Atherosclerosis 263 (2017) 7–14

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Type 1 diabetes increases retention of low-density lipoprotein in the atherosclerosis-prone area of the murine aorta

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ARTICLE INFO

Article history:
Received 24 October 2016
Received in revised form
2 May 2017
Accepted 17 May 2017
Available online 18 May 2017

Keywords:
Low-density lipoprotein
Retention
Type 1 diabetes

ABSTRACT

Background and aims: Individuals with type 1 diabetes mellitus are at high risk of developing atherosclerotic cardiovascular disease, but the underlying mechanisms by which type 1 diabetes accelerates atherosclerosis remain unknown. Increased retention of low-density lipoprotein (LDL) in atherosclerosis-prone sites of the diabetic vascular wall has been suggested, but direct evidence is lacking. In the present study, we investigated whether retention of LDL is increased in atherosclerotic-prone areas using a murine model of type 1 diabetes.

Methods: Fluorescently-labeled human LDL from healthy non-diabetic individuals was injected into diabetic Ins2Akita mice and non-diabetic, wild-type littermates. The amount of retained LDL after 24 h was quantified by fluorescence microscopy of cryosections and by scans of *en face* preparations. Vascular gene expression in the inner curvature of the aortic arch was analyzed by microarray and quantitative polymerase chain reaction.

Results: LDL retention was readily detected in atherosclerosis-prone areas of the aortic arch being located in both intimal and medial layers. Quantitative microscopy revealed 8.1-fold more retained LDL in type 1 diabetic mice compared to wild-type mice. These findings were confirmed in independent experiments using near-infrared scanning of *en face* preparations of the aorta. Diabetic status did not affect arterial expression of genes known to be involved in LDL retention.

Conclusions: Type 1 diabetes increases the ability of the vascular wall to retain LDL in mice. These changes could contribute to the increased atherosclerotic burden seen in type 1 diabetic patients.

1. Introduction

Individuals with type 1 diabetes mellitus are at high risk of developing atherosclerotic cardiovascular disease (ASCVD) independently of traditional CVD risk factors, and the majority of diabetic patients eventually die from ASCVD [1]. Type 1 diabetes infers many metabolic changes that may influence atherogenesis, but the exact mechanisms by which it accelerates atherosclerosis remain unknown [2]. Most importantly, while glucose control with anti-diabetic drugs today is efficient in lowering the incidence of microvascular complications, it is much less effective in retarding macrovascular complications, including atherosclerosis [3].

The arterial wall of type 1 diabetics is characterized by intimal thickening, functional modifications of the endothelium, increased expression of extracellular proteoglycans, smooth muscle cell proliferation and accumulation of glycated proteins [4–7]. It is widely accepted that a key step in early atherogenesis is retention of apolipoprotein B (apoB)-containing lipoproteins, predominantly low-density lipoprotein (LDL), in atherosclerosis-prone areas of the arterial tree (i.e. the response-to-retention hypothesis) [8]. LDL retention is believed to be mediated initially by binding to glycosaminoglycan side chains of proteoglycans within the extracellular matrix [9,10]. Several types of these are found to be upregulated in type 1 diabetes [11–14], indicating that increased LDL retention in pre-lesional arteries in type 1 diabetes may be a mechanism by

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http://dx.doi.org/10.1016/j.atherosclerosis.2017.05.019
0021-9150/

which type 1 diabetes increases atherosclerosis.

However, only few studies have investigated LDL retention in type 1 diabetes [12,15,16]. Mangat et al. found that retention of remnant lipoproteins in the common carotid artery was increased compared to controls, in an *ex vivo* model of type 1 diabetes [12]. However, straight segments exposed to laminar blood flow, such as the common carotid artery, are protected from plaque development and may, therefore, not be relevant as a test bed to study the causal mechanisms of diabetes-accelerated lesion progression. In contrast, Proctor et al. reported that retention of LDL in the thoracic aorta 2 h after infusion of LDL was not affected by hyperglycemia [15]. To date, no one has investigated the impact of diabetes on LDL retention in natural atherosclerosis-prone areas in a model in which type 1 diabetes has been shown to accelerate atherosclerosis.

We and others have previously shown that Akita mice have increased atherosclerosis development when subjected to hypercholesterolemia [17,18]. In the present study, we provide evidence for a mechanism that may contribute to this trait, by showing that diabetes without concomitant hypercholesterolemia increases the ability of atherosclerosis-prone areas to retain LDL.

2. Materials and methods

An expanded Materials and methods section is available in the Supplemental Materials and methods.

2.1. Animals

Type 1 diabetic mice with a mutation in the *Ins2* gene (C57BL/6-Ins2Akita/J; heterozygous) and C57BL/6J mice were obtained from The Jackson Laboratory, Bar Harbor, ME and bred at Aarhus University to generate heterozygous Ins2Akita/J (Akita) and wild-type (WT) littermate controls. Mice were genotyped for the Akita mutation (which abolished a Fnu4HI site) by restriction enzyme digestion of a PCR amplicon from the *Ins2* gene (Primer set: FW- 5′TGC TGA TGC CCT GGC CTG CT 3′ and RV-5′ TGG TCC CAC ATA TGC ACA TG 3′).

All mice enrolled in the experiments were 25–27-week old males. Before sacrifice, mice were fasted for 6 h and blood glucose was measured using a glucometer (CONTOUR®, Bayer Consumer Care AG, Basel, Switzerland). When blood glucose levels exceeded maximum measurable value, the maximum value (600 mg/dl) was used.

Mice were fed a normal chow and given access to food and water *ad libitum*. All procedures were approved by the Danish Animal Experiments Inspectorate.

2.2. Purification, labeling and infusion of human LDL

Preparation and characterization of fluorescent LDL were done as previously described [19]. Briefly, EDTA-plasma from a healthy, non-fasting individual, with a LDL particle count of 1327 nmol/l and a Lp(a) level of 307 mg/dl, was obtained, and lipoproteins were fractionated by ultracentrifugation at 256,000*g* for 20 h, by loading plasma onto a KBr density gradient column (density layers: 1.006, 1.019, 1.063 and 1.21 g/ml). LDL was collected from the 1.063 g/ml density layer and desalted on a PD10 column with PBS before conjugation with Atto 565 or Atto 680 NHS ester fluorochrome (Sigma Aldrich) at pH 8.3. The purity of the LDL preparation was tested by HPLC, which showed only a small contamination of albumin (<1%) (data not shown). Labeled LDL was purified on a PD10 column with PBS. 24 h before the mice were sacrificed, labeled LDL corresponding to 500 μg protein per mouse was injected through the tail vein.

2.3. Metabolism of LDL

To compare clearance of LDL in Akita (n = 4) and WT (n = 8) mice, Atto 565 labeled human LDL (500 μg protein per mouse) was injected into the tale vein. Blood samples were taken 0, 3, 6, 12 and 24 h post injection, and human apoB-100 in plasma was measured using an ELISA (IBL International) with no cross-reactivity to mouse apoB-100.

2.4. Tissue processing and analysis

For fluorescence microscopy quantification of Atto 565 labeled LDL, the aortic arch, descending aorta, abdominal aorta and the right common iliac artery were harvested. Tissues were cryoprotected, snap-frozen and sectioned at 4 μm thickness. To measure retained LDL in the aortic arch using fluorescence microscopy, sections were analyzed for the presence of Atto 565 signal in an Olympus Cell-R wide field microscope system. LDL was quantified in the center of the inner curvature of the aortic arch in 3 sections taken 40 μm apart, using ImageJ version 1.48v (http://rsbweb.nih.gov/ij/). The final intensity was calculated as the mean of intensities on the 3 images. Pixel values from the green channel were subtracted from the red channel (Atto 565), which enabled exclusion of non-specific fluorescence from elastic laminae. For quantification of Atto 680 labeled LDL, *en face* preparations of thoracic aortas were scanned using the 700 nm channel of a LiCor Odyssey Infrared Imaging System.

Endothelial cells were identified by staining with a polyclonal rabbit anti-human von Willebrand Factor (vWF) antibody (A0082, Dako), followed by staining with a FITC goat anti-rabbit secondary antibody (Jackson ImmunoResearch).

2.5. Microarray

Gene expression analysis (RNA) was performed on a small piece of tissue (Ins2Akita n = 6; WT n = 6) of the inner curvature of the aortic arch (between the branchiocephalic- and left subclavian artery). Total RNA was purified using RNeasy Micro Kit following the manufacturer's instructions (Qiagen). The samples were labeled and hybridized to the Affymetrix GeneChip® Mouse Gene 2.0 ST Array (Santa Clara, CA) covering 35,240 RefSeq transcripts, according to the manufacturer's protocol. All samples were run independently and labeled and scanned in a randomized order to avoid batch effects. Data were processed by the IterPLIER algorithm and analyzed using *R*. Student's *t*-tests. 4 pre-defined over-representation analyses (ORA) (Supplemental Table 2) [19] were performed using GO-Elite8 (http://www.genmapp.org/go_elite/), with default settings and all tested genes as denominator file.

2.6. Real-time PCR

Quantitative polymerase chain reaction (QPCR) was used to confirm a change in proteoglycan 4 (*Prg4*) gene expression (*Prg4*: F-5′ AGTTTGGGGTATTCCCTCTCC 3′ and R-5′ TGAATGTTGCCACCTCTCTTGA 3′). A Brilliant III Ultra Fast SYBR Green QRT-PCR Master Mix (Agilent Technologies) was used on a Stratagene Mx3005P (Agilent Technologies) with the MxPro QPCR Software (Agilent Technologies). Conditions for the reaction were as follows: 10 min at 50 °C and 3 min at 95 °C followed by 40 cycles of 20 s at 95 °C and 20 s at 60 °C. In the first analysis, the same tissues that had been used for the microarray were used. In the second analysis, we re-tested for *Prg4* gene expression in a new group of mice (Ins2Akita n = 8; WT n = 6). Expression levels were estimated and normalized to the mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA level.