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Trichostatin A prevents neointimal hyperplasia via activation of Krüppel like factor 4

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ABSTRACT

The proliferation of vascular smooth muscle cells (VSMCs) is an integral part of the mechanism of vascular diseases such as restenosis. Post-translational modifications by histone deacetylase (HDAC) inhibitors play an important role in the regulation of gene expression by inducing cell cycle arrest. However, the role and mechanism of the HDAC inhibitor trichostatin A (TSA) on neointimal proliferation remain unknown. In this study, we investigated the effect and mechanism whereby TSA prevents the proliferation of VSMCs and neointimal hyperplasia induced by balloon injury in rat carotid artery. Local administration of TSA significantly prevented neointimal hyperplasia. TSA dramatically inhibited the proliferation and DNA synthesis of VSMCs in response to FBS or PDGF-BB. Overexpression of Krüppel like factor 4 (KLF4) blocked the cell proliferation and DNA synthesis, as determined by the MTT and [^{3}H]thymidine incorporation assays, whereas knockdown of KLF4 resulted in an increase in VSMC proliferation. In VSMCs, TSA increased the mRNA level and protein expression of KLF4. Treatment with TSA or transfection of KLF4 increased the expression of both p21 and p27 and promoter activity. In addition, the anti-proliferative activity of TSA was recovered in KLF4-knockdown cells.

These data demonstrate that TSA inhibits neointimal thickening and VSMC proliferation via activation of the KLF4/p21/p27 signaling pathway.

1. Introduction

Atherosclerosis is a major pathology in many clinical vascular complications, including myocardial infarction in heart, stroke in brain, and claudication in legs (Adams, 2009; Mukherjee and Cho, 2009; Vinereanu, 2006). The underlying causes of atherosclerosis have been shown to be involved in several different mechanisms, such as low-density lipoprotein (LDL) oxidation, lipid deposition, inflammation, platelet activation, endothelial damage, and proliferation (Berliner et al., 1995; Dong and Goldschmidt-Clermont, 2007; Klingenberg and Hansson, 2009; Langer et al., 2010; Qiao et al., 2010). Platelet activation stimulates atherosclerosis by releasing proinflammatory factors via their binding to leukocytes (Brydon et al., 2006). Endothelial cells (ECs) induce proinflammatory events when they are removed mechanically or by an increase in reactive oxygen species in atherosclerosis (Dong and Goldschmidt-Clermont, 2007; Tardif et al., 2002). In patients with acute myocardial infarction, restenosis occurs after stent implantation (Jaster et al., 2008). In this process, vascular proliferation is a critical factor that can lead to atherosclerosis and in-stent restenosis.

The pharmacological modulation of vascular smooth muscle cell (VSMC) proliferation provides a potential therapeutic strategy for atherosclerosis. Histone deacetylases (HDACs) play a critical role in regulating gene expression and transcription. At present, HDAC inhibitors are promising therapeutic targets for cancer therapy because they can induce cell-cycle arrest. For example, the HDAC inhibitor trichostatin A (TSA) induces cell cycle arrest at the G1 phase in cancerous cell lines. Several HDAC inhibitors including SAHA are approved or are being investigated in clinical trials (Carew et al., 2008). Studies of HDAC inhibitors are underway for the treatment of many other diseases as well, including inflammation and cardiac hypertrophy. Although HDACs can deacetylate histones, recent reports have shown that HDACs may target many nonhistone proteins as well, such as a-tubulin, GATA4, p53, YY1, MEF2D, hsp90, and E2F1 (Gregoire et al., 2007; North et al., 2003; Yao et al., 2001; Zhang et al., 2003). Therefore, HDAC inhibitors are suggested to play a pleiotropic role in a variety of cellular processes such as proliferation, differentiation, and apoptosis.

Krüppel like factor 4 (KLF4) is a transcription factor involved in the regulation of proliferation, differentiation, apoptosis, and carcinogenesis. There are currently 17 identified KLFs, and among them, KLF2, KLF4, KLF5, KLF6, and KLF15 have been shown to be involved in vascular diseases (Suzuki et al., 2005). KLF5 induces cell growth, whereas KLF4 induces cell cycle arrest by activating p21 or by repressing the expression of cyclin B1 (Yoon and Yang, 2004; Zhang et al., 2000a). KLF4 has been shown to function as a tumor suppressor in some cancers such as gastric and colorectal cancer (Wei et al., 2006). In VSMCs, recent studies have shown that KLF4 may be a key mediator in association with

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growth arrest by enhancing or suppressing cell cycle-related genes (Zhang et al., 2000a).

We recently reported that KLF4 negatively regulates cardiac hypertrophy. In our previous report, we observed that the expression of KLF4 was induced by HDAC inhibitors such as TSA or SK7041 in cardiomyocytes (Kee and Kook, 2009). Given the positive relationship between HDAC inhibitors and KLF4, we postulated that KLF4 might regulate cell cycle progression to repress proliferation in VSMCs. In the present study, we examined whether TSA inhibited neointimal hyperplasia in a rat carotid artery balloon injury model. To understand the underlying mechanism by which HDAC inhibitors prevent VSMC proliferation, we characterized KLF4, an anti-proliferative regulator, induced by TSA. Our findings indicate that KLF4 may act as a negative regulator of VSMC proliferation through the induction of both p21 and p27.

2. Materials and methods

2.1. Reagents

Platelet-derived growth factor-BB (PDGF-BB) was obtained from KOMA BIOTECH. Dimethyl sulfoxide (DMSO), TSA, Pluronic gel, and anti-flag were obtained from Sigma. Anti-KLF4 was purchased from Abnova. Anti-p21 and anti-p27 were purchased from B&D Company. Anti-Gapdh was purchased from Santa Cruz.

2.2. Rat vascular smooth muscle cell isolation and A10 cells

Rat aortic VSMCs were isolated from rat thoracic aorta by enzymatic dispersion, as described by Kim et al. (2002). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. VSMCs were used at passages 5 to 10. A10 cells were obtained from American Type Culture Collection (ATCC) and have been used as models of vascular smooth muscle cells (Rao et al., 1997). They were derived from embryonic rat aorta (Kimes and Brandt, 1976). Cells were grown in DMEM with 10% FBS.

2.3. Rat carotid injury model and drug treatment

Eight-week-old male Sprague–Dawley rats (Damul, Daejeon, Korea) weighing 250 g were fed a normal pelleted diet and given water *ad libitum*. All protocols were approved by the Chonnam National University Animal Care and Use Committee.

Animals were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (6.7 mg/kg). After anesthesia a midline incision was made in the animal's neck to expose the distal bifurcation of the right common carotid artery. A 2F Fogarty balloon embolectomy catheter (Baxter, McGraw Park, IL, USA) was placed at puncture of external carotid artery and advanced along the length of the right common carotid artery and retracted three times under mild balloon inflation pressure to endothelial denudation with balloon injury (Clowes et al., 1983; Manderson et al., 1989). Rats were divided into two groups: control (vehicle alone; n = 7) and TSA (n = 7). Immediately after the balloon injury, pluronic gel with vehicle or TSA was applied to circumference of perivascular space of about 10 mm segments of the injured carotid artery, from 5 mm proximal to the bifurcation of the internal and external carotid artery. One hundred microliters of Pluronic gel containing 100 μg TSA or vehicle was applied to the exposed adventitial surface of the injured carotid artery. The Pluronic gel containing TSA was prepared as follows. Thirty percent (w/v) F-127 Pluronic gel solution was prepared and kept at 4 °C for 24 h. One milligram of TSA was dissolved in 100 μl of 100% ethanol, and 10 μl of this ethanolic solution was added to 90 μl of the previously cooled 30% Pluronic gel solution. A 100-μl aliquot of this solution contained 100 μg of TSA. Pluronic gel containing vehicle was prepared as described above.

2.4. Morphometric analysis and immunohistochemistry

For morphometric analysis, carotid arteries were fixed in 10% formalin, dehydrated, and embedded in paraffin. Carotid artery sections (5 μm) were stained with hematoxylin–eosin, and morphometric analysis was performed by using 3 individual sections from the middle of each injured arterial segment by an investigator blinded to the experimental procedure. The intimal/medial area ratios and percentage area stenosis were measured and calculated by using the NIS Elements Imaging Software (Nikon, Japan).

For immunohistochemistry, sections were incubated with anti-KLF4 (Abnova) (1:50) and bound antibodies were detected by using diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA, USA) in bright field images. Sections were lightly counterstained with hematoxylin.

2.5. In vitro VSMC proliferation and [^{3}H]thymidine incorporation assay

The cell growth rates were measured by using the 3-(4,5-dimenthylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and direct cell counting. Briefly, VSMCs were seeded into 96-well culture dishes at a density of 4000 cells/well with DMEM containing 10% FBS and were incubated in serum-free DMEM for 24 h before treatment with PDGF-BB (50 ng/ml) and TSA. After 3 days, MTT was added to the medium. Optical density was determined at 570 nm by use of a microplate reader. For direct cell number counting, A10 mock cells and KLF4 anti-sense cells were seeded into 12 well culture dishes at a density of 60,000 cells/well with DMEM containing 10% FBS. After serum starvation for 24 h, vehicle or TSA in the presence of PDGF-BB was incubated for 48 h. After cells were washed and trypsinized, cell numbers were counted by hemocytometer.

For the [^{3}H]thymidine incorporation assay, VSMCs were plated on 24-well culture dishes at a density of 20,000 cells/well. Cells were incubated in serum-free DMEM medium for 24 h. Cells were stimulated with PDGF-BB for 40 h and were incubated with 1.0 μCi/ml [^{3}H]thymidine for 6–12 h. After fixing with 10% trichloroacetic acid, the cells were extracted in 1.0 M NaOH, and radioactivity was measured in a liquid scintillation counter (Winspectral 1414, Wallac, Turku, Finland).

2.6. Cell transfection, antisense KLF4 stable cell lines, and siKLF4

Transfection assays were performed as described previously (Kee et al., 2008). For transient transfection of Klf4, *pCMVSPORT6–KLF4* construct was introduced to A10 cells by use of NanoFect reagent (Qiagen).

For Klf4-antisense cell lines, *pcDNA6/myc-HisA-antisense Klf4* and vector were transfected into A10 cells. The cells were treated with 5 μg/ml blasticidine (Invitrogen), and positive colonies were selected 2 weeks later. The changes in the expression of Klf4 were confirmed by RT-PCR and immunoblot analysis.

A small interfering RNA (siRNA; 100 nM) targeting rat Klf4 (ON-TARGET *plus* SMARTpool siRNA, Catalog number: L-093552-010, Dharmacon, Lafayette, CO, USA) was transfected into A10 cells by use of DharmaFECT 1 reagent. Nontargeting siRNA (Accell non-targeting negative control, Dharmacon) was used as a negative control.

2.7. Reverse transcriptase-polymerase chain reaction and Western blot

2.7.1. Analysis

Total RNA was isolated with TriZol reagent (Invitrogen Life Technologies), and 1 μg of RNA underwent reverse transcription reaction with the Superscript first strand synthesis system for RT-PCR kit (Invitrogen Life Technologies). mRNA amounts were quantified by semi-quantitative PCR amplification. The following primers were