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The c-Myb functions as a downstream target of PDGF-mediated survival signal in vascular smooth muscle cells

Yilan Chen [a,1], Hong Xu [a,1], Jin Liu [a,1], Chongjie Zhang [b], Achim Leutz [c,d], Xianming Mo [a,*]

[a] *Laboratory of Stem Cell Biology, State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China*
[b] *Department of Immunology, Sichuan University, Chengdu 610041, China*
[c] *Max-Delbruck-Center for Molecular Medicine, 13125 Berlin, Germany*
[d] *Humboldt-University Berlin, Unter den Linden 6, 10099 Berlin, Germany*

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Abstract

Apoptosis of vascular smooth muscle cell (VSMC) is one of the major pathologic features in atherosclerosis. The platelet-derived growth factor (PDGF) pathway has been shown to provide survival signals in VSMCs and PDGF receptors are also highly expressed in VSMCs contained in the plaques of atherosclerosis. However, the downstream targets of PDGF signaling are unclear. In the current study, we show that PDGF signals stimulate the protein expression of c-Myb in human arterial VSMCs. Inhibition of c-Myb function in VSMCs enhanced apoptosis in PDGF treated VSMCs. Our data suggest that c-Myb functions as a downstream target of the PDGF survival pathway and suggest that c-Myb plays an essential role in adult VSMC survival.

Keywords: c-Myb; Vascular smooth muscle cell; Apoptosis; PDGF

Vascular smooth muscle cells (VSMCs) are the main cellular components in the medial layer of adult arteries and secret synthetic products to form the matrix of the arterial medial layer. VSMCs accumulate in the lesion areas of atherosclerosis and arterial restenosis. Recent studies have demonstrated that VSMCs undergo apoptosis in the atherosclerosis plaques [1,2]. Advanced lesion and plaques from patients with unstable symptoms show higher level of apoptosis, suggesting that VSMCs apoptosis contributes to plaque rupture in patients [2,3]. VSMC apoptosis has also been shown to stimulate inflammation, calcification, thrombosis, and aneurysm formation in the plaques [4]. Therefore, prevention of VSMC apoptosis in atherosclerosis plaques may improve the treatment of patients with atherosclerosis.

Many signaling pathways including PDGF signaling are involved in the survival of VSMCs. PDGF is a heparin-binding growth factor of disulfide-bonded A, B, C, and D polypeptides that can assemble into the homodimers PDGF-AA, -BB, -CC, and -DD [5]. The A and B polypeptides also form heterodimers, denoted PDGF-AB. PDGF transduces cellular responses by binding to two related protein tyrosine kinase receptors, PDGF receptor alpha and beta (PDGFRα and β) [6]. PDGF-BB binds to PDGFRα and β with similar affinity, whereas PDGF-AA only binds to PDGFRα. Binding of PDGF to its receptors leads to activation of the receptor tyrosine kinase and to subsequent initiation of cytoplasmic signal transduction pathways that in turn induces migration, proliferation, and differentiation in PDGF-responsive cell types. PDGF receptors are present on VSMCs in atherosclerosis plaques and in restenosis [7]. Within these lesions, PDGF has been identified to be released from activated platelets, smooth muscle cells

* Corresponding author. Fax: +86 28 85164047.
E-mail address: xmingmo@yahoo.com (X. Mo).
[1] These authors contributed equally to this work.

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SMCs, and monocytes and acitvates the PDGF pathways to mediate SMCs proliferation and migration [7]. In vitro studies have demonstrated that truncated PDGFRα, which blocks the activation of both PDGF-AA and -BB, induces apoptosis in human arterial VSMCs, indicating PDGF signaling is important for the survival of VSMCs.

Various studies have suggested that c-Myb plays a central role in cell cycle progression and in VSMC replication [8,9]. Here, we have shown that PDGF-BB promotes c-Myb expression in human arterial VSMCs. Overexpression of c-Myb inhibits apoptosis of VSMCs induced by truncated PDGFRα. Inhibition of c-Myb function induces massive apoptosis in VSMCs treated with PDGF. These results suggest that c-Myb functions as a downstream target of PDGF signaling to maintain the survival of VSMCs.

Methods

Cell culture. VSMCs were derived from human thoracic aorta (Clonetics Corp., San Diego, California, USA) and routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 U/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2. Cells from passage 5 to 9 were used for all studies. The cell purity was assessed by specific morphology and immunostaining with monoclonal antibody specific for smooth muscle α-actin.

Adenovirus infection. Adenoviral vectors Ad5, AdMenT, AdMyb, and AdPDGFRαTr have been described previously [10,11]. Cells were infected with adenovirus incubated overnight with a particle/infectious unit ratio of approximately 10:1. Medium was replaced and cells were counted at different times as indicated.

Western blot analysis. Expression of c-Myb proteins in VSMCs was determined by immunoblot analysis using rabbit antibody against DNA binding domain of c-Myb. The truncated PDGFRα was fused with HA tag and was detected by the anti-HA tag antibody. Cell lysates were prepared and equal amount of cytoplasmic extracts were subjected to Western blot analysis as described previously [12]. After SDS–polyacrylamide gel electrophoresis, the proteins were electrotransfered to nitrocellulose filters. Blots were probed with antibodies against c-Myb, HA, or β-actin as control. HRP labeled antibodies were used as secondary antibodies and blots were developed by ECL.

Assay of apoptosis. Apoptotic rates of cells were determined by Hoechst staining. Cells were cultured on coverslips to 60–80% confluency and then incubated in the different experimental conditions as indicated. The cells were fixed for 15 min and then incubated with 0.5 ml Hoechst 33258 (No. H3569, Invitrogen) dilution (final concentration at 1 μg/ml) for 5 min at room temperature. Cells showing condensed chromatin were determined as apoptotic. The percentage of apoptotic cells was counted and averaged over five fields of microscopic observation in each sample.

Analysis of DNA fragmentation. VSMCs were incubated in the presence or absence of the test compounds in DMEM with serum and harvested after trypsinization. The floating cells were pooled. After washing with PBS cells were resuspended with DNA buffer. The genomic DNA was prepared by proteinase K digestion, phenol–chloroform extraction, ethanol precipitation and dissolved in Buffer containing 10 mM Tris–Cl pH 7.5, 1 mM EDTA. After spectrophotometric determination of the product purity and content, the extracted DNA (10 μg) was separated on 2.5% agarose gels and photographed under ultraviolet light.

Statistical analysis. Results were expressed as means ± standard deviation. Data were evaluated by Student's *t*-test. A value of $P < 0.05$ was considered as statistically significant.

Results

The PDGF signals induce expression of c-Myb protein in VSMCs

The c-Myb protein has been observed to be expressed in VSMCs within the lesion of neointima [13] and atherosclerosis (unpublished data, X. Mo and A. Leutz). Inhibition of c-Myb function can inhibit arterial neointima formation [9]. Since PDGF stimulates VSMCs proliferation and

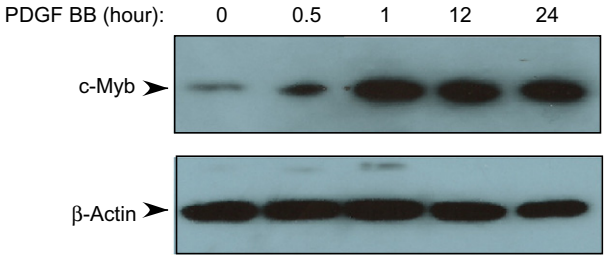

Fig. 1. PDGF-BB promotes expression of the c-Myb protein in human arterial VSMCs. Protein extracts of VSMCs at 0, 0.5, 1, 12 or 24 h after PDGF-BB treatment (50 ng/ml) were subjected to Western blotting and detected by rabbit anti c-Myb antibody. Staining of β-actin was served as a loading control.

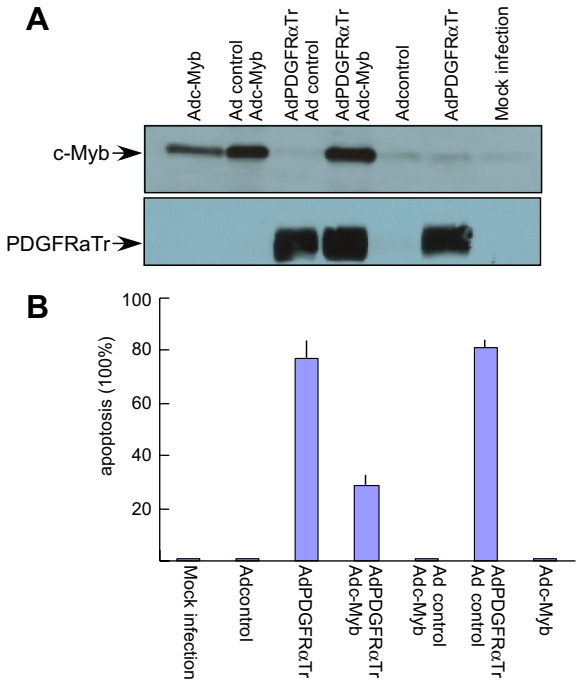

Fig. 2. c-Myb prevents apoptosis in human arterial VSMCs. (A) Western blotting analyses was performed to determine adenoviral protein expression. The VSMCs after coinfection of AdMyb and AdPDGFRαTr overexpressed both proteins. (B) Overexpression of c-Myb diminished apoptosis of VSMCs induced by truncated PDGFRα. The results were derived from counting more than five fields in each sample (80–100 cells per field). The experiments were repeated three times. AdMyb: adenovirus vector carrying full length of human c-Myb. AdPDGFRαTr: adenovirus vector carrying the truncated PDGF receptor α. Adcontrol: Adenovirus 5 309.