The International Journal of Biochemistry & Cell Biology 83 (2017) 56–64

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Hexim1 heterozygosity stabilizes atherosclerotic plaque and decreased steatosis in ApoE null mice fed atherogenic diet

Manya Dhar-Mascareno[a], Inna Rozenberg[b], Jahangir Iqbal[b,1], M. Mahmood Hussain[b], Daniel Beckles[b,c], Eduardo Mascareno[b,*]

[a] Department of Biological Sciences, State University of New York, College at Old Westbury, Old Westbury, New York 11568, USA
[b] Department of Cell Biology, State University of New York Downstate Medical Center, Brooklyn, New York, 11203 USA
[c] Departments of Surgery, Medicine and Cell Biology, State University of New York Downstate Medical Center, Brooklyn, New York, 11203 USA

ARTICLE INFO

Article history:
Received 25 August 2016
Received in revised form
14 December 2016
Accepted 19 December 2016
Available online 21 December 2016

Keywords:
Hexim1
miR145
Smooth muscle α-actin
SOCS3
High fat diet
Atherosclerosis
Triglycerides
Steatosis

ABSTRACT

Hexim-1 is an inhibitor of RNA polymerase II transcription elongation. Decreased Hexim-1 expression in animal models of chronic diseases such as left ventricular hypertrophy, obesity and cancer triggered significant changes in adaptation and remodeling. The main aim of this study was to evaluate the role of Hexim1 in lipid metabolism focused in the progression of atherosclerosis and steatosis. We used the C57BL6 apolipoprotein E (ApoE null) crossed bred to C57BL6 Hexim1 heterozygous mice to obtain ApoE null – Hexim1 heterozygous mice (ApoE-HT). Both ApoE null backgrounds were fed high fat diet for twelve weeks. Then, we evaluated lipid metabolism, atherosclerotic plaque formation and liver steatosis. In order to understand changes in the transcriptome of both backgrounds during the progression of steatosis, we performed Affymetrix mouse 430 2.0 microarray. After 12 weeks of HFD, ApoE null and ApoE-HT showed similar increase of cholesterol and triglycerides in plasma. Plaque composition was altered in ApoE-HT. Additionally, liver triglycerides and steatosis were decreased in ApoE-HT mice. Affymetrix analysis revealed that decreased steatosis might be due to impaired inducible SOCS3 expression in ApoE-HT mice. In conclusion, decreased Hexim-1 expression does not alter cholesterol metabolism in ApoE null background after HFD. However, it promotes stable atherosclerotic plaque and decreased steatosis by promoting the anti-inflammatory TGFβ pathway and blocking the expression of the inducible and pro-inflammatory expression of SOCS3 respectively.

1. Introduction

Atherosclerosis is the main cause of morbidity and mortality among patients with coronary artery diseases (CAD). Several factor may independently contribute to atherosclerosis including lipid metabolism, genetic, and environmental factors that initiate a chronic inflammation of the arterial wall, and apoptosis-induced cell death (Libby and Theroux 2005). Although initially asymptomatic, the establishment of an atherosclerotic plaque entails an inflammatory process with extracellular matrix remodeling, followed by plaque formation. Dependent on plaque content, this process could trigger thrombosis resulting in acute coronary syndromes (ACS) (Libby and Theroux 2005; Napoli et al., 2006; Hansson et al., 2015).

Transforming growth factor β (TGF-β) has been established as an important contributing factor during the progression and remodeling of atherosclerosis (Kamato et al., 2013). TGF-β is an anti-inflammatory cytokine that promotes extracellular matrix (ECM) synthesis in smooth muscle cell (SMC), inhibit the expression of adhesion molecules, proliferation and migration of endothelial cells, and inhibits foam-cell formation in macrophages (Kamato et al., 2013). The contractile phenotype of SMC expresses contractile proteins, including smooth muscle myosin heavy chain (smMHC) and smooth muscle α-actin (SM α-actin). During atherosclerotic plaque formation, smooth muscles cell eventually transitions to a phenotype characterized by a synthetic migratory, proliferative and pro-atherogenic program directing synthesis of cholesterol receptors (LDL and VLDL) and adhesion molecules (Doran et al., 2008). The contractile to synthetic transition determines the type of collagen synthesized and how this collagen contributes to plaque stability. This is brought about by a switch from producing type I

* Corresponding author at: Department of Cell Biology, State University of New York, Downstate Medical Center, 450 Clarkson Ave New York, NY 11203, USA.
E-mail address: emascareno@downstate.edu (E. Mascareno).
[1] Current address: King Abdulaziz Medical City Hospital, Al Hasa, KAIMRC, MNGHA, Al Hasa 31982, Saudi Arabia.

http://dx.doi.org/10.1016/j.biocel.2016.12.010

and type III fibrillar collagen to producing proteoglycan, fibronectin and scattered type I collagen that together enhances the uptake of cholesterol.

The stability of atherosclerotic plaques is a function of a number of factors including the extent of inflammation, neovascularization, hemorrhage, calcification, matrix remodeling, apoptosis and the nature of fibrous-cap, and the lipid-rich core (Silvestre-Roig et al., 2014), and the cell type such as endothelial cells, smooth muscle cells, lymphocytes, neutrophils and macrophages (Napoli et al., 2006). Enhancement of the inflammatory response mediated by lymphocyte and macrophage activity will often leads to increased smooth muscle cell (SMC) apoptosis, decreased fibrous cap, formation and expansion of the necrotic core, all this results in an unstable plaque. SMCs play a key role in promoting a stable plaque because formation of a fibrous cap is dependent on the deposition of ECM proteins including elastin, and collagen.

The expression levels of microRNAs miR143 and miR145 play an inhibitory role during atherosclerosis because these miRNAs promote a contractile SMC phenotype that maintains the expression of contractile but not ECM proteins. Accordingly, decreased expression of miR143 and miR-145 has been observed in the synthetic type of SMC present in established atherosclerotic plaque (O'Sullivan et al., 2011; Wei et al., 2013).

Current evidence indicates that non-alcoholic fatty liver disease (NAFLD) enhances the risk of atherosclerotic plaque formation (Villela-Nogueira et al., 2016), presumably through its ability to promote inflammatory cascades (Targher et al., 2007; Moon et al., 2015). Decreased expression levels of suppressor of cytokine signaling 3 (SOCS3), an established inhibitor of the pro-inflammatory Jak/Stat pathway has been reported to protect against liver steatosis (Ueki et al., 2004). Liver specific deletion of the Jak2 kinase, a key tyrosine kinase that activates downstream transducers of the Jak/Stat pathway such as Stat3, was reported to confer protection against NAFLD in mice fed high fat diet (HFD) (Shi et al., 2012). Together, these findings suggest a role for the Jak/Stat pathway during NAFLD, and although the Jak/Stat pathway is a known transducer of the pro-inflammatory signals, its role during progression of atherosclerosis is not well understood (Dutzmann et al., 2015).

Our laboratory studies Hexim1, an inhibitor of RNA polymerase II dependent transcription elongation. Hexim1 knock-out is embryonic lethal, and Hexim1 heterozygous mice developed normal unless subjected to stress (Huang et al., 2004). Indeed, Hexim1 heterozygous mice are known to modulate events in prostate cancer progression, remodeling of the left ventricle during cardiac hypertrophy and obesity (Dey et al., 2007; Mascareno et al., 2012a, 2012b; Dhar-Mascareno et al., 2016). We have shown that in heart-targeted Angiotensin II transgenic mice carrying one allele for Hexim1 gene, TGF β signaling is enhanced via a CDK9-dependent Smad3 serine phosphorylation mechanism (Alarcon et al., 2009; Mascareno et al., 2012a). Further evidence for Smad3 activation by Hexim1 comes from experiments in which primary embryonic fibroblasts with decreased or null Hexim1 expression showed increased Smad3-S^{208}, which correlated with increased smooth muscle α-actin expression (Mascareno et al., 2012a). Our previous work has also shown that decreased Hexim1 expression regulates obesity by enhancing the leptin-Jak2-Stat3 axis (Dhar-Mascareno et al., 2016), and inhibits adipogenesis (Dhar-Mascareno et al., 2016). Additionally, there is evidence that Hexim1 inhibits smooth muscle cell proliferation and NFκ beta activity (Ouchida et al., 2003).

In this report we asked whether Hexim1 expression levels alter the progression of atherosclerosis. To this end, we used the established apolipoprotein E knock out (ApoE null) mouse as a model of atherosclerosis (Zhang et al., 1994) and crossbred these mice with C57BL6 Hexim1 heterozygous mice (HT). The ApoE null and ApoE null x Hexim1 heterozygous mice (ApoE-HT) were fed HFD and evaluated for lipid profile, atherosclerosis and nonalcoholic liver steatosis (NHLS). Additionally, given the role of Hexim1 in modulating RNA polymerase II dependent transcription elongation, we performed whole transcription analysis in the liver of ApoE null and ApoE-HT mice fed HFD.

Our studies revealed that Hexim1 protein levels modulate the expression of the TGFβ signaling pathway including genes that play a significant role in establishing the SMC contractile phenotype. Hexim1 inhibits the expression of SOCS3, a negative feedback modulator of the cytokine-dependent Jak/Stat signaling pathway that regulates NAFLD. These findings suggest that decreasing Hexim1 expression may provide a molecular pathway to increase plaque stability and decrease NAFLD.

2. Methods

2.1. Animals and diet

C57BL/6 ApoE null mice were purchased from Jackson laboratory, Hexim1 heterozygous mice (HT) were backcrossed onto a pure C57BL/6 background for more than eight generations (Mascareno et al., 2012a). Male mice were maintained in groups of five in a climate-controlled room with a 12:12 light-dark cycle, measuring body weight weekly. Ten weeks old littermate male ApoE and ApoE-HT mice were fed HFD pellets (Diet D12492, Research Diets Inc.) or standard chow pellets (5010 Lab diet) for 12 weeks. HFD provided ~60% of calories as lipids, whereas standard chow provided only 12%.

2.2. PCR screening

Identification of Hexim1 genotype was obtained with the following strategy.

Genomic DNA was obtained from the ear using the hot shot method. PCR was performed with the following primers

forward primer 5′-AACCTCCTCTCCTTGCGCACCAACTC-3′

reverse primer 5′-TACTGTCCTCCTTGGGCACCCGTTCC-3′

Neomycin resistance gene primer 5′-TACCGGTGGATGTGGAATGTGTGCGA-3′. Samples were amplified for 30 cycles (denaturation: 30 s at 94 °C, annealing: 30 s at 68 °C, and elongation: 1 min at 72 °C).

Screening for ApoE genotype was according to Jackson laboratory protocol.

oIMR0180 primer 5′-GCCTAGCCGAGGGAGAGCCG-3′

oIMR0181 primer 5′-TGTGACTTGGGAGCTCTGCAGC-3′

oIMR0182 primer 5′-GCCGCCCCGACTGCATCT-3′

Samples were amplified for 35 cycles (denaturation: 30 s at 94 °C, annealing: 40 s at 68 °C, and elongation: 1 min at 72 °C). Supplementary Fig. S1 depicts the outcome of a PCR screening.

2.3. Plasma and liver lipids

Following overnight fast, blood was collected from mice and total plasma cholesterol and triglyceride levels were measured using commercial kits (Thermo Trace). Fast protein liquid chromatography (FPLC) using a Superose 6 column at a flow rate of 0.2 ml/min and 200 μl fractions was used to separate plasma lipoproteins from pool plasma of ApoE null and ApoE-HT mice (n = 4) fed HFD. Liver tissues (100 mg) were homogenized in a low-salt buffer (1 mM Tris-HCl pH = 7.6, 1 mM EGTA, 1 mM $MgCl_2$), centrifuged and supernatant used to determine protein and lipid extraction. Cholesterol and triglycerides were determined according to manufacturer protocol (Thermo Trace).