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Lipocalin-2 contributes to experimental atherosclerosis in a stage-dependent manner

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ABSTRACT

Background and aims: Lipocalin-2 (Lcn2) is a glycoprotein which can be secreted by immune cells. Several studies in humans have suggested Lcn2 can be used as a biomarker for the detection of unstable atherosclerotic lesions, partly as it is known to interact with MMP-9.
Methods: In this study, we generated *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice to assess the functional role of Lcn2 in different stages of atherosclerosis. Atherosclerotic lesions were characterized through histological analysis and myeloid cell populations were examined using flow cytometry.
Results: We show that *Ldlr*$^{-/-}$ *Lcn2*$^{-/-}$ mice developed larger atherosclerotic lesions during earlier stages of atherosclerosis and had increased circulating Ly6C^{hi} inflammatory monocytes compared to *Ldlr*$^{-/-}$ mice. Advanced atherosclerotic lesions from *Ldlr*$^{-/-}$ *Lcn2*$^{-/-}$ mice had decreased necrotic core area, suggesting Lcn2 deficiency may affect lesion stability. Furthermore, MMP-9 activity was diminished in plaques from *Ldlr*$^{-/-}$*Lcn2*$^{-/-}$ mice.
Conclusions: Altogether, these findings suggest that Lcn2 deficiency promotes lesion growth in earlier stages of the disease while it decreases MMP-9 activity and necrotic core size in advanced atherosclerosis.

1. Introduction

The development of atherosclerosis and subsequent atherosclerotic plaque destabilization are the main underlying pathology of (ischemic) heart disease. Therefore, early detection of unstable atherosclerotic plaques using biomarkers could prove useful to reduce the incidence of acute cardiovascular syndromes. A potential candidate to use as a biomarker for unstable atherosclerosis is lipocalin-2 (Lcn2), which is also known as 24p3 in mice and as neutrophil-gelatinase associated lipocalin (NGAL) in humans.

Lcn2 is a secreted glycoprotein, which was originally identified as a product of human neutrophils [1,2]. Upon bacterial infection, Lcn2 functions as a bacteriostatic agent by sequestering iron from bacterial siderophores, such as enterobactin [3]. During inflammation, Lcn2 can act as an inflammatory mediator by binding N-formylmethionyl-leucyl-phenylalanine and leukotriene B4 (LTB4) [4]. Interestingly, Lcn2 itself can also act as a chemoattractant for neutrophils during infection [5]. Furthermore, Lcn2 can form a complex with matrix metalloproteinase (MMP)-9, thereby preventing it from being inhibited by tissue inhibitor of metalloproteinases-1 [6]. Active MMP-9 inside atherosclerotic lesions can degrade extracellular matrix and may thus contribute to advanced plaque instability [7,8]. By stabilizing active MMP-9, Lcn2 may contribute to the degradation of the fibrous cap and destabilization of atherosclerotic plaques in general [9]. In line, several reports suggest that serum NGAL levels can be used to predict the incidence of cardiac events. Serum NGAL levels were for example shown to correlate with levels of C-reactive protein and to predict major adverse cardiac event (MACE) as well as all-cause mortality in patients with a history of CVD [10–12]. Furthermore, serum NGAL/MMP-9 complex levels are associated with MACE in patients 1 year after coronary angiography [13]. Serum NGAL levels were higher in patients with angiographically confirmed coronary artery disease compared to patients without. Additionally, serum NGAL levels were associated with the number of diseased vessels,

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suggesting serum NGAL levels might be indicative of the severity of disease [14]. Furthermore, patients with symptomatic atherosclerosis in carotid arteries were shown to have higher levels of serum NGAL as compared to asymptomatic patients [15]. mRNA expression of NGAL was also elevated in atherosclerotic plaques of patients with symptomatic carotid atherosclerosis compared to asymptomatic patients [16]. Local NGAL protein levels were also demonstrated to be elevated in unstable *versus* stable plaques and NGAL content correlated specifically with MMP-9 activity [17]. In mouse models for atherosclerosis, Lcn2 was shown to colocalize with MMP-9 in atherosclerotic plaques [18].

Lcn2 is actually a very pleiotropic protein, as it is also associated with the development of metabolic diseases, which can subsequently contribute to cardiovascular disease [19]. Serum NGAL levels were elevated in obese individuals compared to lean controls and correlated with insulin resistance [20]. Furthermore, NGAL expression was shown to be elevated in visceral adipose tissue of obese individuals compared to non-obese controls. In adipose tissue, enzymatic activity of NGAL/MMP-9 complexes was increased in obese individuals compared to lean controls [21]. Despite the evidence from literature which suggests that Lcn2 is associated with the development of atherosclerosis and obesity, there are still many questions regarding the exact pathophysiological role for Lcn2 in atherogenesis. Mechanistic insights gained from experimental models studying Lcn2 in multiple stages of atherosclerosis could contribute to the applicability of Lcn2 as a biomarker for early detection of unstable atherosclerosis and coronary artery disease. In this study, we thus aimed to investigate the contribution of Lcn2 to different stages of diet-induced atherosclerosis. We show here that Lcn2 has a stage-dependent contribution to experimental atherosclerosis as it seems to limit lesion development, whereas it potentially contributes to plaque instability in more advanced stages of atherosclerosis.

2. Materials and methods

2.1. Mice

All animal work was performed according to the guidelines of the European Parliament Directive 2010/63EU and the experimental work was approved by the Animal Ethics committee of Leiden University. *Ldlr* deficient (*Ldlr*$^{-/-}$) mice were originally purchased from Jackson Laboratory and further bred in the Gorlaeus Laboratory in Leiden, The Netherlands. Lcn2 deficient (*Lcn2*$^{-/-}$) mice were kindly provided by Dr. Mak [22] and were backcrossed to *Ldlr*$^{-/-}$ mice to generate *Ldlr*$^{-/-}$ *Lcn2*$^{-/-}$ mice. The animals were kept under standard laboratory conditions and were fed a normal chow diet and water *ad libitum*, unless otherwise stated.

2.2. Microarray on non-constrictive collar-induced carotid atherosclerosis

To determine *Lcn2* gene expression levels during atherosclerotic lesion development, RNA was extracted from atherosclerotic lesions as previously described [23]. In short, *Ldlr*$^{-/-}$ mice were fed a Western-type diet (WTD) (Special Diet Services) two weeks before surgery and throughout the experiment. To determine the gene expression levels in plaques, atherosclerotic carotid artery lesions were induced by perivascular collar placement as described previously [24]. Both common carotid arteries were excised, snap-frozen in liquid nitrogen and stored at −80 °C until further use. Three carotid artery segments carrying carotid plaques from 2, 4, 6, 8 or 10 weeks after collar placement ($t = 2$ until $t = 10$) were pooled for each sample and homogenized by grounding in liquid nitrogen with a pestle [25,26]. Carotid arteries without atherosclerosis from mice which were only fed a WTD for two weeks served as a control ($t = 0$). Per time point, we performed a microarray on three pooled samples.

An additional collar-induced atherosclerosis experiment with a similar design was performed in parallel to generate RNA samples for real-time quantitative PCR analysis as described below. In this setup we again pooled three carotids into one sample and generated the following pooled samples per time point: for $t = 0$ ($n = 4$), for $t = 2,4$ or 6 ($n = 5$) for $t = 8$ ($n = 4$) and for $t = 10$ ($n = 3$). In addition we also stored carotids from *Ldlr*$^{-/-}$ mice that had not been fed a Western type diet and these carotids were used to compare with the $t = 0$ time point, which reflects carotids that were in a hyperlipidemic environment but did not contain atherosclerotic plaques. Total RNA was extracted from the tissue homogenates using Trizol reagent according to manufacturer's instructions (Invitrogen). Gene expression profiles were generated using the Illumina Bead-Chip Whole Genome Microarray platform (ServiceXS).

2.3. Atherosclerosis

Diet-induced atherosclerosis was established by feeding female *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Lcn2*$^{-/-}$ mice from 9 to 12 weeks of age a WTD containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services). Atherosclerosis was induced for either 6 or 12 weeks to study atherosclerotic lesions in an earlier or more advanced stage. At the end-point of the study, the mice were ane-sthetized by subcutaneous injections with ketamine (100 mg/mL), sedazine (25 mg/mL) and atropine (0.5 mg/mL) after which their vascular system was perfused with PBS at a continuous low flow via heart puncture in the left ventricle. The hearts were collected to examine the atherosclerotic lesions in the aortic root through histological and morphometric analysis.

2.4. Histological and morphometric analysis of atherosclerotic lesions

All hearts were embedded in O.C.T. compound (Sakura) and sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the trivalve leaflets, 10 μm sections were collected. Mean plaque size (in μm^2) was calculated from five sequential sections, displaying the highest plaque content, using an Oil-red-O staining (Sigma), which stains neutral lipids. Monocytes and macrophages were visualized using a Moma2 antibody (1:1000, Serotec) and an alkaline phosphatase conjugated secondary antibody (1:100, Sigma). To quantify smooth muscle cell content, smooth muscle cells were visualized by an α-smooth muscle cell actin (αSMA) staining (1:1000, Abcam) and a horseradish peroxidase conjugated secondary antibody. Collagen content inside the plaques was quantified using a Mason's Trichrome staining (Sigma). In the same sections, necrotic areas were identified as intimal a-cellular, debris-like areas. Neutrophils were stained for using the naphtol AS-D chloroacetate esterase staining kit (Sigma). Image quantification was performed blinded for genotype using the Leica Image analysis system (Leica Ltd). Apoptotic cells were stained using the *In situ* Cell Death Detection Kit (Sigma) per the manufacturer's protocol. On the same sections, macrophages were stained using a rat-anti-mouse F4/80 antibody (1:100, Biorad) and an Alexa Fluor 647 conjugated goat-anti-rat IgG as a secondary antibody (1:100, Thermo Fisher Scientific). Nuclei were visualized using Fluoroshield mounting medium with DAPI (Sigma). To quantify apoptosis, TUNEL positive nuclei were quantified inside atherosclerotic lesions. To quantify apoptotic macrophages, TUNEL positive nuclei which were colocalized with F4/80 staining were quantified. Quantification of apoptosis was performed using a Nikon TiE 2000 confocal microscope.