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Research Paper

Human Neutrophil Peptide 1 Limits Hypercholesterolemia-induced Atherosclerosis by Increasing Hepatic LDL Clearance

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ABSTRACT

Increases in plasma LDL-cholesterol have unequivocally been established as a causal risk factor for atherosclerosis. Hence, strategies for lowering of LDL-cholesterol may have immediate therapeutic relevance. Here we study the role of human neutrophil peptide 1 (HNP1) in a mouse model of atherosclerosis and identify its potent atheroprotective effect both upon transgenic overexpression and therapeutic delivery. The effect was found to be due to a reduction of plasma LDL-cholesterol. Mechanistically, HNP1 binds to apolipoproteins enriched in LDL. This interaction facilitates clearance of LDL particles in the liver via LDL receptor. Thus, we here identify a non-redundant mechanism by which HNP1 allows for reduction of LDL-cholesterol, a process that may be therapeutically instructed to lower cardiovascular risk.

1. Introduction

Atherosclerosis represents the most important cause of morbidity and mortality in developed countries. Intrinsically atherosclerotic vascular disease is an inflammatory condition characterized by aberrant lipid metabolism and a maladapted inflammatory response (Libby et al., 2013). Despite the success of lipid-lowering statins, mortality from atherosclerosis-related pathologies remains high and alternative lipid-targeting approaches are being intensely investigated (Rader, 2016). Based on the inverse association of plasma high-density lipoprotein (HDL)-cholesterol levels and cardiovascular events (Assmann et al., 2002) the 'HDL hypothesis' was formulated wherein an increase in HDL-cholesterol would lead to reduction in adverse cardiovascular events. While preclinical studies with HDL infusion, apolipoprotein (Apo) A1 overexpression or inhibition of cholesterylester transfer protein resulted in inhibition or regression of atherosclerosis (Badimon et al., 1990; Tangirala et al., 1999; Kühnast et al., 2015), recent randomized clinical trials using HDL-cholesterol-raising drugs were largely disappointing (AIM-HIGH Investigators et al., 2011; Barter et al., 2007; Schwartz et al., 2012) hence challenging the importance of HDL cholesterol levels in cardiovascular disease. In contrast, clinical trials of low-density lipoprotein (LDL) lowering drugs as well as careful studies of human genetics of LDL-cholesterol and their relationship to cardiovascular risk have unequivocally established LDL as a causal risk factor. Recent strategies to inhibit proprotein convertase subtilisin/kexin type 9 (PCSK9) have corroborated the efficacy of LDL lowering therapies, although neurocognitive side effects, parenteral delivery routes and costs question the long-term feasibility of this approach (Sabatine et al., 2015). Thus, alternative LDL-cholesterol lowering strategies may be beneficial to a large cohort of patients with hypercholesterolemia (Rader, 2016).

While atherosclerosis-related inflammation is thought to be predominantly macrophage-driven, recent evidence points towards the

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importance of neutrophils (Drechsler et al., 2010). These stimulate atherosclerotic lesion formation by releasing the granule protein cathelicidin which paves the way for inflammatory monocytes (Döring et al., 2012). The most abundant neutrophil-derived granule proteins, however, are human neutrophil peptides (HNPs) comprising approximately 5% of total neutrophil protein. HNPs are antimicrobial polypeptides which exert various inflammatory effects when released extracellularly (Choi et al., 2012). As an example, HNPs can stimulate macrophage polarization towards an inflammatory phenotype (Soehnlein et al., 2008) or enhance microvascular permeability (Bdeir et al., 2010). On the other hand, HNPs are strongly cationic and hence show promiscuous, charge-dependent interactions. In this context heteromers comprised of CCL5 and HNP1 were recently shown to strongly stimulate recruitment of classical monocytes (Alard et al., 2015). In addition, HNP1 was shown to interact with Lp(a) (Bdeir et al., 1999) but the pathophysiological relevance of such interaction remains unclear. Here, we study the role of HNP1 in hypercholesterolemia-induced atherosclerosis and witness its strongly protective effect. HNP1-dependent atheroprotection related to enhanced hepatic clearance of HNP1-LDL complexes, a mechanism that could be therapeutically targeted by repetitive HNP1 delivery.

2. Materials and Methods

2.1. Atherosclerosis Studies

HNP1$^{tg/tg}$ mice (Bdeir et al., 2010) were intercrossed with *Apoe*$^{-/-}$ mice to generate double mutant mice. All genetically modified animals were backcrossed to C57Bl/6 background for at least 10 generations. Female mice were fed a high-fat diet (HFD) (21% fat and 0.15% cholesterol, ssniff) for four weeks to induce early atherosclerotic lesions. In a separate set of experiments, female *Apoe*$^{-/-}$ mice (from The Jackson Laboratory) were treated with HNP1 (10 μg, i.v., every other day, Bachem) or vehicle (PBS, i.v.) during the last four weeks of an eight week period of HFD feeding. While mice in Fig. 1 were obtained from our own breeding facility, mice in Fig. 2 were obtained from a commercial breeder which may explain the differences in atherosclerotic lesion sizes after 4 weeks of HFD feeding.

2.2. Histology, Immunohistochemistry, and Immunofluorescence

The extent of atherosclerosis was assessed in aortic roots by HE staining, lipid depositions were assessed following Oil Red O staining. To define neutrophil and monocyte/macrophage numbers in atherosclerotic plaques, frozen sections of aortic roots were stained with antibodies to Ly6G (1A8, BD Biosciences) and Mac2 (Cedarlane) with a 1:100 dilution. After incubation with a secondary antibody for 30 min at room temperature, sections were analyzed. Nuclei were counterstained by 4′,6-Diamidino-2-phenylindol (DAPI). Liver sections from mice treated with Dil-LDL (Purified human LDL labelled with DiI (1,1′-dioctadecyl- 3,3,3′,3′-tetramethylindocarbocyanine perchlorate), Kalen) in presence or absence of HNP1 were stained with anti-LDLR (Abcam, EP1553Y). A Leica DM4000 microscope with a 25/×0.95 water emersion objective (Leica Microsystems) and a Leica DFC 365FX camera were used to capture images. Leica Qwin Imaging software (Leica Ltd.) was employed for image analysis.

2.3. Lipid Fractionation

For lipoprotein separation, samples from 10 animals per group were pooled (0.25 ml) and subjected to fast performance liquid chromatography (FPLC) gel filtration on two Superose 6 columns connected in series as described previously (Hofmann et al., 2008).

2.4. Flow Cytometry

Blood leukocyte counts were quantified by flow cytometry. Staining of single cell suspensions of blood was conducted using combinations of

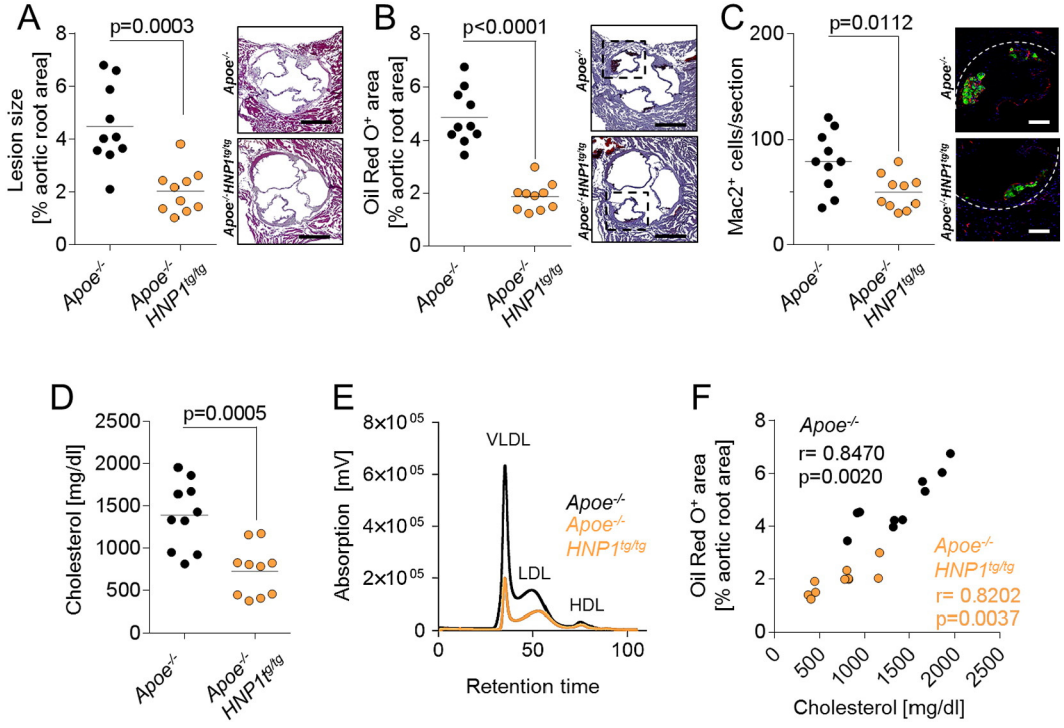

Fig. 1. Transgenic expression of HNP1 protects from atherosclerosis. *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*HNP1*$^{tg/tg}$ mice were fed a high-fat diet for 4 weeks. (A) Quantification of atherosclerotic lesion sizes in HE-stained aortic root sections. (B) Assessment of lipid deposition in in Oil Red O-stained aortic root sections. (C) Analysis of Mac2^{+} cells indicating macrophage accumulation. Fluorescence images represent macrophages. Valves are zoomed in as indicated by dashed boxes in panel (B). (D) Plasma cholesterol levels. (E) FPLC-assisted fractionation of plasma lipids. (F) Pearson correlation of plasma cholesterol levels and lipid deposition (Oil Red O^{+} area) in aortic root sections. Data in A–D were analyzed by unpaired *t*-test. Scale bars represent 500 μm (A, B) or 100 μm (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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