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Evidence of extensive atherosclerosis, coronary artery disease and myocardial infarction in the *ApoE*$^{-/-}$:*Ins2*$^{+/Akita}$ mouse fed a western diet

Daniel E. Venegas-Pino [a, c], Andrew Lagrotteria [b, c], Pei-Wen Wang [c], Jaiya Morphet [c], Cassaundra Clapdorp [c], Yuanyuan Shi [c], Geoff H. Werstuck [a, b, c, *]

[a] Department of Medicine, McMaster University, Hamilton, Ontario, Canada
[b] Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada
[c] Thrombosis and Atherosclerosis Research Institute, McMaster University, Hamilton, Ontario, Canada

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ABSTRACT

Background and aims: Diabetic patients with no history of cardiac infarction have a prevalence of coronary atherosclerosis and a risk of heart attack equivalent to euglycemic patients who have coronary atherosclerosis and have suffered a prior myocardial infarction. Although several murine models of diabetes have been established, none of these show indications of cardiac events. In an attempt to establish a diabetic mouse model with coronary atherosclerosis and myocardial injury, we have fed hyperglycemic *ApoE*$^{-/-}$:*Ins2*$^{+/Akita}$ mice a western diet to enhance the dyslipidemic phenotype.
Methods: Five-week-old *ApoE*$^{-/-}$:*Ins2*$^{+/Akita}$ mice and *ApoE*$^{-/-}$ controls were fed a diet, 0.15% cholesterol and 21% anhydrous milk lipids, until 25 weeks of age. Changes in lifespan, clinical and metabolic parameters were evaluated as well as atherosclerosis and heart injury.
Results: In comparison to male *ApoE*$^{-/-}$, male *ApoE*$^{-/-}$:*Ins2*$^{+/Akita}$ mice presented with chronic hyperglycemia (30.8 ± 1.2 mM *vs.* 9.3 ± 0.5 mM) accompanied by extremely high levels of total plasma cholesterol (49.3 ± 6.3 mM *vs.* 30.1 ± 1.5 mM) and triglycerides (11.6 ± 1.7 mM *vs.* 2.36 ± 0.18 mM). These mice have atherosclerosis at multiple vascular sites, including aortic sinus, ascending and descending aorta, brachiocephalic artery and coronary arteries. In addition, myocardial infarcts and a significant reduction of the lifespan (close to 20% of survival *vs.* other groups) were observed. Distinctively, both strains of female mice presented a parallel increase in plasma lipids, atherosclerosis, and no effects on mortality.
Conclusions: We have established a diabetic mouse model, the western-diet-fed male *ApoE*$^{-/-}$:*Ins2*$^{+/Akita}$ mouse, with profound cardiovascular disease involving extensive atherosclerosis, coronary artery disease and myocardial infarct resulting in shortened lifespan.

1. Introduction

The prevalence of diabetes mellitus and impaired glucose tolerance are increasing dramatically and will include over 15% of the global adult population by the year 2025 [1]. This increase is driven by the continued worldwide trends toward increased urbanization, aging populations, disproportionate diets, lack of physical activity, and other unhealthy lifestyles. Diabetes mellitus has been associated with a cardiovascular disease mortality rate that exceeds 70%, and patients with type 2 diabetes who have no history of cardiac infarction have a prevalence of coronary atherosclerosis and a risk of heart attack equivalent to euglycemic patients who have coronary atherosclerosis and have suffered a prior myocardial infarction [2–4].

To facilitate the investigation and identification of the relevant molecular and physiological mechanisms that link diabetes and atherosclerosis, several different mouse models of diabetes-induced atherosclerosis have been developed [5–9]. The Ins2$^{+/Akita}$ mouse represents a genetic alternative to chemically induced

* Corresponding author. Geoff H Werstuck Thrombosis and Atherosclerosis Research Institute, 237 Barton Street East, Hamilton General Campus, McMaster University Hamilton, Ontario L8L 2X2, Canada.
E-mail address: Geoff.Werstuck@taari.ca (G.H. Werstuck).

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hyperglycemia. This mouse carries a spontaneous mutation (C96Y) in the *Insulin 2* gene that disrupts a disulfide bond between the insulin A and B chains. The resulting insulin protein cannot be properly processed leading to endoplasmic reticulum (ER) stress and beta cell dysfunction [10]. It has been postulated that ER stress-induced beta cell death is the major cause of insulinopenia in the $Ins2^{+/Akita}$ mouse [11–13]. Subsequent studies have suggested that the formation of mutant proinsulin-derived aggregates sequester the wild type proinsulin leading to the ER retention and degradation of mutant-wild proinsulin as the cause of the decrease in circulating insulin [14].

When crossed into an $ApoE^{-/-}$ genetic background and fed a regular chow diet, $Ins2^{+/Akita}$ male mice develop accelerated atherosclerosis [15]. We have independently created an $ApoE^{-/-}$:$Ins2^{+/Akita}$ mouse strain in a *C57BL/J6* genetic background and confirmed the accelerated atherosclerosis [16]. In addition, we have noted striking sex-dependent differences in this mouse model that were not previously reported. Female $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice are only transiently hyperglycemic and do not develop relative dyslipidemia compared to age and sex matched $ApoE^{-/-}$ controls. Male $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice are chronically hyperglycemic, develop enhanced cholesterolemia and apparent insulin resistance. In spite of the advanced atherosclerosis quantified at the aortic sinus of the $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice, no effects on mortality and coronary arteries were detected at up to 25 weeks of age. Here, we hypothesize that the combination of chronic hyperglycemia with a diet rich in fat content will produce a more severe model of accelerated atherosclerosis that may affect the cardiac anatomy and/or physiology of the $ApoE^{-/-}$:$Ins2^{+/Akita}$ mouse.

2. Materials and methods

2.1. Mice

Male $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice were crossed with female $ApoE^{-/-}$:$Ins2^{+/+}$ mice to produce $ApoE^{-/-}$:$Ins2^{+/Akita}$ and $ApoE^{-/-}$:$Ins2^{+/+}$ littermates that were used in the following experiments. Genotypes were confirmed by PCR using primers specific for *Ins2* and *ApoE* genes [16]. All the mice used in this study were fed a regular chow diet (RD) which corresponds to 18% of calories derived from fat (2018 Teklad Global 18% Protein Rodent Diet; Harlan Teklad). At 5 weeks of age, mice were randomly allocated to continue receiving the regular chow diet or to receive a western diet (WD) with a high fat content (Teklad Adjusted Calories TD 97363; Harlan Teklad) containing 0.15% cholesterol and 21% anhydrous milk lipids, which correspond to 42% of calories derived from fat. Survival curves were based upon the outcomes of RD-fed $ApoE^{-/-}$ mice, RD-fed $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice, WD-fed $ApoE^{-/-}$ mice and WD-fed $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice. Mice were euthanized once they reached endpoint or 25 weeks of age. Endpoint was defined as exhibiting one or more of the following symptoms: severe duress involving laboured and very slow breathing rate, unsteady gait, or hunched posture.

Clinical parameters, atherosclerosis and myocardial complications were assessed. Mice were anesthetized and blood samples were extracted. After cervical dislocation, the vasculature was rinsed with 5 mL of 0.9% saline. Liver and fat pad were removed and the vasculature was perfusion fixed with 10% neutral buffered formalin (NBF). All the organs were extracted and stored in 10% NBF at 25 °C. All the procedures were pre-approved by the McMaster University Animal Research Ethics Board.

2.2. Atherosclerosis in the aortic sinus

The harvested hearts were cut transversely along the plane of the aortic sinus and the top portions were embedded in paraffin. Paraffin-embedded hearts were sectioned using a microtome and 4.5 μm serial sections were collected on glass slides until atherosclerotic lesions were no longer observed [17]. The area, volume and necrotic core of the lesions were quantified on Masson's trichrome stained sections (Sigma) [16–18]. Calcified lesion areas were detected and quantified by von Kossa staining [19]. The macrophage presence was quantified in the lesions by immunofluorescent staining for Mac-3 (BD Pharmingen). Macrophage polarization was assessed with anti-inducible nitric oxide synthase (iNOS, Abcam) and anti-mannose receptor C type 1 (MRC1, Abcam) antibodies. Smooth muscle cells were evaluated using an anti-α-smooth actin antibody (Santa Cruz Biotechnology). Apoptosis was assessed with an antibody against cleaved caspase 3 (Cell Signalling). All images were captured with an Olympus DP71 digital camera (Olympus) mounted on a Leitz Laborlux S bright field microscope (Leica Microsystems), and assessed using Image J software.

2.3. Coronary artery atherosclerosis and myocardial infarction

The ventricles were processed and embedded in paraffin blocks. The entire tissue was cross sectioned and 10 μm sections were collected onto slides. Slides containing consecutive sections that account for the entire ventricles were stained with Masson's Trichrome to identify atherosclerotic lesions in the coronary arteries and infarcted areas in the ventricles. For each section, the total number of coronary arteries was determined, and each artery was assessed with respect to the degree of occlusion due to atherosclerotic plaque. A grade of 0% occlusion, <50% occlusion, >50% occlusion and 100% occlusion was assigned and an atherosclerotic profile for each heart, indicating the proportion of diseased arteries, was generated [20]. Cross sectioned heart montages were generated using Fiji software to determine ventricle volume and to assess myocardial tissue and estimate total injury. Ischemic heart condition was evaluated by immunohistochemistry for hypoxia-inducible factor 1-α (HIF-1α, Novus Biologicals) and complement component C9 (CCC9, Boster Biological Technology). Apoptosis was evaluated using an ApopTag Fluorescein detection kit (Millipore Sigma). Cardiomyocytes undergoing differentiation were estimated by counting those positively stained for α-smooth actin.

2.4. Atherosclerosis in brachiocephalic arteries

Dissected arteries were manually processed as follows: 10% NBF for 1 h at 42 °C, 70% ethanol for 30 min at 40 °C, 85% ethanol for 30 min at 40 °C, 100% ethanol for 45 min at 40 °C, 100% ethanol for 45 min at 40 °C, 100% ethanol for 45 min at 40 °C, xylene for 1 h at 40 °C, and xylene for 1 h at 40 °C. The tissue was then embedded in paraffin and sectioned into 8 μm serial sections that were stained and analyzed as described above. The antibody against von Willebrand factor (vWF, Dako) was used to detect microvessels.

2.5. Atherosclerosis in en face aortas

Fixed whole aortas were cleaned of surrounding muscle and adventitial fat. They were longitudinally opened and stained for lipid content with Sudan IV (Sigma). Images of the whole aorta were captured using a L320 digital camera (Nikon) and the percentage of the atherosclerotic area was assessed.

2.6. Analysis of plasma

Fasting (6 h) blood glucose levels were measured using a glucometer (LifeScan). Plasma lipid levels were determined using the