Animal Models of Diabetic Complications Consortium
(U01 HL087946)

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"Dislipidemia, Lipoic Acid and Diabetic Vascular Complications in Humanized Mice"

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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. As we reported last year, we generated mice that are homozygous for apoE3 or apoE4, heterozygous for human LDLR and mouse LDLR, and also carry an Akita mutation (3hAkta and 4hAkita). At 2 months of age, both 3hAkta and 4hAkita males already had fasting glucose levels above 400 mg/dl, reflective of diabetes. However, there were no significant differences between 3hAkta and 4hAkita mice in regards to glucose, cholesterol or triglyceride levels. At 4 months of age, the diabetic 3hAkta 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 3hAkta males, although they are still within normal range (110±30 mg/dl vs 60±5 mg/dl, $P<0.02$). We also noticed that the life span of 4hAkita males was significantly shortened compared to 3hAkta 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 artic root sections from the two surviving 6 mo old 4hAkita males were examined, both of them had clear atherosclerotic alterations. Thus 4hAkita mice demonstrate an initiation of atherosclerosis due to diabetes in the absence of severe diabetes-induced hyperlipidemia (cholesterol levels <125 mg/dl). 4hAkita mice represent the first mouse model in which diabetes is required for atherosclerosis development.

Unfortunately, we have had a breeding problem of this strain of mice, and the reduced lifespan remained a problem. However, we are currently characterizing three additional 4hAkita mice to validate above observations, and monitoring some younger animals in the colony. We have also sent breeders to the Jackson laboratory with a hope to overcome the breeding problem with an expert help from them.

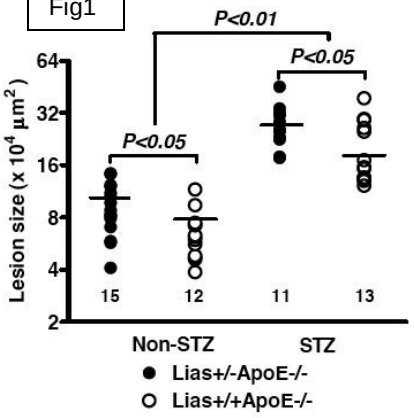
1-2. Interaction between diet and apoE isoforms in LDLR-/- mice expressing human apoE3 or apoE4. LDLR-/- male mice expressing human apoE4 isoform (4KO) develop significantly larger atherosclerosis than those expressing human apoE3 isoform (3KO) when they were made diabetic with STZ injection at 2 months of age. Our study of lipoprotein metabolism suggests that diabetic 4KO mice appear to have mild impairment of postplandial plasma lipid clearance and accumulate TG in the liver. However, the diabetic dyslipidemia is overall mild, although significant, may not explain all the increase in atherosclerosis observed in 4KO mice compared to 3KO mice. We therefore have begun to shift our attention to the role of apoE isoforms on glucose metabolism and insulin resistance. This is based on our observation that mice expressing apoE4 gain less weight compared to mice expressing apoE3 when fed a western type diet, but are more susceptible to develop impaired glucose tolerance. We also found that mice expressing apoE4 respond poorly to thiazolidinediones, a class of insulin sensitizer and PPARgamma agonists.

To examine whether the diet-induced obesity and insulin resistance would affect the atherosclerosis development, we fed 3KO and 4KO mice (n=8 in each group) with a diet high in sucrose (70% of calories) or a diet high in lard (60% of calories), and compared the gain of body weights, adipose tissue weights, and insulin sensitivities with those fed regular mouse chow (60% calories from starch). At the end of 2 months on diets, atherosclerotic plaque sizes at their aortic root areas were also assessed. None of the animals fed the high sucrose diet gained excessive weight. Plasma glucose clearance following an oral glucose overload was normal, although their plasma insulin levels were about 5 times higher than in chow fed mice regardless of their apoE genotype. Importantly, however, the 4KO mice on the high sucrose diet had 3 times larger atherosclerotic plaques than the 3KO mice. Animals on a high lard diet gained weight, and the gain was significantly more in 3KO than in 4KO males. The 3KO mice had also impaired insulin sensitivity more than the 4KO mice on the high lard diet. Thus the effects of apoE isoforms on metabolic phenotypes are exaggerated in mice fed a diet high in fat. We are currently examining the effects of the metabolic consequences caused by the high lard diet on atherosclerosis with a hope to assess how apoE isoforms interact with diet in determining insulin resistance and atherosclerosis.

2-1. Enhanced atherosclerosis in male, but not in female, apoE deficient 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-/- males (93±8 x10^3µm^2, n=15) was about 1.5X that in Lias+/+Apoe-/- mice (64±6 x10^3µm^2, n=12, P<0.05). Importantly, the increase was not statistically significant in females. RNA analyses of aortic tissues showed that the expression of Sod1 and Sod2 genes in Lias+/-Apoe-/- mice was significantly lower than in Lias+/+Apoe-/- mice, while the expression of IL6 trended higher. This suggests that the reduced endogenous antioxidant enzymes are likely contributing to the enhanced inflammatory response and atherosclerotic plaque development. While the effects of reduced Lias gene expression on the markers of oxidative stress in females were to the same directions as in males but the difference was smaller and not siginificant, suggesting that presence of female-specific protection mechanism against the consequences of reduced LA, including that for the plaque development. We have completed this study and published the results during the last year.

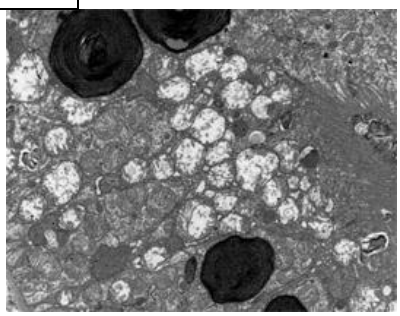
2-2. Atherosclerosis in Lias+/-Apoe-/- mice made diabetic by a STZ treatment. We have also made excellent progress in assessing whether 50% reduction of Lias expression will enhance the diabetes-induced atherosclerosis. Both Lias+/-Apoe-/- and Lias+/+Apoe-/- males were treated with STZ at 2 months of age and hyperglycemia was monitored for 4 months. Plasma levels of glucose and cholesterol were significantly higher in the diabetic Lias+/-Apoe-/- mice (385±11 and 843±36 mg/del respectively) than in Lias+/+Apoe-/-mice (352±10 and 709±35 mg/dl respectively) but triglycerides not significantly different between the two groups. The ratio of GSH/GSSH in erythrocytes in the diabetic Lias+/-Apoe-/- mice was significantly lower than that in the diabetic Lias+/+Apoe-/- mice (P<0.02), suggesting that the Lias+/-Apoe-/- mice were experiencing increased oxidative stress response to hyperglycemia. After 4 mo of diabetes, the lesion size in the diabetic Lias+/-Apoe-/- mice (275±25 x10^3µm^2, n=11) was larger than in the diabetic Lias+/+Apoe-/- mice (215±23 x10^3µm^2, n=13, p=0.04). While diabetes increased plaque size by about 3 fold, the increase was proportional to atherosclerosis in non-diabetic mice (Figure 1). Thus, 50% reduction of endogenous LA production and diabetes appear to have independent additive effects in exaggerating atherosclerosis.

Fig1



2-3. Enhanced dibetic nephropathy in Lias+/-Akita mice. We found that Lias+/-Akita mice (F1 between 129 and B6J) have mildly lower plasma glucose levels than Lias+/+Akita mice. However, they excrete two fold more urinary albumin compared to Lias+/-Akita mice (100 ± 18 μg/day vs. 50 ± 12 μg/day, $P<0.01$). Histologically, a significantly more glomerular basement membrane thickening and mesangial expansion were observed at six months of age. EM in the Figure 2 shows many of mitochondria within the proximal tubular cells are disorganized and damaged, and cells contain multiple inclusion bodies (electron-dense swirls), that are indicative of glycolipid accumulations in lysosomes. Although podocytes appear not significantly altered and no fibrosis was evident, expression in kidney cortex of the gene for nephrins are significantly reduced (30%) while those for TGFβ and collagen IV were both increased (150%). Importantly, superoxide generation in proximal tubules is significantly increased, despite of increased expression of both *Sod1* and *Sod2* genes (130% and 170% respectively). These data indicate that a 50% reduction in the expression enhances diabetic nephropathy. Unexpectedly, we also observed that the systolic blood pressure of the Lias+/-Akita mice is higher than that of Lias+/+Akita mice at 6 months of age. (115±4mmHg, n=18, vs 104±3mmHg n=16, $p<0.01$). We do not know at present how this blood pressure change relates to the kidney pathology or causative of the damage. We are currently examining whether younger Lias+/-Akita mice show elevated blood pressure and whether or not Lias+/- mice have elevated blood pressure in general.

Figure2



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. We will investigate how lipoprotein uptake in macrophage influences atherosclerosis in diabetic animals.
2. We will continue to study diet-apoE isoform interaction on atherosclerosis in the setting of type 2 diabetes.
3. We will complete renal histology and EM of STZ-treated Lias+/-Apoe-/- mice and on Lias+/-Akita mice in order to assess the effects of reduced endogenous LA production in diabetic complication in kidneys.
4. We will expand and develop diabetes models using Lias-H and Lias-L mice to study diabetic complications. STZ will be used to induce diabetes in mice, while cross between Lias-L and Akita is in progress at the Jackson Laboratory.
5. We will investigate the effect of Lias (and/or lipoic acid) on blood pressure regulation more in detail.

2. Collaboration:

With other AMDCC Pis : none

With JAX: In collaboration with the Jackson Laboratory, we have made Lias-Hi and Lias-Low lines of mice on C57BL/6J background, and have established colonies at UNC. The steady state mRNA levels from the H-allele is about 2X normal, while that from the L-allele is about 0.3X, showing that the success of our overall scheme of varying the endogenous Lias gene expression. By breeding Lias-Low/+ mice on C57BL/J background with Lias-KO/+ mice on 129SvEv background, we generated mice with Lias-low/KO combined mutant. The mRNA levels in these mice are about 15% that of the wild type littermates.

We are currently examining metabolic and antioxidant status of the Lias-Low/low homozygotes.

With the MMPCs:

Tom Coffman: We have recently sent apoE-/- mice on 129/SvEvTac background to Dr. Coffman's group to breed them with Akita mice on the same background. This is to assess the effects of diabetes on atherosclerotic plaque development in aortic arch, for which apoE-/- mice on this strain background is suited. In addition, since this genetic background is also good for modeling diabetic nephropathy, we will be assessing the effects of hyperlipidemia on kidney pathology.

With other non-AMDCC Pis: none

Address previous EAC comments:

1. Great progress overall. The LDLR;ApoE3/E4 transgenic models turned out to be really exciting. As indicated in the plans further characterization of this model should be given high priority. Presumably Dr. Maeda will also have a closer look at the lipid profiles; with the observed reduction in HDL one might be curious whether the lipid profiles differ between E3 and E4.

Yes, we will complete the analysis of the lipoprotein profiles. FPLC shows that plasma HDL levels are reduced in both 3hAkita and 4hAkita mice compared to Akita mice without human LDLR. Cholesterol in the VLDL fraction is higher in 4hAkita than in 3hAkita, although not a lot. Postplandial lipid clearance in 4hAkita mice is not different from in 4h mice although is faster than in 4Akita mice without human LDLR. In our future studies, we will be concentrating on the differences between 4hAkita and 4Akita mice.

2. Dr. Maeda should work with the consortium to expand the breeding of the model? What about STZ-induced diabetes in the E4-Transgenics?

Breeders (E4/E4,Akita+ and E4/E4,hLDLR/+) have been sent to the Jackson Laboratory to expand the colonies. At UNC, we are also attempting to generate E4/E4,hLDLR/hLDLR double homozygotes to use as a breeder to increase the efficiency of the production of and 4hAkita and their littermate controls, 4Akita mice. We have not tested STZ-induced diabetes in the 4h mice because of a consideration that genetic model would give us more uniform diabetes. However, I do agree that the STZ-induced diabetes is a valid alternative.

3. LIAS+/- mice have been crossed with the apoE(-/-) mouse and have shown higher atherosclerosis in males but not in females. This is associated with increased cholesterol and TGs in both animals. Male animals have evidence of higher oxidative stress. STZ treatment of these mice (in relatively small numbers) shows a trend to accelerated atherosclerosis with diabetes, however the proportional increase compared to LIAS+/+ is the same. These studies should at least be taken to completion to confirm if this observation holds true with larger numbers.

As described under 2-2 above, we have repeated the study and with the numbers of animals >11 in each group, we are convinced that the effect on atherosclerosis of the level of the *Lias* gene expression and of diabetes are independent and additive. We are currently not sure why this is so, but it suggests that the increased atherosclerosis caused by a 50% reduction of the *Lias* gene expression is not due to its effects on oxidative stress but rather its effects on energy metabolism and mitochondrial function. We also need to consider our preliminary finding that Lias+/-Akita mice have higher blood pressure compared to Lias+/+Akita mice (see above) as a potential contributor, and plan to examine blood pressures of STZ treated Lias+/-apoE-/- mice.

4. The plan to investigate renal complications is encouraged as enhanced oxidative stress may be more important in other settings. Other tissues (paws, eyes) could be sent to Consortium collaborators to screen for complications. The LIAS-H and –L mouse models are potentially exciting developments.

Our studies described above (under 2-3) have shown that the Lias+/-Akita mice (F1 between 129 and B6) have enhanced diabetic nephropathy compared to the Lias+/+Akita mice. Our findings indicate that reduced endogenous antioxidant capacity in Lias+/-Akita mice contributed to the accelerated development of diabetic complication in other organs than in the kidneys, and we agree with the EAC comment that it would be worthwhile to save other tissues and send them to consortium collaborators to screen other complications. In this regard, we are generating, in collaboration with Jax, *Lias$^{low/-}$ins2$^{Akita/+}$* (LiasL/-,Akita) mice that express 15% normal levels of lipoic acid synthase. These mice would be of use as a model for distribution.

3. Publications:

- Yi XW, Kim K, Yuan W, Xu, L, Kim HS, Homeister JW, Key NS, Maeda N. Mice with heterozygous deficiency of lipoic acid synthase have an increased sensitivity to lipopolysaccharide-induced tissue injury. J. Leukocyte Biol. 2009 Jan;85(1):146-53. PMCID: PMC2626770
- Johnson LA, Maeda N. Macrovascular complications of diabetes in atherosclerosis-prone mice. Expert Rev. Endocrinol. Metab. 2010. 5(1), 89-98. PMCID in progress
- Yi X, Xu L, Kim K, Kim H-S, Maeda N. Genetic reduction of endogenous alpha-lipoic acid synthesis modestly increases atherosclerosis in male but not female apolipoprotein E deficient mice. Atherosclerosis 2010, Mar 10. [Epub ahead of print] NIHMSID 202552
- Arbones JM, Johnson LA, Altenburg MK, Kim HS, Maeda N. Impaired adipogenic response to thiazolidinediones in mice expressing human apolipoprotein E4. FASEB J. 2010, in press.

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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:

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2. Collaboration:

With other AMDCC PIs

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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