MCP-induced protein 1 suppresses TNF-α-induced VCAM-1 expression in human endothelial cells

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Endothelial inflammation plays a critical role in the development and progression of cardiovascular disease, albeit the mechanisms need to be fully elucidated. We here report that treatment of human umbilical vein endothelial cells (HUVECs) with tumor necrosis factor (TNF) α substantially increased the expression of MCP-induced protein 1 (MCPIP1). Overexpression of MCPIP1 protected ECs against TNFα-induced endothelial activation, as characterized by the attenuation in the expression of the adhesion molecule VCAM-1 and monocyte adherence to ECs. Conversely, small interfering RNA-mediated knock down of MCPIP1 increased the expression of VCAM-1 and monocyteic adherence to ECs. These studies identified MCPIP1 as a feedback control of cytokines-induced endothelial inflammation.

1. Introduction

A crucial step in atherogenesis is the arterial recruitment of inflammatory cells from the circulation and their transendothelial migration into the subendothelial space of large arteries where they differentiate into macrophages and become functionally active [1,2]. In response to inflammatory stimuli, such as tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), and interferon γ, endothelial cells (ECs) undergo inflammatory activation, resulting in an increased surface expression of cell adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin, which contributes to the recruitment of inflammatory cells to arterial wall and their transmigration across the wall [3,4]. The activated ECs also secret cytokines and chemokines such as monocyte chemotactic protein 1 (MCP-1), which is a potent inducer for monocyte attachment to ECs and migration into subendothelial space [5,6]. Mice deficient in MCP-1 or its receptor CCR2 had significant reduced atherosclerotic lesions, suggesting that MCP-1/CCR2 interaction has a role in monocyte recruitment in atherosclerosis [7,8].

MCP-induced protein 1 (MCPIP1, also known as ZC3H12A) is a recently identified gene in human peripheral blood monocytes treated with MCP-1 [9,10]. The gene undergoes rapid and potent transcription induction upon stimulation with proinflammatory molecules, such as TNFα, MCP-1, IL-1β, and lipopolysaccharide (LPS) [9–13]. Further studies showed that MCPIP1 plays an important role in both physiological and pathological processes related to inflammation [10,11]. In the experiments on cultured cells, MCPIP1 was proved to be a negative regulator of macrophage activation [10]. In a recent report on mice, MCPIP1 deficiency leads to a complex phenotype including severe anemia, autoimmune response and severe inflammatory response and most mice died within 12 weeks of birth [11]. These results suggest that MCPIP1 may critically control inflammation and immunity and would be a potential therapeutic target for treatment of human inflammatory diseases such as atherosclerosis.

Abbreviations: MCPIP1, MCP-induced protein 1; HUVECs, human umbilical vein endothelial cells; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; NF-κB, nuclear factor-κB; EC, endothelial cell; IL-1β, interleukin 1β; MCP-1, monocyte chemotactic protein 1; LPS, lipopolysaccharide

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In this study, we showed that MCPIP1 expression is induced by inflammatory cytokines TNFα and IL-1β and that overexpression of MCPIP1 suppresses cytokine-induced expression of VCAM-1, as well as monocyte adhesion to human ECs. These results indicate that MCPIP1 also negatively regulates proinflammatory activation of vascular ECs.

2. Materials and methods

2.1. Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) and human CD14+ monocytes were acquired from Lonza Walkersville Inc. (Walkersville, MD) and cultured in EC basal medium-2 and lymphocyte growth media-3 respectively according to the manufacturer instructions. The human acute monocytic leukemia cell line THP-1 was obtained from American Type Culture Collection and was maintained as described previously [10]. Human MCPIP1 expression plasmid was described previously [10]. Human VCAM-1-Luc plasmid was originally generated by Dr. W.C. Aird’s Laboratory (Beth Israel Deaconess Medical Center, Boston) and kindly provided by Dr. Mukesh K. Jain (Case Western Reserve University, Cleveland, Ref. [14]). The MCPIP1 rabbit polyclonal antibody was described previously [10]. VCAM-1 (sc-13160) and actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PAI-1, iNOS and ICAM-1 antibodies were from Cell Signaling Technology. Anti-VCAM-1-Fluorescein, anti-ICAM-1-Fluorescein and VCAM-1 monoclonal antibody were purchased from R&D Systems, Inc. Human recombinant TNFα, IL-1β and actinomycin D were purchased from Sigma (Saint Louis, MI).

2.2. Immunohistochemistry

Adult, male C57BL/6 wild-type and ApoE−/− mice were fed with high-cholesterol diet for 4 weeks. Arterial segments were isolated and fixed in 4% paraformaldehyde. Paraffin (5 μm) sections were immunostained with rabbit anti human MCPIP1 antibody using the avidin-biotin peroxidase method. This investigation has been approved by the animal use and care committee of Peking University Health Science Center and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

Human atherosclerotic plaque tissue was collected from an anonymous patient with consent as described in an approved human subject protocol from the UCSC Human Subjects Committee in coordination with Cottage Hospital (Study number 06-59) and conforms to the principles outlined in the “Declaration of Helsinki”. Human atherosclerotic sections were co-stained with 1:50 diluted rabbit anti human MCPIP1 antibody (GeneTex, GTX110807) and 1:100 diluted FITC conjugated mouse anti-smooth muscle cell (SMC)-Actin antibody (Sigma, F3777) or FITC conjugated anti-CD31, followed by incubation with Alexa fluor 568 conjugated goat anti rabbit IgG antibody (Invitrogen, A11036). Images were taken using Samsun Fluoview confocal system using the no primary slides as negative control.

2.3. Measurement of cell-surface expression of adhesion molecules

The cell-surface expressions of VCAM-1 and ICAM-1 on HUVECs were performed essentially as described [15] but with some modifications using cell ELISA. HUVECs were seeded in 96-well plate and transfected with HA-MCPIP1 or control vector using transPass HUVEC transfection reagent (NEB). 24 h later the cells were stimulated with TNFα for overnight. The stimulated cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde for 30 min at 4°C. FBS (1% (v/v) in PBS) was used as a blocking reagent. After washing with PBS, cells were incubated with FITC conjugated anti-VCAM-1 or anti-ICAM-1 monoclonal antibody for 1 h at 4°C. The cell-surface expressions of VCAM-1 and ICAM-1 were measured with a fluorometer (Flix800, BioTek Instrument, Inc.) at 485 nm excitation wavelength and 535 nm emission wavelength.

2.4. Western blot and Q-PCR

HUVECs were transfected with MCPIP1-EGFP or HA-MCPIP1 as well as their control vectors by electroporation using Amazix Electroporation Unit following the manufacturer's instruction. 24 h later, the transfected cells were exposed to the indicated stimuli. Total protein or RNA was harvested for Western blot or Q-PCR analysis as described previously [10].

2.5. Luciferase assay

HUVECs were seeded into 12-well plate and transfected with Fugene 6 Transfection Reagent (Roche Applied Science) following the manufacturer's instruction. The total amount of plasmid DNA was kept constant within each experiment. Luciferase activity was measured by luciferase Assay System (Promega) and normalized to β-galactosidase activity by cotransfecting the pCMV-β-gal plasmid in all experiments. All transfections were performed in triplicate and at least repeated two times.

2.6. mRNA stability assay

HUVECs were transfected with MCPIP1-EGFP or EGFP control vector by electroporation. After 8 h stimulation with TNFα (10 ng/ml), transcription was stopped by adding 5 μg/ml actinomycin D. The cells were harvested and RNA was isolated at different time points as indicated. The mRNA levels of VCAM-1 were measured by Northern blot as described previously [10].

2.7. Short-interfering RNA

The pre-designed siRNA targeting to human MCPIP1 as well as its negative control were purchased from Santa Cruz Biotech. (CA). The siRNA was transfected into HUVECs by electroporation using Amazix Electroporation Unit following the manufacturer's instruction. 24 h later, the cells were treated with or without TNFα (10 ng/ml) for 8 h. Then the cells were harvested and protein was isolated to assess for MCPIP1 knockdown and VCAM-1 expression.

2.8. Monocyte adhesion assay

THP-1 cells or human CD14+ monocytes were labeled with fluorescein isothiocyanate using a PKH67 fluorescent staining kit (Zynaxis, Inc., Malvern, PA) according to the instructions of the manufacturer. After the HUVECs were stimulated and washed, 2.5 × 10^5 fluorescein isothiocyanate-labeled THP-1 cells were added to each well and allowed to interact for 60 min at 37°C. Unbound cells were removed by gently washing with cold PBS. Images were taken using a Nikon Fluorescence Microscopy (Zeiss, Thornwood, NY). Adherent cells were lysed with 50 mmol/l Tris (pH 8.4)/0.1% sodium dodecyl sulfate, and the fluorescence was measured by a fluorometry.

2.9. Statistics

Data were expressed as mean ± S.D. For comparison between two groups, the unpaired Student’s test was used. For multiple comparisons, analysis of variance followed by unpaired
Student’s test was used. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Expression of MCPIP1 in human and mouse atherosclerotic lesion

To investigate whether MCPIP1 expression might be relevant to atherosclerosis, we examined MCPIP1 expression in mouse and human atherosclerotic lesions. As shown in Fig. 1A, in the artery wall from C57BL/6 wild-type mice, no positive staining was showed with MCPIP1 antibody. However, MCPIP1 expression was significantly increased in the atherosclerotic plaque (especially in endothelial layer) from ApoE\(^{-/-}\) mice. Consistently, MCPIP1 mRNA was increased by 2.5 folds in the aorta from ApoE\(^{-/-}\) mice compared with that from C57BL/6 wild-type mice (Fig. 1B). High expression of MCPIP1 can also be identified in human atherosclerotic lesions, in which most of MCPIP1 positive cells are smooth muscle cell specific marker SM actin positive (Fig. 1C). Some CD31 positive cells also expressed MCPIP1 in human atherosclerotic lesions (Fig. 1D). These results indicated that smooth muscle cells are the major population responsible for MCPIP1 expression in human atherosclerotic lesion, although this protein may also be expressed by other cell types such as ECs.

3.2. Upregulation of MCPIP1 by inflammatory cytokines in human ECs

To investigate the role of MCPIP1 in vascular ECs, we examined the expression of MCPIP1 in HUVECs in response to TNF\(\alpha\), IL-1\(\beta\), LPS and oxLDL stimulation. Similar to macrophages, we found that TNF\(\alpha\), IL-1\(\beta\) and LPS, but not oxLDL strongly upregulated MCPIP1 expression in HUVECs, as determined by Western blot analysis (Fig. 2A). Further study showed that TNF\(\alpha\)-induced MCPIP1 expression in HUVECs in a time- and dose-dependent manner. The time course of MCPIP1 expression, when incubated with 10 ng/mL TNF\(\alpha\), showed a maximal induction of MCPIP1 expression 8 h after TNF\(\alpha\) addition (Fig. 2B). At the concentration of as low as 2.5 ng/\(\mu\)l, TNF\(\alpha\)-induced MCPIP1 protein expression by fivefold. The maximal induction of TNF\(\alpha\) on MCPIP1 expression was observed at the concentration of 5 ng/mL, with increased MCPIP1 expression by 5.5-fold (Fig. 2C). Likewise, MCPIP1 mRNA was upregulated in HUVECs in a time-dependent fashion by TNF\(\alpha\), as determined by Real-time RT-PCR (Fig. 2D). Our observation that MCPIP1 can be induced in cultured ECs by TNF\(\alpha\) or IL-1\(\beta\) suggests that it may regulate cellular responses to these stimuli.

![Fig. 1. Expression analysis of MCPIP1 in mouse and human atherosclerotic lesions. (A) Representative photomicrographs of immunochemical staining with anti-MCPIP1 antibody. Three segments showed similar results. (B) MCPIP1 mRNA level in aorta from C57BL/6 and ApoE\(^{-/-}\) mice measured by Q-PCR. Mean ± S.D., \(n = 5\). \(* P < 0.01\) vs C57BL/6 group. (C) Immunofluorescence staining with anti-MCPIP1 antibody (1:50, red), and a smooth muscle-specific marker, anti-smooth muscle actin (α-SM actin, green) and nuclear staining (Dapi, blue) of the human carotid atherosclerotic lesion were performed. Negative control indicates non-immunized IgG matching the host species and concentration of anti-MCPIP1 antibody. (D) Immunofluorescence staining with anti-MCPIP1 antibody (1:50, red), and an EC-specific marker, anti-CD31 (green) and nuclear staining (Dapi, blue) of the human carotid atherosclerotic lesion were performed.](image-url)
3.3. MCPIP1 suppresses cytokine-induced VCAM-1 expression in HUVECs and attenuates monocyte adhesion to the activated HUVECs

Next, we examined the effects of MCPIP1 on cytokine-induced VCAM-1 and ICAM-1 expression in HUVECs. As shown in Fig. 3A and B, TNFα significantly induced the cell-surface expression of VCAM-1 and ICAM-1 by more than sixfolds in HUVECs. Overexpression of MCPIP1 markedly attenuated TNFα-induced expression of VCAM-1 in a dose-dependent manner, but not affect ICAM-1 expression, as determined by cell ELISA (Fig. 3A and B). MCPIP1 overexpression did not affect HUVEC viability as determined by trypan blue exclusion test (Fig. 3C). Western blot further confirmed that MCPIP1 overexpression suppressed TNFα-induced VCAM-1 expression, but not affect ICAM-1 expression. Meanwhile, it was observed that PAI-1 and iNOS expression in HUVECs were also suppressed by MCPIP1 (Fig. 3D). To determine the functional consequence of MCPIP1’s effect on VCAM-1 expression, we examined the effect of MCPIP1 on THP-1 and human CD14+ monocyte adhesion to the activated HUVECs. When HUVECs were stimulated with TNFα (10 ng/mL), THP-1 cell adhesion was substantially increased and the increased adhesion of THP-1 cells to the TNFα-stimulated HUVECs was suppressed by 50% by MCPIP1 overexpression (Fig. 3E). Similar to THP-1 cells, TNFα-induced adhesion of human CD14+ monocytes to HUVECs was also suppressed by MCPIP1 expression in a dose-dependent manner (Fig. 3F). To determine that VCAM-1 is critically involved in the monocyte adhesion to HUVECs, anti-VCAM-1 monoclonal antibody was added into the HUVECs. As shown in Fig. 3G, anti-VCAM-1 also dose-dependently reduced the adhesion of human monocytes to the activated HUVECs. Taken together, these results further suggest that MCPIP1 functions as a negative regulator of the cytokine-induced inflammatory responses in vascular ECs.

3.4. SiRNA-mediated knocking-down of MCPIP1 increases cytokine-induced VCAM-1 expression in HUVECs and enhances THP-1 cell adhesion to the activated HUVECs

To further study the significance of MCPIP1 in endothelial activation, we performed loss-of-function studies, using the RNA interference technique. Transfection of MCPIP1 siRNA substantially inhibited MCPIP1 expression by ≈60%, which results increased expression of TNFα-induced VCAM-1 expression in HUVECs (Fig. 4A and B). Furthermore, silencing of MCPIP1 expression significantly enhanced THP-1 cell adhesion to the TNF-α-activated HUVECs (Fig. 4C and D), further suggesting the involvement of MCPIP1 in the regulation of cytokine-induced endothelial activation.
3.5. MCPIP1 does not affect mRNA stability of VCAM-1, but inhibits the promoter activation of human VCAM-1 gene

The expression of adhesion molecules VCAM-1 is tightly regulated at both transcriptional and posttranscriptional levels. One recent report showed that MCPIP1 (ZC3H12A) functions as a RNase to regulate mRNA degradation [11]. To explore the mechanisms of MCPIP1 effect on the expression of VCAM-1, we first evaluated the potential influence of MCPIP1 on the mRNA degradation of VCAM-1 in TNFα-stimulated HUVECs. In the experiment shown...
in Fig. 5A, the cells were stimulated with TNF-α (10 ng/ml, Sigma) for 8 h and then treated with actinomycin D (5 μg/ml, Sigma). The RNA was isolated at different time points as indicated. Overexpression of MCPIP1 does not affect the mRNA degradation of VCAM-1. Then, we asked whether MCPIP1 regulates the promoter activity of VCAM-1. For these studies, we used a 1.3 kb fragment of the human VCAM-1 promoter. The promoter contains all of regulatory elements necessary for TNF-α responsiveness in vivo [14,16]. As shown in Fig. 5B, forced expression of MCPIP1 dose-dependently inhibited TNF-α-induced promoter activity of VCAM-1. These results strongly suggest that MCPIP1 negatively regulates adhesion molecules gene expression by inhibition of their transcription.

4. Discussion

This study provides evidence that MCPIP1 acts as a negative regulator for the proinflammatory action of TNF-α on vascular ECs in vitro. In particular, MCPIP1 could counteract the increase in monocyte adhesion to ECs attributable to TNF-α, which is crucial to the inflammatory reaction. This anti-inflammatory effect of MCPIP1 is correlated with the inhibition of the induction of VCAM-1 in response to TNF-α. Our data also shows increased expression of MCPIP1 in atherosclerotic lesion, which underlines the importance of this novel protein in plaque progression.

Vascular disease such as atherosclerosis involves predominantly macrophages, T cells, ECs, and smooth muscle cells that interact with each other in the vessel wall [17]. Recently, we have identified MCPIP1 as a critical regulator of proinflammatory activation of macrophages. In response to inflammatory stimuli, such as LPS and TNF-α, the expression of MCPIP1 is markedly induced in monocytes and macrophages. Indeed, overexpression of MCPIP1 in macrophages has been shown to inhibit inflammatory cytokine expression, but not affect the surface receptors expression as well as oxidized LDL uptake in these cells. In ECs, MCPIP1 overexpression has been shown to increase cell survival and angiogenesis [18]. However, the function of endogenous MCPIP1 in endothelial activation has not been previously studied. Here, we demonstrate that MCPIP1 may exert additional activities of potential benefit for the vessel wall through suppressing monocyte adhesion to the activated endothelium, which is a key process in the initiation of atherosclerosis. Thus, we conclude that MCPIP1 may contribute to the resolution of vascular inflammation by constituting a negative feedback loop in proinflammatory activation of macrophages and ECs.

Interestingly, it is noted that MCPIP1 represses TNF-α-induced VCAM-1 expression, but no significant inhibition is seen on ICAM-1 expression. This is an intriguing observation, although the basis for this differential inhibition has not been fully elucidated by our studies. Similar phenomenon was also observed by other group [14].

The exact mechanisms that MCPIP1 reduced the expression of VCAM-1 are not clear. Recent reports show that MCPIP1 (Zc3h12a) may contain Rnase activity to promote the mRNA degradation of some proinflammatory cytokines [11,19]. We observed that overexpression of MCPIP1 did not affect the mRNA degradation of
VCAM-1 and ICAM-1. We did observed that overexpression of MCPIP1 inhibited TNFα-induced human VCAM-1 promoter activity. Ample evidence suggests that proinflammatory cytokines such as TNFα and IL-1β induce nuclear factor-kB (NF-kB) activation in ECs, which mediates their proatherogenic effects. These include induction of adhesion molecules such as VCAM-1 and ICAM-1, thus promoting monocyte adhesiveness to the endothelium. Indeed, MCPIP1 has been reported to inhibit NF-kB activation by an as yet unknown mechanism in HEK293 cells and macrophages. Our recent studies further identified MCPIP1 as a deubiquitinating enzyme that negatively regulates NF-kB and JNK signaling by targeting to TNF receptor associate factor family (unpublished data). Taken together, our results suggest that MCPIP1 may inhibit cytokines-initiated NF-kB signaling and thus limits VCAM-1 gene transcription.

We have conducted serial experiments to understand how MCPIP1 controls LPS and cytokines-initiated proinflammatory signaling pathways and the resulting manuscript has been submitted to elsewhere. Overall, the data reported herein provide the evidence that the MCPIP1 is a potent negative regulator of the TNFα-induced EC activation. Thus, specifically modifying MCPIP1 activity may have potential application in the treatment of inflammatory vascular disorders.

5. Conflict of interest

None declared.

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References