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PTEN differentially regulates expressions of ICAM-1 and VCAM-1 through PI3K/Akt/GSK-3β/GATA-6 signaling pathways in TNF-α-activated human endothelial cells

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ABSTRACT

Phosphotase and tensin homolog deleted on chromosome 10 (PTEN) is a potent negative regulator of PI3K/Akt pathway. Here, we tried to elucidate the role of PTEN in the regulation of endothelial adhesion molecules, vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1, induced by TNF-α in human endothelial cells (ECs). Transfection with PTEN overexpressing vector resulted in the significant decrease in phosphorylation of Akt in TNF-α-treated ECs. PTEN strongly inhibited VCAM-1 but not ICAM-1, however this inhibitory effect was reversed by co-trasfection with constitutively active-Akt (CA-Akt-HA) in TNF-α-stimulated ECs. Additionally, silencing of PTEN with specific siRNA showed significant increase of phosphor-Akt compared with TNF-α alone treated ECs. siPTEN significantly upregulated VCAM-1 but was indifferent to ICAM-1 in TNF-α-treated cells. Further, chromatin immunoprecipitation (ChIP) assay showed that PTEN targets GATA-6 but not IRF-1 binding to VCAM-1 promoter. In addition, GATA-6 is associated with glycogen synthesis kinase-3beta (GSK-3β) which is in turn regulated by PTEN-dependent Akt activity. Finally, PTEN significantly prevented monocyte adhesion to TNF-α-induced ECs probably through VCAM-1 regulation. It is concluded that PTEN selectively inhibits expression of VCAM-1 but not ICAM-1 through modulation of PI3K/Akt/GSK-3β/GATA-6 signaling cascade in TNF-α-treated ECs.

1. Introduction

The activation of the vascular endothelium, the increased adhesion of circulating monocytes to the injured endothelial layer, and their subsequent infiltration into the vessel wall and differentiation into macrophages are critical early events in the development of atherosclerosis [1]. Endothelial cells recruit monocytes by selectively expressing various cell surface adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) [2]. It is well known that both VCAM-1 and ICAM-1 are highly expressed in atherosclerotic lesions and both are involved in attachment of leukocytes [3]. However, the pattern of the expression of VCAM-1 and ICAM-1 is quite different. It has been demonstrated that VCAM-1 is expressed in lesion predisposed areas only, whereas, ICAM-1 extends to not predisposed areas [2]. For a long time comparative study of what molecule is more important VCAM-1 or ICAM-1 in the development of atherosclerosis was impossible due to embryonic lethality of VCAM-1 null mice [4]. For the first time, Cybulsky et al. performed the deletion of two ligand binding sites in VCAM-1 avoiding embryonic lethality but significantly affecting the functionality of VCAM-1. It has been concluded that VCAM-1 plays more important role in the early atherosclerosis than ICAM-1 [5]. According to these genetic experiments, it seems that specific inhibition of VCAM-1 is perspective for future therapeutic intervention.

Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, is commonly found in atherosclerotic lesions [6,7]. TNF is

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DMEM, dulbecco's modified eagle's medium; ECL, enhanced chemoluminescence; GSK-3β, glycogen synthesis kinase-3beta; ICAM-1, intracellular adhesion molecule-1; IRF-1, interferon regulatory factor-1; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; SDS, sodium dodecyl sulfate; PTEN, phosphotase and tensin homologue deleted on chromosome ten; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene difluoride; TBS-T, Tris-buffered saline/Tween 20; VCAM-1, vascular adhesion molecule-1.

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a heterotrimeric cytokine that binds to two receptors, TNF-RI and TNF-RII. As most information regarding TNF signaling is derived from TNF-RI, the role of TNF-RII is likely underestimated [8]. This binding causes a conformational change to occur in the receptor, leading to dissociation of the inhibitory protein silencer of death domain (SODD) from the intracellular death domain. This dissociation enables the adaptor protein tumor necrosis factor receptor type 1-asocciated death domain (TRADD) to bind the death domain, serving as a platform for subsequent protein binding. Following TRADD binding can initiate multifaceted signaling pathways such as the activation of NF-κB through TNF receptor-associated factor (TRAF2) and receptor interacting protein (RIP) recruitment, mitogen-activated protein kinases MAPKs, and death signaling by binding of TRADD to Fas-associated protein with death domain (FADD) [9–11].

Previously, our group suggested that PI3K/Akt signaling can also be activated under TNF-α treatment and play a detrimental role in atherosclerosis [12]. Moreover, recently we demonstrated that modulation of PTEN activity can selectively regulate VCAM-1 over ICAM-1 expression in activated endothelial cells [13]. In this way, it seems that modulation of PTEN activity can provide beneficial effect against cardiovascular disorders [13]. However, the molecular mechanism of PTEN was not yet elucidated. Here we provide evidence that PTEN negatively regulates VCAM-1 expression by modulation of PI3K/Akt/GSK-3β/GATA-6 signaling cascade in TNF-α activated ECs. Thus, this finding further suggests that modulation of PTEN activity can be a target for treatment of early atherosclerotic events.

2. Materials and methods

2.1. Materials

Tissue culture medium 199, fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), glutamine and collagenase were purchased from Gibco-BRL (Rockville, MD). Anti-ICAM-1 (sc-7891), anti-VCAM-1 (sc-8304), anti-PTEN (sc-7974), anti-GATA-2 (sc-9008), anti-GATA-6 (sc-9055), anti-IRF-1 (sc-497), anti-GSK-3β (sc-9166) and phosphor-GSK-3β (Ser 9) (sc-11757) antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phosphor-Akt (9271), anti-Akt (9272) antibodies were supplied by Cell Signaling Technology (Beverly, MA). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) and wortmannin were purchased from Calbiochem (San Diego, CA). All other chemicals were supplied by Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

Human vascular endothelial cells (HUVECs) were obtained from Cascade Biologics (Portland, OR) and U937 human monocyte was obtained from Korea Cell Line Bank (KCLB, Seoul, Korea). Cells were grown in a humidified 5% CO_2 incubator as described previously in Ref. [13].

2.3. Western blot

Whole cell lysates were harvested and concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford, IL). To detect VCAM-1, ICAM-1, PTEN, phosphor-Akt, Akt or GATA-6, the total protein was electrophoresed on polyacrylamide gel. The gels were transferred to polyvinylidene difluoride (PVDF) membranes by semidry electrophoretic transfer. Then the PVDF membranes were blocked overnight at 4 °C in 5% bovine serum albumin (BSA). The cells were incubated with primary antibodies diluted 1:500 in Tris/buffered saline/Tween 20 (TBS-T) containing 5% BSA for overnight in 4 °C and then incubated with secondary antibody at room temperature for 1 h. The signals were detected by ECL.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) for ICAM-1 and VCAM-1 detection

Total RNA was extracted from HUVEC by a single-step guanidine thiocyanate–phenol–chloroform extraction procedure, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A negative control without reverse transcriptase was done to verify that amplification did not ensue from residual genomic DNA. PCR amplification was carried out on cDNA equivalent to 100 ng of starting mRNA, using specific oligonucleotide primers for ICAM-1 (forward, 5′-TATGGCAACGACTCCTTCT-3′ and reverse, 5′-CATTCAGCGTCACCTTGG-3′), GATACAACCGTCTTGGTCAGCCC-3′ (sense) and 5′-CGCATCCTTCAACTGGCCTT-3′ (antisense) for VCAM-1 and GAPDH (forward, 5′-ATCACCATCTTCCAGGAGCGAGA-3′ and reverse, 5′-CAAAGTTGTCATGGATGACCTT-3′). The PCR products were electrophoresed on 2% ethidium bromide stained agarose gels.

2.5. Immunoprecipitation

To identify the protein–protein interactions between GATA-6 and GSK-3β, GATA-2 and GSK-3β, IRF-1 and GSK-3β or GATA-6 and Akt, HUVECs were subjected to lysis in the immunoprecipitation buffer supplemented with 0.1% (w/v) SDS, 0.1 mM Na_3VO_4, and protease inhibitors. Cells were sheared by brief sonication on ice and cellular debris was removed by centrifugation at 12,000 × *g* for 10 min. Lysates were cleared initially by incubation with protein A/G-sepharose for 1 h at 4 °C. Lysates were incubated with a specific polyclonal GATA-6 antisera or a preimmune rabbit IgG at a final concentration of 4 μg/mL each for 4 h at 4 °C. Protein A/G-sepharose was then added for 4 h at 4 °C. Immune complexes were collected by centrifugation, washed eight times with 1 mL of the immunoprecipitation buffer lacking Na_3VO_4 and protease inhibitors, then disrupted by boiling in 1% SDS. Then samples were subjected to western blotting against GSK-3β or Akt antibodies.

2.6. Plasmid construction

Please see the provided in the online data supplement regarding to method in detail.

2.7. Transfection

Wild type-ICAM-1, wild type-VCAM-1-luciferase, VCAM-1-luciferase with mutated IRF-1 site (mIRF-1), with mutated GATA site (mGATA) were constructed as described above. Transient transfection was performed using Superfect© from QIAGEN (Valencia, CA) according to the manufacturer's protocol. Please see the provided in the online data supplement regarding to method in detail.

2.8. Luciferase assay

After these treatments, the cells were washed twice with cold PBS, lysed in a passive lysis buffer provided in the dual luciferase kit (Promega, Madison, WI) and assayed for luciferase activity using a TD-20/20 luminometer (Tuner Designs, Sunnyvale, CA) according to the manufacturer's protocol. All transfections were done in triplicate. Data are presented as the ratio between Firefly and *Renilla* luciferase activities.

2.9. siRNA technique

siRNAs against human PTEN and scramble were provided from Santa Cruz Biotechnology (Santa Cruz, CA) and performed according to manufacturer's protocol.