Journal of Molecular and Cellular Cardiology 84 (2015) 84–94

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Loss of p27 phosphorylation at Ser10 accelerates early atherogenesis by promoting leukocyte recruitment via RhoA/ROCK

P. Molina-Sánchez [1], R. Chèvre [1], C. Rius, J.J. Fuster [2], V. Andrés *

Laboratory of Molecular and Genetic Cardiovascular Pathophysiology, Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain

ARTICLE INFO

Article history:
Received 19 September 2014
Received in revised form 23 March 2015
Accepted 14 April 2015
Available online 20 April 2015

Keywords:
Atherosclerosis
Endothelial cell
Leukocyte recruitment
p27
RhoA

ABSTRACT

Reduced phosphorylation of the tumor suppressor p27^{Kip1} (p27) at serine 10 (Ser10) is a hallmark of advanced human and mouse atherosclerosis. Apolipoprotein E-null mice defective for this posttranslational modification (apoE$^{-/-}$p27Ser10Ala) exhibited increased atherosclerosis burden at late disease states. Here, we investigated the regulation of p27 phosphorylation in Ser10 at the very initial stages of atherosclerosis and its impact on endothelial-leukocyte interaction and early plaque formation. Hypercholesterolemia in fat-fed apoE$^{-/-}$ mice is associated with a rapid downregulation of p27-phospho-Ser10 in primary endothelial cells (ECs) and in aorta prior to the development of macroscopically-visible lesions. We find that lack of p27 phosphorylation at Ser10 enhances the expression of adhesion molecules in aorta of apoE$^{-/-}$ mice and ECs, and augments endothelial-leukocyte interactions and leukocyte recruitment *in vivo*. These effects correlated with increased RhoA/Rho-associated coiled-coil containing protein kinase (ROCK) signaling in ECs, and inhibition of this pathway with fasudil reduced leukocyte-EC interactions to control levels in the microvasculature of p27Ser10Ala mice. Moreover, apoE$^{-/-}$p27Ser10Ala mice displayed increased leukocyte recruitment and homing to atherosusceptible arteries and augmented early plaque development, which could be blunted with fasudil. In conclusion, our studies demonstrate a very rapid reduction in p27-phospho-Ser10 levels at the onset of atherogenesis, which contributes to early plaque build-up through RhoA/ROCK-induced integrin expression in ECs and enhanced leukocyte recruitment.

1. Introduction

Atherosclerosis and associated ischemic events are the leading cause of morbidity and mortality in Western societies and are predicted to soon become the leading health problem worldwide [1]. Several cardiovascular risk factors have been identified (dyslipidemia, hypertension, diabetes, smoking, aging, sedentary life style, etc.), which promote a chronic inflammatory state that leads to dysfunction of the endothelial cell (EC) monolayer lining the inner arterial surface [2]. One of the earliest manifestations of atherosclerosis is the expression of adhesion molecules by ECs, which triggers the recruitment of circulating leukocytes that normally do not interact with the 'healthy' vessel wall [3–9]. Leukocytes are considered important mediators of all phases of atherosclerosis, from the initiation and progression of asymptomatic lesions to the establishment of complex vulnerable plaques that can rupture and provoke acute ischemic events. Luminal recruitment appears to be the central route of leukocyte infiltration in the subendothelial space during early atherosclerosis since at these stages no *vasa vasora* can be detected, contrary to their reported abundance in late-stage atheromata [10]. Leukocytes roll and adhere to the endothelium to finally extravasate, and monocytes (the predominant leukocyte subset recruited in the damaged vessel wall) differentiate into macrophages that critically contribute to plaque development by secreting an array of inflammatory mediators [3–9].

Studies in animals and humans have identified excessive neointimal cell proliferation as a characteristic of atherosclerosis; consequently, regulation of hyperplastic growth of neointimal cells by tumor suppressors has emerged as a prominent mechanism regulating plaque

Abbreviations: apoE$^{-/-}$, apolipoprotein E-null; apoE$^{-/-}$p27Ser10Ala, apolipoprotein E-null p27 Serine-10-Alanine; BM, bone marrow; CKI, cyclin-dependent kinase inhibitor; DMEM, Dulbecco's modified eagle medium; EC(s), endothelial cell(s); ECGS, endothelial cell growth supplement; EDTA, ethylenediaminetetraacetic acid; ERM, ezrin/radixin/moesin; E-Sel, E-selectin; FBS, fetal bovine serum; GFP, green fluorescent protein; HBSS, Hank's Balanced Salt Solution; HFD, high fat diet; HRP, horseradish peroxidase; ICAM-1, intercellular adhesion molecule-1; IV, intravenous; L-Sel, L-selectin; mAECs, mouse aorta endothelial cells; Mafia, Macrophage Fas-Induced Apoptosis; P-Sel, P-selectin; p27, p27^{Kip1}; p27-phospho-Ser-10, p27 phosphorylation at serine 10; p27Ser10Ala, p27 serine-10-alanine; PBS, phosphate buffered saline; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; ROCK, Rho-associated coiled-coil containing protein kinase; Ser10, serine 10; SDS, sodium dodecyl sulfate; TNFα, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

* Corresponding author at: Laboratory of Molecular and Genetic Cardiovascular Pathophysiology, CNIC, Melchor Fernández Almagro 3, 28029 Madrid, Spain. Tel.: +34 91 453 12 00x1502; fax: +34 91 453 12 65.
E-mail address: vandres@cnic.es (V. Andrés).
[1] Authors with equal contribution.
[2] Current address: Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, USA.

http://dx.doi.org/10.1016/j.yjmcc.2015.04.013
0022-2828/

development [11]. The oncosuppressor $p27^{Kip1}$ (p27) is a cyclin-dependent kinase inhibitor (CKI) that attenuates the proliferation of both vascular smooth muscle cells and macrophages, and also neointimal thickening in animal models of vasculoproliferative disease [11–16]. Interestingly, a micro-RNA-based strategy has been recently developed to selectively inhibit vascular smooth muscle cell hyperplasia while preserving EC proliferation and function, which attenuates restenosis post-angioplasty but allows complete reendothelialization [17].

Compelling evidence has emerged supporting a role for p27 beyond cell cycle control, including modulation of actin cytoskeleton, cell motility, and gene transcription [18–22]. A number of important functional aspects of p27 are regulated through its phosphorylation status [23–31]. We recently reported that sparse phosphorylation of p27 at serine 10 (p27-phospho-Ser10), the most common post-translational modification of p27 [23], is a hallmark of mouse and human atherosclerosis [32]. Moreover, apolipoprotein E-null mice defective for this post-translational modification ($apoE^{-/-}$p27Ser10Ala) did not show alterations in neointimal cell proliferation, yet they had an increased atherosclerosis burden at advanced disease states, at least in part due to increased macrophage foam cell formation [32].

Since p27-phospho-Ser10 is involved in neointimal thickening by modulating proliferation-independent processes, we hypothesized that it could also promote EC dysregulation, which is thought to initiate and sustain atherosclerosis development [2,5–7]. To address this issue, we have used complementary approaches with primary ECs and genetically-engineered mice. Our results demonstrate that loss of p27-phospho-Ser10 occurs rapidly in fat-fed $apoE^{-/-}$ mice and promotes VCAM-1 expression in ECs and leukocyte adhesion *in vivo*. Further, this defect also stimulates the formation of incipient atherosclerotic lesions through activation of the RhoA/Rho-associated coiled-coil containing protein kinase (ROCK) pathway.

2. Material and methods

2.1. Mice and diets

$apoE^{-/-}$p27Ser10Ala mice were generated by crossing non-phosphorylatable p27Ser10Ala knock-in mice [30] with $apoE^{-/-}$ mice (The Jackson laboratory, Madison, WI). Macrophage Fas-Induced Apoptosis (Mafia)-$apoE^{-/-}$ mice [3] were generated by crossing Mafia transgenic mice [33] (which express GFP in the myeloid lineage) with $apoE^{-/-}$ mice. Mafia $apoE^{-/-}$p27Ser10Ala mice were generated by crossing Mafia-$apoE^{-/-}$ mice with $apoE^{-/-}$p27Ser10Ala mice. All mice were on C57BL/6 background. Mice were maintained on a low-fat standard diet (2.8% fat; Panlab, Barcelona, Spain). For studies of diet-induced atherosclerosis, mice were placed on atherogenic high-fat diet (HFD) (10.8% total fat, 0.75% cholesterol, S4892-E010, Ssniff, Germany) for the indicated periods of time. *In vivo* studies were performed with 3-month-old mice. Care of animals was in accordance with institutional guidelines and regulations.

2.2. Blood and serum collection and cholesterol analysis

Mouse blood was extracted by submandibular puncture as described [34]. To obtain serum, 100 μl of blood was collected in polypropylene tubes, the blood was allowed to clot by leaving it undisturbed at room temperature for 30 min, and the clot was removed by centrifugation at 2000 ×g for 15 min at 4 °C. The serum was recovered and stored at −80 °C until analyzed. Total and free cholesterol levels were measured using the automated analyzer Dimension RxL Max Clinical System (Siemens) in serum from mice which were starved overnight.

2.3. Mouse anesthesia and euthanasia

For intravital microscopy experiments (see below), mice were anesthetized with a mixture of ketamine and medetomidine (50 mg/kg and 0.5 mg/kg, respectively; intraperitoneal injection). Mice were euthanized by carbon dioxide inhalation.

2.4. Isolation and culture of mouse aorta endothelial cells (mAECs)

mAECs from p27Ser10Ala mice and wild-type counterparts (both on C57BL/6 backgrund) were isolated as described [35]. Briefly, 8 mice (8 weeks of age) were culled and aortas were rapidly harvested. After removing adipose tissue and adventitia layer, aortas were cut into 1 mm rings. Aortic rings were placed in gelatin (0.5%) pre-coated plates and incubated in mAEC medium: DMEM:F12 (Lonza) containing 1% of penicillin/streptomycin, L-glutamine, 10 mM Hepes, Fungizone, 10% fetal bovine serum (FBS), 0.1 mg/ml of heparin (Sigma-Aldrich) and 50 μg/ml of endothelial cell growth supplement (ECGS, BD). After 7–12 days, mAECs were selected with CD102 antibody (Purified Rat Anti-Mouse CD102-ICAM-2 Monoclonal Antibody, BD Pharmigen) and with a secondary antibody linked to magnetic beads (Dynabeads Sheep anti-Rat IgG, Invitrogen) using a magnetic platform (DynaMag-15 Magnet, Life Technologies). These cells (passage 0) were expanded in gelatin-coated plates containing mAEC medium. All cells used for assays were between passages 4 and 7.

2.5. Isolation of bone marrow (BM) cells

BM cells were harvested from $apoE^{-/-}$, $apoE^{-/-}$p27Ser10Ala and Mafia-$apoE^{-/-}$ mice as described [36]. Briefly, femurs and tibiae were obtained from mice after carefully removing the surrounding skeletal muscle and fat tissue. Both ends of each bone were trimmed to expose the interior and the BM was flushed with Hank's Balanced Salt Solution (HBSS) containing 2 mM EDTA using a 1-ml insulin syringe with a 27 G needle. The collected BM was disaggregated by pipetting and red blood cells were eliminated by 5 min incubation with cold lysis buffer (KH_4Cl 0.15 M, $KHCO_3$ 0.01 M, EDTA.N_2 0.01 M, pH 7.4). After washing with PBS, BM nucleated cells were kept on ice until used.

2.6. Leukocyte adhesion in cremasteric vessels

Mice were anesthetized and the cremaster muscle was dissected free of surrounding tissues and exteriorized onto an optical clear viewing pedestal. The muscle was cut longitudinally with a cautery and held extended at the corners of the exposed tissue using surgical suture. To maintain the correct temperature and physiological conditions, the muscle was perfused continuously with warmed Tyrode's buffer. Four hours before surgery, animals were injected with 100 μl of TNFα (0.5 μg in 0.3 ml saline) to promote leukocyte–endothelial cell interactions. The cremasteric microcirculation was then observed using a Leica DM6000-FS intravital microscopy with an Apo 40× NA 1.0 water-immersion objective equipped with a DFC350-FX camera. LAS-AF software was employed for acquisition and image processing. Five randomly-selected arterioles were analyzed per mouse, and leukocyte adhesion was measured in 150-μm vessel segments for 5 min. When indicated, IgG isotype control (AB-108-C, R&D Systems) or polyclonal anti-VCAM-1 antibodies (sc-1504, Santa Cruz Biotechnology) were injected intravenously prior to intravital microscopy (100 μg/mouse).

2.7. Intravital imaging of carotid artery and rolling

Mice were anesthetized, the neck was shaved, and animals were immobilized in decubitus position. The right carotid artery was exposed and carefully dissected from the surrounding tissues [37] to perform intravital imaging as described [3]. In brief, sutures were used to maintain the salivary gland and adjacent muscles away from the artery, and the right vague nerve was carefully separated from the artery. Throughout the procedure, warm saline was applied to the tissues. The carotid artery was stabilized and placed under the water-dipping objective of an epifluorescence microscope with constant perfusion with warm