycerides were not significantly different between the two groups. The ratio of GSH/GSSH in erythrocytes in the diabetic *Lias*^{+/-}*Apoe*^{-/-} mice, on the other hand, was significantly lower than that in the diabetic *Lias*^{+/+}*Apoe*^{-/-} mice ($P < 0.04$), suggesting that the *Lias*^{+/-}*Apoe*^{-/-} mice were experiencing increased response to hyperglycemia. The lesion size in the diabetic *Lias*^{+/-}*Apoe*^{-/-} mice ($245 \times 10^3 \mu\text{m}^2$, $n=8$) was larger than in the diabetic *Lias*^{+/+}*Apoe*^{-/-} mice ($194 \times 10^3 \mu\text{m}^2$, $n=12$), but the difference was not significant with this small number of mice. While atherosclerosis was accelerated by diabetes, the increase (60%) was proportional to atherosclerosis in non-diabetic mice, suggesting that the reduction of endogenous lipoic acid at this level does not further exaggerate diabetic cardiovascular complications. We will continue to examine whether the increased/decreased production of endogenous lipoic acid production cause of this enhancement.

Plans

1. We will expand Akita diabetic mice expressing human apoE4 and human LDLR. As described above, this model is important since they do not lack any factors, and are consequently more human-like. Our preliminary observations show that 4hAita mice develop atherosclerosis without a significant hyperlipidemia. We will examine the roles of postprandial lipidemia, HDL production, and oxidative stress in diabetic atherosclerosis using this model.
2. We will complete analyses of antioxidant defense system in diabetic *Lias*^{+/-}*apoE*^{-/-} mice. Renal histology and EM will be completed on STZ-treated *Lias*^{+/-}*apoE*^{-/-} mice and on *Lias*^{+/-}Akita mice.
3. We will expand and develop diabetes models using *Lias*^{-H} and *Lias*^{-L} mice to study diabetic complications. STZ-induced diabetes models will be used.

2. Collaboration:

With other AMDCC Pis : none

With JAX:

2-2. Lias High-Low mice. An exciting development during the last funding period is the production of the Lias-H homozygotes in collaboration with the Jackson Laboratory. These mice initially produce stabilized transcripts of the *Lias* gene using the 3'UTR sequence of bovine growth hormone, but will change to produce unstable transcripts using 3'UTR from the cFos gene after Cre-mediated recombination is induced. We mated the Lias-H mice with Tg(Ella-cre) mice which expresses Cre-recombinase in testis and therefore the offspring (Lias-L) will globally express low levels of Lias. One homozygous Lias-L mouse was born recently, and is healthy. Some of the Lias-H homozygotes and Lias-L heterozygotes have been sent to UNC to establish colonies of these lines. The preliminary data suggest that the steady state mRNA levels from the H-allele is about 3X normal, while from the L-allele is about 0.5X, showing that the success of our overall scheme. We are currently examining the Lias expression and general phenotypes of the compound mutant mice carrying Low-allele and KO allele by breeding Lias+/L mice with Lias+/- mice. Additionally, although an increased production of endogenous lipoic acid could be beneficial, it may also cause the disturbance of energy metabolism, and may cause adverse effects. We will therefore characterize the Lias-H homozygotes with this in mind.

Mating of the L/+ mice with Akita mice has also been commenced at JAX. Also at JAX, it was found that ES cell line used to generate the mutants is B6/N, and therefore lacks deletion mutation in the *Nnt* gene which encodes the mitochondrial proton pump, nicotinamide nucleotide transhydrogenase (NNT). (The Jackson Laboratory's C57BL/6J (B6/J) strain contains the *Nnt* spontaneous deletion.) Aston-Mourney et al found that a positive correlation ($r^2 = 0.90$, $p < 0.01$) between NNT activity and first-phase insulin secretion in five mouse strains they examined, emphasizing a potential importance of this enzyme in beta cell function (Diabetologia, 2007). We are currently making sure to place the Lias High-low mutation on C57BL/6J.

With the MMPCs: none

With other non-AMDCC Pis: none

3. Address previous EAC comments:

On the effects of age. As a part of AMDCC efforts, we compiled the published data of atherosclerosis enhancement in the STZ treated apoE^{-/-} mice and LDLR^{-/-} mice. The comparison of data from the diabetic apoE-deficient mice is somewhat easier than those of LDLR because most of the investigators used regular mouse chow as diet in their experiments. All the experiments showed that diabetes enlarged the atherosclerotic lesion size by about 50% to 300%. Overall impression is that the degrees of enhancements were larger when mice were made diabetic earlier (at about 6 to 8 weeks of age) than later (8 to 12 weeks of age). Similarly, the degrees of enhancement were larger when atherosclerosis was evaluated by en face (i.e. scoring the areas of entire aortic tree covered by the plaque) than when average areas of plaques within the cross section of the vessels at the aortic roots were scored. We previously made apoE^{-/-} mice diabetes at 24 weeks of age, and examined the plaques at the aortic root of mice after 5 months diabetes. Plaques were about 30% larger than non-diabetic control mice. These observations suggest a possibility that diabetes accelerates initiation and growth during the early stages of plaque development perhaps by involving increased inflammatory response. We note that the larger plaques were more matured than smaller plaques in general, but, when the plaques of similar sizes were compared, there were no histological differences in the plaque components between the diabetic mice and non-diabetic apoE^{-/-} mice.

Two to three fold increase in atherosclerosis sizes were observed in diabetic LDLR^{-/-} mice compared to non-diabetic controls. Notably, most of the experiments used diets containing high in cholesterol and fat, and diabetic LDLR^{-/-} mice had higher than 1000 mg/dl of plasma cholesterol, making it difficult to dissect effects of diabetes from effects of plasma cholesterol. In some studies, however, no increase in atherosclerosis was observed. For example, Reaven et al. previously reported that atherosclerosis in the LDLR^{-/-} mice, made diabetic with a single high dose of STZ and maintained on high fat diet for 6 months, did not differ from those in the control, non-diabetic, LDLR^{-/-} mice. The reason for this is not clear, but the plasma glucose levels in these diabetic mice were not as high as in other experiments because they have been treated with a low dose of insulin infusion, and the plasma cholesterol levels were not significantly different between diabetic and nondiabetic mice.

Specific comments: EAC comments attached

Conditional Lias construct sent to JAX but to-date germline transmission has not been established—continue with current plan. When available, this model should be investigated for all AMDCC complication

With the help of JAX, we have established both Lias-Hi and Lias-Lo mouse line as described in 2-2 above.

Lias^{+/-} mice on 129/SvEv background mated with Akita on B6 background. Generalized oxidative stress was slightly higher in Lias^{+/-}Akita compared to Lias^{+/+}Akita with no obvious

renal phenotype. While not necessarily encouraging, experiments investigating the effects on atherosclerosis should be completed. This may offer further rationale for the High/Low expressing mice.

We agree that albumin urea in these mice were not strikingly high compared to *Lias*^{+/+} Akita mice. However, we recently performed TEM of the kidneys from *Lias*^{+/-} Akita mice and found that in *Lias*^{+/-} Akita kidneys showed a significant podocyte effacement. In contrast, podocyte effacement was negligible in the kidneys of *Lias*^{+/+} Akita littermates. Podocyte effacement in apoE^{-/-} made diabetic with STZ was also negligible although these mice had much worse mesangial expansion and higher urinary albumin excretion than in Akita mice. It would be of great importance if the podocyte damage and urinary albumin excretion can be separable. We are currently pursuing this issue more carefully.

We have studied atherosclerosis in *Lias*^{+/-} apoE^{-/-} mice as described above under 2-1. We found that atherosclerosis was significantly but mildly increased in males but not in females. We have made male *Lias*^{+/-} apoE^{-/-} mice diabetic by treating with STZ. Atherosclerosis was enhanced by diabetes but interaction between genotype and diabetes was not significant, although we need to study a larger number of mice.

Human apoE isoform mice are progressing. The general approach is attractive to try and mimic diabetic dyslipidemia. With the current preliminary results encouraging, it will be interesting to see effects on atherosclerosis ± diabetes; ± Lias modulation.

We are very encouraged with our recent finding that Akita mice with human apoE4 isoform and over-expressing human LDLR develop atherosclerosis. These mice have been fed a normal mouse chow, which contains low-fat, low-cholesterol, and the 4 hour-fasted plasma cholesterol levels are below 125 mg/dl. To our knowledge, this is the first mouse model that develop atherosclerosis without hypercholesterolemia, and only when they are diabetic.

4. Publications:

1. Arbones-Mainar JM, Johnson LA, Altenburg MK, Maeda N. Differential modulation of diet-induced obesity and adipocyte functionality by human apolipoprotein E3 and E4 in mice. *Int J Obes (Lond)*. 2008 Oct;32(10):1595-605. PMID: 18725890
2. Lance Johnson and Nobuyo Maeda. Macrovascular complications of diabetes in atherosclerosis- prone mice. *Expert Reviews of Endocrinology and Metabolism*. 2009 Submitted.

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(U01 XX#####)

Part B:

Update by Individual Project Leaders
(if applicable)

Project 1 (if applicable): “**Title**”

Responsible Investigator: **Name**

1. Project Accomplishments:

Hypothesis

Progress toward stated milestones

Plans for the Upcoming Year

2. Collaboration:

With other AMDCC PIs

With Jax

With the MMPCs

With other non-AMDCC PIs

3. Publications:

Please list

Project 2 (if applicable): “Title”

Responsible Investigator: Name

1. Project Accomplishments:

Hypothesis

Progress toward stated milestones

Plans for the Upcoming Year

2. Collaboration:

With other AMDCC PIs

With Jax

With the MMPCs

With other non-AMDCC PIs

3. Publications:

Please list

Project 3 (if applicable): “Title”

Responsible Investigator: Name

1. Project Accomplishments:

Hypothesis

Progress toward stated milestones

Plans for the Upcoming Year

2. Collaboration:

With other AMDCC PIs

With Jax

With the MMPCs

With other non-AMDCC PIs

3. Publications:

Please list