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Genetic and environmental risk factors for atherosclerosis regulate transcription of phosphatase and actin regulating gene *PHACTR1*

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ABSTRACT

Background and aims: Coronary artery disease (CAD) risk is associated with non-coding genetic variants at the phosphatase and actin regulating protein 1(*PHACTR1*) gene locus. The *PHACTR1* gene encodes an actin-binding protein with phosphatase regulating activity. The mechanism whereby PHACTR1 influences CAD risk is unknown. We hypothesized that *PHACTR1* would be expressed in human cell types relevant to CAD and regulated by atherogenic or genetic factors.

Methods and results: Using immunohistochemistry, we demonstrate that PHACTR1 protein is expressed strongly in human atherosclerotic plaque macrophages, lipid-laden foam cells, adventitial lymphocytes and endothelial cells. Using a combination of genomic analysis and molecular techniques, we demonstrate that PHACTR1 is expressed as multiple previously uncharacterized transcripts in macrophages, foam cells, lymphocytes and endothelial cells. Immunoblotting confirmed a total absence of PHACTR1 in vascular smooth muscle cells. Real-time quantitative PCR showed that *PHACTR1* is regulated by atherogenic and inflammatory stimuli. In aortic endothelial cells, oxLDL and TNF-alpha both upregulated an intermediate length transcript. A short transcript expressed only in immune cells was upregulated in macrophages by oxidized low-density lipoprotein, and oxidized phospholipids but suppressed by lipopolysaccharide or TNF-alpha. In primary human macrophages, we identified a novel expression quantitative trait locus (eQTL) specific for this short transcript, whereby the risk allele at CAD risk SNP rs9349379 is associated with reduced PHACTR1 expression, similar to the effect of an inflammatory stimulus.

Conclusions: Our data demonstrate that *PHACTR1* is a key atherosclerosis candidate gene since it is regulated by atherogenic stimuli in macrophages and endothelial cells and we identify an effect of the genetic risk variant on PHACTR1 expression in macrophages that is similar to that of an inflammatory stimulus.

1. Introduction

Coronary artery disease (CAD) is caused by atherosclerosis, a form of chronic inflammation. Deposition of pro-inflammatory lipoproteins in arterial walls is associated with the development of atherosclerotic plaques that can obstruct blood flow and trigger the formation of occlusive thrombi [1,2]. CAD is a complex disease with a strong heritable component [3]. Genome-wide association studies (GWAS) have identified 58 genomic regions where single nucleotide polymorphisms (SNPs) are associated with CAD risk [4,5]. Most of these disease-associated variants are in non-coding DNA and likely exert their effect predominantly by altering transcription factor binding to regulatory DNA elements [4,6–8]. A number of CAD risk loci contain genes that were not previously implicated in coronary artery disease and for which no mechanism

Abbreviations: CAD, coronary artery disease; GWAS, genome wide association studies; SNP, single nucleotide polymorphism; PHACTR1, phosphatase and actin regulator 1; oxLDL, oxidized low density lipoprotein; PP1, protein phosphatase 1; PBMC, peripheral blood mononuclear cells; TSS, transcription start site; RACE, rapid amplification of cDNA ends; HAEC, human aortic endothelial cells; LPS, lipopolysaccharide; NLS, nuclear localization signal; DAB, diaminobenzidine.

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has been identified to account for the risk associated with the reported genetic variant. Understanding the effect of these genetic risk variants on the expression and function of the genes that they regulate will advance our understanding of atherosclerosis and could lead to new therapeutic targets.

Several studies have independently replicated an association between CAD and SNPs at a genetic locus containing the phosphatase and actin regulator 1 (*PHACTR1*) gene [9–12]. SNPs at the *PHACTR1* locus are associated with the specific phenotypes of early onset myocardial infarction, coronary artery calcification [11,12] and with an intermediate phenotype of impaired central hemodynamic indices, indicating abnormal vascular stiffness [13]. The *PHACTR1* locus is pleiotropic since the protective alleles of the CAD risk SNPs are associated with an increased risk of ischemic stroke caused by cervical artery dissection, a form of non-atherosclerotic vascular disease [14]. The variants reported in these studies lie in an intronic region of *PHACTR1* over 250 kb away from any other gene. A genetic fine mapping study suggested that rs9349379 was the most likely causative variant at the locus and it was associated with expression of *PHACTR1* mRNA in composite right coronary artery tissue [15]. An expression quantitative trait locus (eQTL) study assaying gene expression in diverse human tissues showed that rs9349379 also affected *PHACTR1* mRNA expression in aortic artery and tibial artery tissue [16]. In a whole genome epigenetic and expression study, we recently demonstrated that *PHACTR1* is one of the most highly upregulated genes in macrophages exposed to oxLDL [17].

The *PHACTR* family consists of 4 genes encoding proteins that interact directly with both actin and protein phosphatase 1 (PP1) [18]. *PHACTR1* was originally cloned from a rat brain cDNA library using a yeast two-hybrid system with PP1 as bait [18]. The human *PHACTR1* gene is on chromosome 6 and a transcript with a 1743 bp open reading frame has been cloned from human brain [19]. The resulting 580 amino acid protein has 4 highly conserved actin-binding RPEL domains and both mouse and rat orthologues have been shown to bind actin [18,20]. Human PHACTR1 protein contains nuclear localization signal (NLS) motifs at both ends of the protein that, in the mouse ortholog, have been shown to facilitate importin-dependent nuclear translocation in response to serum stimulation [20]. Binding of the RPEL domains to G-actin maintains PHACTR1 in a cytoplasmic location [20]. Serum induces Rho-dependent remodeling of actin from G- to F-actin and translocation of PHACTR1 into the nucleus [20].

PP1 is part of a family of serine/threonine phosphatases that are present in both the nucleus and cytoplasm [21]. The enzymatic specificity of PP1 enzymes is achieved in part by their association with accessory proteins [22]. PHACTR1 may function as an accessory protein since PHACTR1 binds to PP1 and inhibits its activity *in vitro* [18]. PHACTR1 may also affect actin structure as siRNA-mediated PHACTR1 knockdown in human umbilical vein endothelial cells (HUVEC) reduces F-actin filament numbers and repartitioning as well as increasing cell protrusion dynamics [23]. Although the precise cellular role of PHACTR1 remains to be determined, overexpression and siRNA-mediated knockdown experiments suggest a role in cell motility and vascular morphogenesis [20,23,24].

Atherosclerosis involves the interplay of genetic factors with atherogenic stimuli, such as modified low-density lipoprotein, in cell types including macrophages, lymphocytes, endothelial cells and vascular smooth muscle cells [1,25–27]. The role of PHACTR1 in these cells and the mechanism whereby it alters CAD risk are unknown. Understanding the role of PHACTR1 is complicated by the fact that the human *PHACTR1* gene is predicted to encode multiple transcripts that have not been characterized. In this study, we hypothesized that PHACTR1 is regulated by atherogenic or inflammatory stimuli and that its expression is influenced by CAD-associated genetic variants. After establishing the expression of PHACTR1 protein in atherosclerotic lesions and the profile of human *PHACTR1* transcripts in primary cell types involved in atherosclerosis, we determined the responses of these transcripts to inflammatory stimuli and to atherogenic lipid. The role of PHACTR1 in CAD was highlighted by its *in vivo* abundance in macrophages and foam cells in human atherosclerotic plaque. The CAD risk allele at SNP rs9349379 was associated with significantly reduced expression of a short transcript in macrophages such that the risk genotype mirrors the effect of an inflammatory stimulus.

2. Materials and methods

2.1. Ethical approval

Ethical approval for the study was obtained from the NHS Research Ethics Committee (South Central-Hampshire B, reference 13/SC/0392) and all participants provided written informed consent.

2.2. Cell isolation, culture and reagents

Primary human coronary artery vascular smooth muscle cells (Invitrogen, Carlsbad, CA) were cultured in Medium 231 (Invitrogen) and Smooth Muscle Growth Supplement (Invitrogen). Primary human aortic endothelial cells (Invitrogen) were cultured in Medium 200 (Invitrogen) with Low Serum Growth Supplement. $CD14^+$ monocytes, T cells ($CD3^+$) and B cells ($CD19^+$) were isolated from the peripheral blood of healthy human volunteers. Blood was centrifuged over Ficoll-Paque PLUS (GE Healthcare LifeSciences, Buckinghamshire, UK) to isolate peripheral blood mononuclear cells (PBMCs). Subsets of cells were isolated using positive selection with magnetic bead-conjugated antibodies (Miltenyi Biotec, Bergisch Gladback, Germany). Individual populations were confirmed to be >95% pure using flow cytometry for the markers used for positive selection. The cells remaining after $CD14^+$ cells were magnetically separated from PBMCs were termed $CD14^+$-depleted PBMCs and constitute a mixed predominantly lymphocytic population. $CD14^+$ monocytes were differentiated into macrophages by 7 days of culture in RPMI 1640 medium with 10% fetal calf serum, 4 mM L-glutamine, 50 units/ml penicillin and 50 mcg/ml streptomycin (Sigma, St Louis, MO), supplemented with 50 ng/ml macrophage colony stimulating factor (M-CSF, eBioscience, San Diego, CA). Foam cells were generated by treating macrophages with human oxLDL (50 mcg/ml) for 48 h. Foam cell formation was confirmed by oil red O staining for intracellular neutral lipid by light microscopy. Macrophage and foam cell viability was shown to be >98% using the Invitrogen LIVE/DEAD microscopy kit.

Ultracentrifugation of freshly isolated human plasma using a discontinuous potassium bromide gradient was used to purify LDL with density 1.019–1.063 g/ml [28]. LDL was extensively dialyzed against PBS, and incubated with 25 μM $CuCl_2$ at 37° for 18 h to produce oxidized-LDL. To terminate oxidation 1 mM EDTA was added and oxLDL was stored at 4°. The TBARS assay was used to confirm that LDL was oxidized (Caymen Chemical, Ann Arbor, Michigan). Careful precautions were taken to ensure low endotoxin levels and LDL was tested for endotoxin using the gel clot method after heating to 75° for 15 min to remove the plasma inhibitor (Associates of Cape Cod, East Falmouth, MA). LDL was used at levels of <0.1 EU/ml.

2.3. Genotyping of rs9349379

Individuals providing blood samples were genotyped by the