Downregulation of Pin1 in human atherosclerosis and its association with vascular smooth muscle cell senescence

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ABSTRACT

Objective: Pin1 is prevalently overexpressed in human cancers and implicated to regulate cell growth and apoptosis. Thus far, however, no role for Pin1 has been described in modulating vascular smooth muscle cell (VSMC) senescence.

Methods: Immunohistochemistry and Western blotting were used to assess Pin1 protein level in human normal and atherosclerotic tissues. β-galactosidase staining, cumulative population doubling level, telomerase activity, and relative telomere length measurement were used to confirm VSMC senescence. The expressions of Pin1 and other genes involved in this research were analyzed by quantitative reverse-transcription polymerase chain reaction and Western blotting in VSMCs. Apolipoprotein E gene-deleted mice (ApoE$^{-/-}$) fed a high-fat diet were treated with juglone or 10% ethanol, respectively, for 3 weeks. The extent of atherosclerosis was evaluated by Oil Red O, Masson trichrome staining, and immunohistology.

Results: Pin1 protein level decreased in human atherosclerotic tissues and VSMCs, synchronously with increased VSMC senescence. Adenoviral-mediated Pin1 overexpression rescued cellular senescence in atherosclerotic VSMCs, with concurrent down-regulation of P53, p21, growth arrest and DNA-damage-inducible protein 45-alpha (Gadd45a), phosphorylated retinoblastoma (p-pRb), p65 and upregulation of cyclin subfamilies (cyclin B, D, and E), and cyclin-dependent kinase subfamilies (2, 4, and 6), whereas Pin1 knockdown resulted in the converse effects, indicating that VSMC senescence mediated by Pin1 is an integrated response to diverse signals. In vivo data from ApoE$^{-/-}$ mice showed that treatment of juglone led to accelerated atherosclerosis development.

Conclusions: Altogether this work supports a role for Pin1 as a vital modulator of VSMC senescence, thereby providing a novel target for regulation and control of atherosclerosis. (J Vasc Surg 2017;■:1-11.)

Clinical Relevance: We found that decreased Pin1 protein level in human atherosclerotic tissues and vascular smooth muscle cells (VSMCs) was related to increased VSMC senescence, and in vivo data from apolipoprotein E$^{-/-}$mice showed that treatment of a Pin1 inhibitor led to accelerated atherosclerosis development. This research indicated that interventions targeted at Pin1, such as cell-specific drugs, are potential novel approaches to the retardation of atherosclerosis, in which VSMC senescence has a prominent role. Patients with atherosclerosis may benefit from pharmacologic interference with Pin1.

Cellular senescence limits the proliferation of normal cells and shows a series of changes in morphology and physiology, including a flat and enlarged morphology, increased acidic β-galactosidase (Gal) activity, and altered gene expression pattern, including P53.[1,2] Atherosclerosis has been regarded as a chronic inflammatory disease, and several potential mechanisms have been reported, but accumulating evidence has suggested a pivotal role of vascular cell senescence in atherogenesis.[3] As the most abundant cell intrinsic to the vessel wall, vascular smooth muscle cells (VSMCs) play a major role in the pathologic progression of atherosclerosis.[4,5]

Pin1 is the only enzyme that can induce a conformational change in a specific protein through mediating the isomerization of specific phosphorylated Ser/Thr-Pro motifs in a subset of proteins. Such activity suggests a novel and tightly controlled mechanism regulating a series of protein functions during physiologic and pathologic conditions.[6,7] Acting as a molecular timer, Pin1 potentates cell cycle progression, cell division, and cellular senescence.[2,8]

Pin1 acts as an important modulator in the etiopathogenesis of Alzheimer disease and many human cancers.[9] Our recent studies uncovered the provocative role of Pin1 in regulating VSMC proliferation,[10,11] but the role of this critical regulatory molecule in VSMC senescence has not been previously examined. Interestingly, the present study observed decreased Pin1 expression level in atherosclerotic tissues and VSMCs concurrently with enhanced

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VSMC senescence. We therefore sought to determine the contribution, if any, of reduced Pin1 expression in human VSMC senescence and the resultant effect on atherosclerosis.

METHODS

An expanded Methods section is available in the Supplementary Methods (online only).

Samples of atherosclerotic femoral arteries and healthy arteries were obtained from patients undergoing bypass surgery or traumatic amputation, respectively, under informed consent using protocols approved by Renji Hospital Institutional Review Board. Immunohistochemistry and Western blotting were performed according to the manufacturer's instructions.

For cell culture studies, primary VSMCs were isolated from the arterial tissues. Cell senescence, cumulative population doubling level, and related signaling pathways were determined. For in vivo studies, apolipoprotein E gene-deleted (ApoE$^{-/-}$) mice fed a high-fat diet received juglone or only ethanol 3 weeks before euthanasia and tissue harvest.

All data are presented as the mean ± standard error of the mean. Statistical comparison was performed using the Student *t*-test or one-way analysis of variance with a Bonferroni multiple comparison post hoc test. A value of $P < .05$ was considered statistically significant.

RESULTS

Pin1 is downregulated in VSMCs in human atherosclerosis. We first examined Pin1 expression in human atherosclerotic femoral arteries and healthy femoral arteries. There was no statistical difference in patient characteristics between groups, including age, gender, the percentage of patients with hypertension or diabetes mellitus, or who smoked cigarettes or had taken statins within the last 3 months (Supplementary Table I, online only). Immunohistochemistry analysis showed Pin1 protein expression was markedly reduced in atherosclerotic tissues compared with that of the corresponding controls (Fig 1, *A*). Because arterial segments comprise the vessel intima, media, and adventitia with a heterogeneous mixture of cells, we cultured VSMCs from human atherosclerotic or normal femoral arteries. Pin1 protein expression were significantly decreased in atherosclerotic VSMCs vs normal VSMCs (Fig. 1, *B* and *C*) using Western blotting.

Cellular senescence is accelerated in atherosclerotic VSMCs and inhibited by Pin1 overexpression. Previous studies have shown that vascular cellular senescence might contribute to atherosclerosis. Therefore, senescence-associated (SA)-β-Gal staining was applied to enumerate senescent VSMCs. As shown in Fig 2, we observed that the percentage of cells positive for SA-β-Gal was increased in atherosclerotic VSMCs. In agreement, telomerase activity and relative telomere length were suppressed in atherosclerotic VSMCs. We also found a marked decrease of proliferative lifespan occurred in atherosclerotic VSMCs (Fig 2, *D*), which is also a marker of senescence. Interestingly, overexpression of Pin1 rescued atherosclerotic VSMCs from senescence (Fig 2, *G*), and Pin1 knockdown further accelerated senescence in atherosclerotic VSMCs (Fig 2, *H*). Thus, Pin1 expression was reduced in atherosclerotic VSMCs associated with promoted cell senescence.

Pin1 mediates VSMC senescence. To further explore the mechanisms by which Pin1 prevents VSMC senescence, we examined several important senescent regulators in VSMCs. As shown in Fig 3, the protein and messenger (m)RNA levels of P53, p21, and growth arrest and DNA-damage-inducible protein 45-alpha (Gadd45a) were augmented in atherosclerotic VSMCs, concomitantly associated with downregulated Pin1 expression. The expression of growth arrest and DNA-damage-inducible protein 45-beta (Gadd45b) remained unchanged in atherosclerotic VSMCs. however. We further examined whether exogenous expression of Pin1 affected the expression of P53, p21, Gadd45a, and Gadd45b. The results showed Pin1 overexpression caused a marked decrease in levels of P53, p21, and Gadd45a in atherosclerotic VSMCs, despite a substantially unchanged expression of Gadd45b, with concurrent reduced VSMC senescence. In contrast, Pin1 knockdown in atherosclerotic VSMCs triggered upregulation of the expression of P53, p21, and Gadd45a, but not Gadd45b.

We then asked whether induction of senescence in VSMCs was prevented independently of Pin1 expression. By knocking down P53 in VSMCs from atherosclerotic or normal arteries, we observed that suppression of P53 alleviated VSMC senescence in the presence or absence of Pin1 (Supplementary Fig 1, online only). To test the hypothesis that Pin1 regulates VSMC senescence through a P53 mechanism, we first transfected VSMCs with adenovirus-mediated (Ad)-Pin1 together with Ad-LacZ or Ad-P53 and determined cell senescence by SA-β-Gal staining. Compared with the control,

ARTICLE HIGHLIGHTS

- **Type of Research:** Prospective experimental study
- **Take Home Message:** Decreased Pin1 protein level in human atherosclerotic tissues and VSMCs was related to increased VSMC senescence, and in vivo data from apolipoprotein E$^{-/-}$ mice showed that treatment of a Pin1 inhibitor led to accelerated atherosclerosis.
- **Recommendation:** The authors suggest that determination of the mechanism by which Pin1 controls VSMC senescence may find a target for treatment of vascular disease.