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Activation of CD137 signaling accelerates vascular calcification in vivo and vitro

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ABSTRACT

Objectives: Vascular calcification is a characteristic feature of atherosclerosis and is considered as an independent predictor of cardiovascular risk. CD137 signaling has previously shown to be involved in atherosclerosis. However, the possible role of CD137 signaling in regulation of vascular calcification has not been reported. In the present study, we investigated the effect of CD137 signaling on vascular calcification in ApoE$^{-/-}$ mice and in vascular smooth muscle cells (VSMCs) of mice.

Methods: Calcium deposition and muscle fibers in vivo or vitro were identified by von-Kossa and Masson's trichrome staining respectively. Alkaline phosphatase (ALP) activity was measured by the ALP assay Kit. The presence of bone morphogenic protein 2 (BMP2) and runt-related transcription factor 2 (Runx2) was detected by real-time PCR, Western blot and immunofluorescence in vitro or vivo.

Results: Our data shows that activation of CD137 signaling by intraperitoneal injection of agonist-CD137 antibody increased the areas of vascular calcification. Activation of CD137 signaling also increased the expression of BMP2 and Runx2 in the atherosclerotic plaques. In vitro, activation of CD137 signaling also aggravated VSMC calcification, while blocking CD137 signaling could alleviate agonist-CD137 induced VSMC calcification. In addition, the levels of calcium, BMP2 and Runx2, indicators of calcification, were all significantly elevated in agonist-CD137 group in VSMCs.

Conclusion: Our data revealed a previously unrecognized role of CD137 signaling in vascular calcification in vivo and vitro and provides a novel target for prevention and treatment of atherosclerosis in the future.

1. Introduction

Vascular calcification, which refers to the ectopic deposition of calcium phosphate crystal in cardiovascular tissue, is a common characteristic of advanced atherosclerotic lesion and a well-known independent predictive risk factor of subsequent cardiovascular morbidity and mortality [1]. Recent evidence suggested that vascular smooth muscle cell (VSMC) differentiation to osteogenic cell and express bone related proteins with concomitant down-regulation of SMC contractile protein play a pivotal role in vascular calcification [2,3]. However, the molecular mechanisms that regulate VSMC osteogenic transdifferentiation are complex and poorly understood.

Accumulated evidence has demonstrated that inflammation may play an important role in VSMC osteogenic transdifferentiation [4]. CD137, a member of the tumor necrosis factor receptor superfamily (TNFRSF), is mainly expressed in a variety of immune cells which include natural killer (NK) cells, neutrophils, CD4 + CD25 + regulatory T (Treg) cells, resting monocytes, and dendritic cells (DCs). However, under proinflammatory conditions, it is also expressed in some non-immune cells, such as VSMCs and endothelial cells [5,6]. Olofsson and colleagues showed that CD137 is expressed in human atherosclerotic plaques and promotes the development of plaque [7]. Another study demonstrated that CD137 not only regulates T-cell activation as a co-stimulatory receptor, but also mediates atherosclerosis. Deficiency of CD137 reduces atherosclerosis in mice on both chow and high-fat diets [8]. In addition, our previous study showed that CD137-CD137L signaling pathway played a positive role in facilitating atheromatous plaque formation and progression. Furthermore, inhibition of CD137-CD137L signaling significantly inhibited the formation of atherosclerotic lesions in apolipoprotein E-deficient (ApoE$^{-/-}$) mice [9,10].

Based on these studies, we hypothesize that CD137-CD137L signaling pathway plays a critical role in the progression of atherosclerotic calcification. In this study, our study demonstrates that activation of CD137 signaling exacerbated vascular calcification in vivo and aggravated VSMC calcification in vitro. The mechanisms may involve increasing VSMC osteogenic differentiation.

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2. Materials and methods

2.1. Animals

15 ApoE$^{-/-}$ mice aged 8 weeks were purchased from vital river laboratories (Distributor of Jackson Laboratory, Beijing, China). All the animals were housed under a 12-h light-dark cycle, and 23 ± 2 °C under 55 ± 10% humidity, in normal cages with free access to water and provision of high fat foods. At the age of 13 weeks, the mice were randomly divided into the following groups: agonist-CD137 group, anti-CD137 group, and the control group. Mice in each group were intraperitoneally injected with 200 μg agonist-CD137 antibody (R&D), 200 μg anti-CD137 antibody + 200 μg agonist-CD137 antibody, and 200 μg IgG 2b (eBioscience) at 13, 15, and 17 weeks of age respectively. At 19 weeks of age, the mice were euthanized using 8% chlorate hydrate and aortas were pushed with phosphate-buffered saline through the left ventricle. Aortas, from the proximal ascending aorta to the bifurcation were freed from connective tissue under a dissection microscope. Aortas were fixed in 10% formaldehyde in PBS overnight, and further embedded in paraffin, and then the paraffin sections were cut out from the aortic arch to the thoracic artery. We chose the same position (2 mm above the aortic valve, Fig. 1A) of the short-axial slice among groups as the representative image. All animal experiments were reviewed and approved by the Animal Care and Use Committee of Jiangsu University.

2.2. Analysis of vascular lesions in ApoE$^{-/-}$ mice

Hematoxylin/eosin (H&E) was used to assess tissue architecture. Additionally, sections were stained with the following staining kits according to supplied protocol: von-Kossa, Masson's trichrome, and ALP. Digital images of arterials were captured using an Olympus microscope, and quantitative analyses of indicated stains were performed using image-pro plus 6.0 (IPP6.0) software.

2.3. Immunohistochemical analysis

We performed immunohistochemical staining on 5 μm thick paraffin-embedded sections, which were prepared from 4% formaldehyde-fixed tissue. The following primary antibodies were used: anti-Runx2 (immunoway) and anti-BMP2 (immunoway). After incubation with primary antibody overnight at 4 °C, horseradish peroxidase-conjugated secondary antibody was used in the second step followed by 3,3-diaminobenzidine to visualize the antigen. Nuclei were stained with hematoxylin. Negative controls were routinely employed.

2.4. VMSC culture

Primary VSMCs were obtained from mouse thoracic aorta by Patch-attaching method as previously described [11] and identified by immunofluorescence staining for smooth muscle specific α-actin antibody (α-SMA, Sigma). Briefly, adventitia and intima were striped from segments of thoracic aorta, and the remaining tunica media was cut to 1–3 mm^3 pieces. Then, the small pieces were cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose, 4.5 g/L; Gibco) supplemented with 20% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. After migrated from the explants, cells were collected and maintained in growth medium. Passage 4–6 VSMCs were used in experiments.

2.5. In vitro calcification and quantification of VSMCs

Calcification of VSMCs was induced as previously described. Briefly, VSMCs were calcified with medium containing DMEM, 6% FBS, and 10 mmol/L β-glycerophosphate (β-GP). To induce CD137 expression, cells were treated with TNFα (10 ng/ml) for 24 h [11]. Then, the following incubations were performed: agonist-CD137 (10 μg/ml), anti-CD137 (10 μg/ml) + agonist-CD137 (10 μg/ml), and IgG 2b (10 μg/ml). The calcification

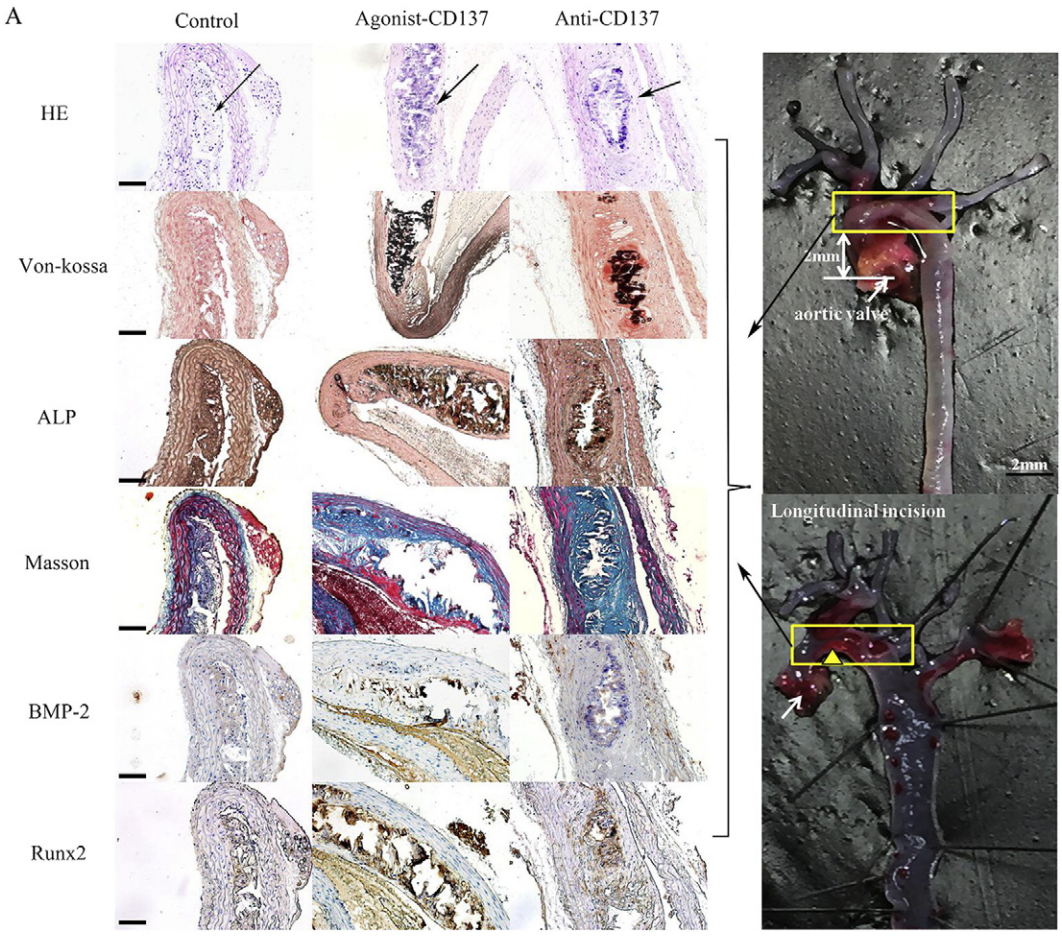

Fig. 1. Activation of CD137 signaling promotes atherosclerotic calcification and accelerates osteogenic cell formation in ApoE$^{-/-}$ mice. (A) En face aorta with oil red O staining: from the proximal ascending aorta to the thoracic aorta. Short-axial slice of the aorta (yellow box area), 2 mm above the aortic valve (white arrows), red oil o staining positive area (yellow triangle). Histological analysis of aortas, 200× magnification: Hematoxylin/eosin (H&E) staining is used to assess tissue architecture; black arrow denotes osteogenic-like cells. The calcification was checked by von-Kossa staining. For further characterization of the calcified lesions, the lesions were stained for alkaline phosphatase (ALP) and fibers. Masson's trichrome staining shows that muscle fibers and collagen fibers are stained red and blue, respectively. The expression of BMP2 and Runx2 was stained by immunohistochemistry. Scale bar: 100 μm. The quantization bar graph of the plaque areas was below the pictures (D). (B) Quantitation of von-Kossa staining. (C) Quantitation of BMP2 and Runx2 expression. IOD: integrated optical density. Data are shown as mean ± SD, $n = 5$. In all panels, * and ** denote significance with $p < 0.05$ and $p < 0.01$ respectively, by Tukey's procedure.