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Effect of impaired glucose tolerance on atherosclerotic lesion formation: An evaluation in selectively bred mice with different susceptibilities to glucose intolerance

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A B S T R A C T

Objective: Impaired glucose tolerance (IGT) is an independent risk factor for atherosclerotic cardiovascular disease. However, due to the lack of appropriate animal models, the underlying mechanisms for IGT-induced atherosclerosis remain to be elucidated *in vivo*. We recently used selective breeding to establish 2 mouse lines with distinctively different susceptibilities to diet-induced glucose intolerance, designated selectively bred diet-induced glucose intolerance-resistant (SDG-R) and SDG-prone (SDG-P), respectively. Here, we assessed atherosclerotic lesion formation in these mice.

Methods: Female SDG-R and SDG-P mice were fed an atherogenic diet (AD; 1.25% cholesterol, 0.5% sodium cholate, and 36% energy as fat) for 20 weeks (8–28 weeks of age). Oral glucose tolerance tests were performed during the AD-feeding period. Atherosclerotic lesion formation was quantitatively analyzed in serial aortic sinus sections by oil red O staining. Plasma lipids were measured after the AD-feeding period.

Results: Glucose tolerance was impaired in SDG-P mice as compared to SDG-R mice over the 20-week AD-feeding period. No significant differences were observed in any plasma lipid measurement between the 2 mouse lines. Aortic sinus atherosclerotic lesion formation in SDG-P mice was approximately 4-fold greater than that in SDG-R mice.

Conclusion: In 2 mouse lines with different susceptibilities to diet-induced glucose intolerance, IGT accelerated atherosclerotic lesion formation. These mice may therefore serve as useful *in vivo* models for investigating the causal role of IGT in the pathogenesis of atherosclerosis.

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1. Introduction

Individuals with diabetes have an increased risk for atherosclerotic cardiovascular events, including myocardial infarction, stroke, and peripheral vascular disease [1,2]. In individuals with pre-diabetes as well, impaired glucose tolerance (IGT) is an independent risk factor for cardiovascular disease [3–9]. Although the benefit of intensive glycemic control for improved cardiovascular outcomes did not yield statistically significant results in 2 large prospective studies (the Diabetes Chronic Complications Trial [DCCT] in young subjects with type 1 diabetes [10] and the United Kingdom Prospective Diabetes Study [UKPDS] in subjects with newly diagnosed type 2 diabetes [11]) during the original study periods, long-term follow-up of those subjects revealed that intensive glycemic control early in the course of diabetes reduced the occurrence subsequent cardiovascular events [12,13]. These intriguing observations of the so-called “legacy effect” or “metabolic memory” imply that glucose intolerance in pre-diabetes or early-stage diabetes may play a pivotal role in the initiation of the atherosclerotic process.

A number of animal models have been used to investigate the role of hyperglycemia in atherosclerosis [14,15]. The predominant models used have been genetically atherosclerosis-prone, hypercholesterolemic mice (apolipoprotein E [apoE]- or LDL receptor [LDLR]-deficient mice) in combination with chemical destruction of pancreatic β-cells (by streptozotocin) or crossbreeding with genetically obese type 2 diabetic models (*db/db* or *ob/ob*). However, severe hypercholesterolemia in apoE- or LDLR-deficient mice often masks the effect of hyperglycemia on the atherosclerotic process [16,17]. In addition, both streptozotocin-treated mice and

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genetically obese mice show severe hyperglycemia even under fasting conditions. Thus, these mice serve as appropriate models for established diabetes rather than for IGT early in the course of diabetes.

Recently, we established 2 mouse lines with distinctively different glucose tolerance by selective breeding. In brief, using C57BL/6, C3H, and AKR as background strains, mice exhibiting superior or inferior glucose tolerance after high-fat feeding were selectively bred and designated as selectively bred diet-induced glucose intolerance-resistant (SDG-R) and SDG-prone (SDG-P), respectively [18]. Since SDG-P mice show evident glucose intolerance as compared with SDG-R mice without any apparent difference in fasting blood glucose levels, these mice may serve as appropriate models for studying IGT-related disorders, including atherosclerotic cardiovascular complications. In this study, we assessed atherosclerotic lesion formation in these novel mouse lines.

2. Methods

2.1. Animals and diets

Female SDG-R and SDG-P mice (14th–19th generations [18]) bred at the Institute for Animal Reproduction (Kasumigaura, Japan) were used (see Supplementary Fig. S1 for detailed breeding history). Female mice are known to be more susceptible to atherosclerotic lesion formation than males [19,20]. The mice were fed a standard rodent chow (MF; Oriental Yeast, Tokyo, Japan) until 8 weeks of age. Subsequently, the diet was changed to an atherogenic diet (AD) containing 1.25% cholesterol, 0.5% sodium cholate, and 36% energy as fat (F2HFD1; Oriental Yeast: see Supplementary Table S1 for detailed composition). The mice were maintained on the AD for 20 weeks in standard housing (3 or 4 mice per cage) with a 14-h light (06:00–20:00 h)/10-h dark cycle. Food intake, body weight, and random-fed blood glucose levels were monitored every 4 weeks (at 16:00 h). Daily food intake per mouse was calculated from 1-week food consumption per cage. Circadian blood glucose levels were measured on 1 day in the 14th week of AD feeding; measurements were taken every 4 h. This study was conducted under approval from the institutional animal care and use committee of Nippon Medical School.

2.2. Oral glucose tolerance test (OGTT)

Glucose tolerance was evaluated by OGTT. At 1 week before (under standard rodent chow), 10 week after, and 19 week after the start of AD feeding, overnight-fasted mice were administered a 20% glucose solution (40, 50, and 60 mg glucose/mouse, respectively) by oral gavage. The dose was based on the average body weight at each time point (approximately 2 g/kg). Blood samples were obtained by tail bleeding. Blood glucose was measured with a glucose sensor (Glutest Neo Super; Sanwa Kagaku Kenkyusho, Nagoya, Japan). The insulin concentration of the plasma was measured by ELISA (Ultra Sensitive Mouse Insulin kit; Morinaga Institute of Biological Science, Yokohama, Japan).

2.3. Plasma lipid analysis

At the end of the 20-week AD-feeding period, blood was collected from the inferior vena cava of overnight-fasted mice under anesthesia. Total cholesterol, HDL-cholesterol (sodium phosphotungstate-magnesium chloride precipitation method), triacylglycerols, and non-esterified fatty acids in the blood plasma were measured using commercial kits (Wako Pure Chemical, Osaka, Japan).

2.4. Evaluation of atherosclerotic lesion formation

Atherosclerotic lesion formation in the aortic sinus was quantitatively analyzed based on the method of Paigen et al. [20] with modifications. After perfused *in situ* with saline followed by 4% formaldehyde in PBS from left ventricle, the heart was isolated and further fixed overnight with 4% formaldehyde in PBS. It was then cut at a plane parallel to atrial appendages and the upper part including aortic root was embedded in OCT compound (Sakura Finetek, Tokyo, Japan). Cryostat sections were cut from the left ventricular outflow tract and discarded until 3 valve cusps were shown. Then, 45 serial cross-sections (10-μm thickness) of aortic sinus were prepared (i.e., covered a distance of 450 μm). Of the 45 serial sections, every 5 sections (total of 9 sections each separated by 50 μm) were stained with oil red O and counterstained with hematoxylin. The oil red O-stained sections were examined under a light microscope (AX80; Olympus, Tokyo, Japan) with cellSens imaging software (ver. 1.4.1; Olympus). The oil red O-stained area was determined manually from the photomicrograph images [21] on Photoshop Elements software (ver. 9.0.3; Adobe Systems, San Jose, CA). For each mouse, the oil red O-stained area of the 9 sections was averaged and expressed as the mean lesion size.

Immunohistochemical staining was performed to confirm macrophage infiltration into the atherosclerotic lesions. In brief, serial sections to the oil red O-stained ones were stained with MOMA-2 rat monoclonal antibody to mouse macrophages (AbD Serotec, Oxford, UK) using Vectastain Elite ABC kit (Vector, Burlingame, CA) followed by hematoxylin counter stain.

2.5. Statistical analysis

Values are presented as mean ± SEM. Values of $p < 0.05$ by Student's *t*-test were considered statistically different between the SDG-R and SDG-P groups.

3. Results

3.1. Body weight, food intake, and tissue weight

Over the 20-week AD-feeding period, SDG-P mice had a greater body weight than SDG-R mice (Fig. 1A). Food intake of SDG-P mice was also higher than that of SDG-R mice during most of the feeding period (Fig. 1B). At the end of the feeding period, SDG-P mice showed a greater gonadal fat mass as compared with SDG-R mice, whereas no differences were observed in liver weight (Table 1).

3.2. Glucose tolerance and random-fed blood glucose

In the OGTT, no significant differences were observed in fasting blood glucose levels between the SDG-R and the SDG-P mouse groups at 1 week before, 10 weeks after, and 19 weeks after the start of AD feeding (Fig. 2A–C; 0 min). However, SDG-P mice showed higher blood glucose levels at 30 min and subsequent times after glucose injection as compared with SDG-R mice during the 20-week AD-feeding period (Fig. 2A–C). Although a statistical difference was observed in the fasting insulin levels before the start of AD feeding, no such differences were observed in post-challenge insulin levels over the feeding period (Fig. 2E–G).

Random-fed blood glucose levels were higher in SDG-P mice than in SDG-R mice over the AD-feeding period (Fig. 3A). In addition, the circadian blood glucose profile revealed that SDG-P mice had higher blood glucose levels throughout the day with greater fluctuations than those seen in SDG-R mice (Fig. 3B). The standard deviations of individual circadian blood glucose levels (6 time

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