Myocardin-related transcription factor A cooperates with brahma-related gene 1 to activate $P$-selectin transcription

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Abstract

Expression of $P$-selectin in injured or activated endothelia cells serves as a permissive step towards leukocyte recruitment and perpetuation of inflammation in the pathogenesis of atherosclerosis. $P$-selectin can be induced by pro-inflammatory stimuli via the transcription factor NF-$k$B, but the epigenetic mechanisms remain incompletely understood. Previously we reported that myocardin-related transcription factor A (MRTF-A) mediates the transactivation of a slew of adhesion molecules by oxidized low-density lipoprotein (oxLDL), likely through a crosstalk with brahma-related gene 1 (BRG1), a chromatin remodeling protein. Here, we show that MRTF-A was both sufficient and necessary for the transactivation of $P$-selectin gene in endothelial cells treated with TNF-$\alpha$. Depletion of MRTF-A using small interfering RNA (siRNA) abrogated the binding of BRG1 on the $P$-selectin promoter. Overexpression of BRG1 up-regulated the activity of $P$-selectin promoter activity while BRG1 knockdown attenuated $P$-selectin expression. Finally, BRG1 silencing suppressed the accumulation of acetylated histone H3 and methylated histone H3K4, and altered the binding of NF-$k$B on the $P$-selectin promoter. Therefore, our data demonstrate an essential role for MRTF-A and BRG1 in $P$-selectin transactivation in endothelial cells.

Keywords: myocardin-related transcription factor A (MRTF-A), brahma-related gene 1 (BRG1), $P$-selectin, endothelial cell

Introduction

Under physiological conditions, the endothelial layer of the vessels does not support a firm interaction with circulating leukocytes. In response to various injurious signals, expression of adhesion molecules by endothelial cells allows attraction, retention, and trans-membrane migration of leukocytes, thereby initiating vascular inflammation$^{[1]}$. This pathophysiological process constitutes a pivotal step in the pathogenesis of a host of cardiovascular diseases including atherosclerosis$^{[2]}$, pulmonary hypertension$^{[3]}$, and myocardial infarction$^{[4]}$.

Adhesion of leukocytes to the vessel wall is mediated by a group of structurally and functionally diverse membrane proteins called adhesion molecules$^{[5]}$. This family of proteins includes intercellular adhesion molecules (ICAMs), vascular adhesion molecules (VCAMs), and selectins. Previous studies indicated that each adhesion molecule preferentially mediates the adhesion of a sub-set of circulating leukocytes to the vessel wall. For instance, $P$-selectin is

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known to specifically support the adhesion of platelets while ICAM-1 is necessary for the adhesion of lymphocytes\(^{[6,7]}.\) While the basal expression level of \(\text{P-selectin}\) is low in endothelial cells, its transcription rate can be rapidly turned on by a number of pro-inflammatory factors including tumor necrosis factor (TNF-\(\alpha\)), lipopolysaccharide (LPS), and hypoxia.

Myocardin-related transcription factor A, or MRTF-A, was first identified as a co-factor for serum response factor (SRF) to promote the transcription of muscle-specific genes\(^{[8]}\). MRTF-A deficiency in mice does not disturb organogenesis, but seems to protect the rodents from atherosclerosis\(^{[9]}\), pulmonary hypertension\(^{[10]}\), and myocardial infarction\(^{[11]}\). In addition, in vitro studies conducted in cultured endothelial cells demonstrate that MRTF-A is required for the transactivation of \(\text{ICAM-1, VCAM-1, and E-selectin genes}^{[12]}\). We report here that MRTF-A is also responsible for \(\text{P-selectin transactivation in TNF-\(\alpha\)}\) treated endothelial cells by recruiting the chromatin remodeling protein BRG1 to alter the chromatin structure.

**Materials and methods**

**Cell culture and treatment**

Human immortalized umbilical vein endothelial cells (EAhy926, ATCC, Manassas, VA, USA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone). Prior to treatment, cells were serum starved overnight and then treated with TNF-\(\alpha\) (R&D, Minneapolis, MN, USA).

**Plasmids, transient transfection, and reporter assay**

FLAG-tagged MRTF-A construct, FLAG-tagged BRG1 construct, \(\text{P-selectin promoter-luciferase construct (-1379/-13)}\), short hairpin RNA (shRNA) construct for MRTF-A, small interfering RNA (siRNA) sequences for human MRTF-A and BRG1 have been previously described\(^{[12,13]}\). Transient transfections were performed with Lipofectamine 2000 (Invitrogen). Luciferase activities were assayed 24-48 hours after transfection using a luciferase reporter assay system (Promega, Madison, WI, USA). Experiments were routinely performed in triplicate wells and repeated three times.

**Protein extraction and Western blotting assays**

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% Triton X-100) with freshly added protease inhibitors (Roche, Burgess Hill, west Sussex, UK). Western blot analyses were performed with anti-MRTF-A, anti-BRG1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-\(\beta\)-actin (Sigma, St. Louis, MO, USA) antibodies.

**RNA isolation and real-time PCR**

RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen). Real-time PCR was performed on an ABI Prism 7500 system. Primers and Taqman probes, used for real-time reactions, were purchased from Applied Biosystems.

**Chromatin Immunoprecipitation (ChIP)**

Chromatin Immunoprecipitation (ChIP) assays were performed essentially as described before\(^{[14]}\). In brief, chromatin in control and treated cells were cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mmol/L NaCl, 25 mmol/L Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with a protease inhibitor tablet and PMSF. DNA was fragmented into ~500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-MRTF-A, anti-BRG1, anti-NF-kB/p65 (Santa Cruz Biotechnology), anti-acetyl histone H3, and anti-trimethylated histone H3K4 (Millipore, Billerica, MA, USA). Precipitated genomic DNA was amplified by real-time PCR using previously described primers\(^{[15]}\).

**Statistical analysis**

One-way ANOVA with post-hoc Scheffe analyses were performed using an SPSS package. \(P \text{ values smaller than} .05 \text{ were considered statistically significant.}^{[16]}\)

**Results**

MRTF-A activates \(\text{P-selectin transcription in endothelial cells}\)

We have previously shown that MRTF-A mediates the transactivation of \(\text{ICAM-1, VCAM-1, and E-selectin genes}\) in endothelial cells in response to the stimulation of oxLDL\(^{[12]}\) and hypoxia\(^{[10]}\). Since \(\text{P-selectin}\) is also involved in the interaction between vascular endothelium and circulating leukocytes, we hypothesized that MRTF-A might activate \(\text{P-selectin transcription. We transfected a} \text{P-selectin promoter-luciferase fusion construct into EAhy926 cells with or without an MRTF-A expression construct. As shown in Fig. 1A, MRTF-A up-regulated} \text{P-selectin promoter activity in a dose-dependent manner. In addition, MRTF-A markedly enhanced the activation of the} \text{P-selectin promoter by TNF-\(\alpha\)} \text{(Fig. 1B). On the contrary, knockdown of MRTF-A}\)
Fig. 1 MRTF-A is essential for the transactivation of P-selectin promoter by TNF-α in endothelial cells. A: A P-selectin promoter construct was transfected into EAhy926 with increasing amounts of MRTF-A expression construct. Luciferase activities were normalized to the control group and expressed as relative luciferase unit (RLU). B: A P-selectin promoter construct was transfected into EAhy926 with or without MRTF-A followed by treatment with TNF-α. Luciferase activities were normalized to the control group and expressed as RLU. C: A P-selectin promoter construct was transfected into EAhy926 with shRNA plasmid targeting MRTF-A (shMRTF-A) or a control shRNA plasmid (non-target) followed by treatment with TNF-α. Luciferase activities were normalized to the control group and expressed as RLU.

Fig. 2 MRTF-A is essential for BRG1 recruitment. A: EAhy926 cells were transfected with siRNA targeting MRTF-A or scrambled siRNA (SCR) followed by treatment with TNF-α. The mRNA transcript levels of P-selectin were measured by qPCR. Knockdown efficiency of MRTF-A siRNA was verified by qPCR and Western blotting. B: EAhy926 cells were transfected with siRNA targeting MRTF-A, or scrambled siRNA (SCR), followed by treatment with TNF-α. ChIP assays were performed with anti-MRTF-A or anti-BRG1. Precipitated DNA was amplified by primers flanking the P-selectin gene proximal promoter.
expression by a shRNA plasmid attenuated TNF-α-induced activation of the P-selectin promoter (Fig. 1C). Therefore, MRTF-A can indeed activate P-selectin transcription in endothelial cells.

**MRTF-A is essential for BRG1 recruitment**

Next, we asked whether MRTF-A might be essential for the induction of endogenous P-selectin messages by TNF-α in endothelial cells. We used siRNA to deplete endogenous MRTF-A; MRTF-A depletion down-regulated P-selectin induction by more than 50% in the presence of TNF-α (Fig. 2A). Furthermore, TNF-α stimulated the occupancy of MRTF-A on the P-selectin promoter (Fig. 2B). Therefore, MRTF-A may be an activator of P-selectin transcription by directly binding to the P-selectin promoter.

Prior studies have demonstrated that the chromatin remodeling protein BRG1 is required for MRTF-A-dependent transcription in vascular smooth muscle cells, macrophages, and endothelial cells. BRG1 binding on the P-selectin promoter was up-regulated by TNF-α; depletion of MRTF-A with siRNA simultaneously blocked the binding of MRTF-A and BRG1 to the P-selectin promoter (Fig. 2B). Collectively, these data suggest that MRTF-A might recruit BRG1 to activate P-selectin transcription.

**BRG1 contributes to P-selectin transactivation**

To verify the functional interaction between MRTF-A and BRG1 in transactivating the P-selectin gene, we performed the following reporter assays. Co-expression of wild type (WT), but not an enzyme deficient (ED), form of BRG1 with MRTF-A additively activated the P-selectin promoter (Fig. 3A). On the other hand, siRNA-mediated depletion of BRG1 ameliorated MRTF-A-induced P-selectin promoter activation (Fig. 3B). Similarly, BRG1 WT instead of BRG1 ED enhanced activation of P-selectin promoter by TNF-α (Fig. 3C). Finally, BRG1 silencing suppressed P-selectin promoter activation by TNF-α (Fig. 3D). Together, these data suggest that BRG1 might be an integral part of TNF-α-induced, MRTF-A-mediated P-selectin transactivation.

**BRG1 alters the chromatin structure surrounding P-selectin promoter**

We then tackled the mechanism whereby BRG1 might contribute to P-selectin transactivation. As shown in Fig. 4A, BRG1 depletion by siRNA down-regulated the induction of endogenous P-selectin mRNA transcripts by TNF-α. BRG1 siRNA also prevented the binding of BRG1 to the P-selectin promoter (Fig. 4B). In the meantime, BRG1 silencing eliminated the enrichment of two preeminent histone markers indicative of transcriptional activation, namely acetylated histone H3 and trimethylated histone H3K4, on the P-selectin promoter.

The sequence-specific transcription factor NF-κB/p65 has been shown to mediate TNF-α induced P-selectin transcription. Indeed, TNF-α stimulation promoted the binding of p65 on the P-selectin promoter in EAhY926 cells in a time course-dependent manner: significant p65 binding started to appear on the P-selectin promoter as early as three hours post-treatment, peaked at six hours and declined at nine hours (Fig. 4C). BRG1 depletion, however, impaired p65 binding; without BRG1, p65 binding peaked less and declined more (Fig. 4C). Combined, these data suggest that BRG1 might regulate P-selectin
transcription by altering histone modification and p65 binding kinetics on the P-selectin promoter.

Discussion

We show evidence here that MRTF-A and BRG1 cooperate to activate P-selectin transcription in vascular endothelial cells. In light of our previous findings that suggest MRTF-A can activate the transcription of other adhesion molecules\cite{10,12}, these new data allude to the possibility that targeting MRTF-A in endothelial cells could alleviate leukocyte adhesion and thus vascular inflammation in human diseases\cite{22}.

Our data suggest that MRTF-A activates P-selectin gene in endothelial cells. It is known that P-selectin expression is detected in platelets and that P-selectin is required for platelet function\cite{23}; one wonders whether MRTF-A might also drive P-selectin expression in platelets. Indeed, Krause and colleagues have recently shown that
MRTF-A, along with its family member MRTF-B, play essential roles in megakaryocyte maturation and platelet formation, acting as a co-factor for SRF. It would be of great interest to determine whether the deficiency in platelet formation in MRTF-AB-null mice can be rescued by the introduction of exogenous P-selectin.

Mounting evidence has made it abundantly clear that MRTF-A may act as a bridge between the epigenetic machinery and the basal transcription machinery. We show here that MRTF-A activates P-selectin by recruiting BRG1 to the promoter region. BRG1 can sense a number of pro-inflammatory stimuli, including LPS, hypoxia, nutrition excess, and TNF-α, to program cellular response. Of note, BRG1 is absolutely required for vasculogenesis, as germ line deletion of systemic BRG1 or endothelial BRG1 in mice causes lethality due to extensive defects of the vasculature. On the other hand, adult mice with induced deletion of BRG1 in endothelial cells appear to be normal under physiological conditions.

Consistent with our previous findings that BRG1 could forge a crosstalk with histone modifying enzymes, we demonstrate here that BRG1 depletion led to the disappearance of acetylated histone H3 and methylated histone H3K4 from the P-selectin promoter. ChIP-seq analyses have indicated a co-enrichment of BRG1, AcH3, and H3K4Me3 in a cell-specific and differentiation-dependent manner. It remains to be determined how BRG1 modulates histone modification on a genome-wide scale in inflammation-challenged endothelial cells. Another interesting finding in the present study is that BRG1 deficiency altered the binding of p65, although it is not clear whether this is due to BRG1-dependent nucleosome displacement or changes in histone modifications or both. Ding et al. recently found that vitamin D receptor (VDR) binding deprives certain regions of the chromatin of acetylated histones. This renders them less permissible for SMAD3 to bind, which explains the anti-fibrogenic effect of VDR. BRG1 might operate in a similar mode to modulate p65 binding.

In summary, we report here an epigenetic mechanism by which MRTF-A regulates P-selectin transcription. Future investigations employing spatiotemporally controlled animal models and ChIP-seq analysis would shed more light on the role of MRTF-A in regulating the vascular transcriptome.

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