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MKP-7, a negative regulator of JNK, regulates VCAM-1 expression through IRF-1

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ABSTRACT

Cell adhesion molecules (CAMs) are involved in a variety of pathologies including cancer, inflammation, pathogenic infections and autoimmune disease. In particular, VCAM-1, rather than ICAM-1, plays a major role in the initiation of atherosclerosis and tumor progression. Therefore, we attempted to elucidate differential mechanisms that regulate VCAM-1 and ICAM-1 expressions. Down-regulation of JNK by a specific inhibitor (SP600125) or dominant negative (DN) JNK1 plasmid enhanced TNF-α-induced VCAM-1 but not ICAM-1 expression. Moreover, transfection with a JNK1-overexpressing vector resulted in the inhibition of VCAM-1 expression stimulated by TNF-α in HUVECs, suggesting that JNK negatively regulates TNF-α-induced VCAM-1 expression in endothelial cells (ECs). Next, we investigated whether JNK signaling affects IRF-1 and/or GATA6, which are transcription factors that mediate TNF-α induction of VCAM-1 but not ICAM-1. The DN-JNK1 plasmid-transfected cells enhanced TNF-α up-regulation of IRF-1 whereas JNK1-overexpressing cells displayed down-regulation; however, neither DN-JNK1 transfection nor JNK1 overexpression affected GATA6 protein levels in the nuclear fraction. Chromatin immunoprecipitation (ChIP) assay confirmed that the inhibition of JNK by DN-JNK1 transfection increases the binding of IRF-1 to the VCAM-1 promoter whereas the overexpression of JNK1 inhibits IRF-1 binding to the VCAM-1 promoter. However, neither DN-JNK1 nor JNK1 overexpression altered GATA6 affinity for the VCAM-1 promoter region. We also examined whether MKP-7 affects ICAM-1 or VCAM-1 by regulating JNK. TNF-α-induced phosphor-JNK levels increased after 5 min, peaked at 10 min, and decreased after 30 min. Interestingly, MKP-7 protein levels increased after 30 min, when phosphor-JNK induction by TNF-α was decreased. In addition, silencing MKP-7 with specific siRNA resulted in an increase in phosphor-JNK and inhibited the expression of VCAM-1 but not ICAM-1. Moreover, silencing MKP-7 caused the down-regulation of IRF-1 protein levels and binding to the VCAM-1 promoter. Thus, we suggest that MKP-7, a negative regulator of JNK, regulates VCAM-1 expression in activated endothelial cells through IRF-1 but not GATA6.

1. Introduction

Cell adhesion molecules (CAMs) are involved in a broad range of normal physiological processes, including cell–cell and cell–matrix interactions, cell migration, cell cycle, and signaling as well as morphogenesis during development and tissue regeneration [1]. The pivotal role of CAMs is emphasized by the fact that CAMs are involved in a variety of pathologies including cancer, inflammation, pathogenic infections, and autoimmune disease [1].

Vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) are closely related in structure and function. Both are cytokine-inducible Ig gene superfamily members that bind leukocyte integrins [2]. However, VCAM-1 expression is largely restricted to lesions and lesion-predisposed regions whereas ICAM-1 expression extends into uninvolved aorta and lesion-protected regions [3], suggesting that VCAM-1 and ICAM-1 have different functions in lesion initiation. Moreover, Cybulsky et al. reported that VCAM-1 plays a major role in the initiation of atherosclerosis, although the expression of both VCAM-1 and ICAM-1 is regulated in atherosclerotic lesions [4]. In addition, it has been reported that some highly metastatic human melanoma cells migrate and adhere to VCAM-1 rather than to ICAM-1 [5] and that tumor cells can escape T-cell immunity by overexpressing VCAM-1 [6]. These

Abbreviations: ANOVA, analysis of variance; AP-1, activator protein-1; ChIP, chromatin immunoprecipitation; ECL, enhanced chemiluminescence; ECGS, endothelial cell growth supplements; ERK, extracellular regulated kinase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; ICAM, intracellular adhesion molecule; IL-1, interleukin-1; IRF-1, IFN regulatory factor-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MKP, MAPK phosphatase; NF-κB, nuclear factor κB; SDS, sodium dodecyl sulfate; PCNA, proliferating cell nuclear antigen; PKC, protein kinase C; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SP-1, specificity protein 1; TBS-T, Tris-buffered saline/Tween 20; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

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observations suggest that the expression of VCAM-1 in tumors promotes T-cell migration away from tumors, resulting in decreased accumulation of T cells in the tumor microenvironment. Therefore, it is important to discover therapeutic strategies that have specific suppressive effects on VCAM-1 rather than ICAM-1. To this end, we have attempted to elucidate differential mechanisms that regulate VCAM-1 and ICAM-1 expressions. TNF-α-dependent VCAM-1 induction is regulated at the gene level by the activity of transcription factors such as NF-κB, activator protein-1 (AP-1), specificity protein-1 (SP-1), IFN regulatory factor-1 (IRF-1), and GATA. The existence of functional IRF-1 and GATA binding motifs in the VCAM-1 gene promoter region distinguishes VCAM-1 from other CAMs [7–12].

Previously, we have reported that PI3K/Akt signaling is involved in VCAM-1 but not ICAM-1 induction by TNF-α [13] and, further, that PTEN negatively regulates TNF-α-induced VCAM-1 expression by modulating the PI3K/Akt/GSK-3β/GATA6 signaling cascade [14]. We also found that MAPKs differentially regulate ICAM-1 and VCAM-1 [15]. Interestingly, in the present study, we discovered that specific inhibition of JNK in endothelial cells enhances TNF-α-mediated VCAM-1 induction.

MAPK activity is regulated at multiple levels. One critical aspect of this regulation is the reversible phosphorylation of MAPKs. Negative regulation of MAPKs is achieved by the dephosphorylation of the TXY motif by phosphatases [16,17]. Recently, the MAPK phosphatases (MKPs), a family of dual-specificity protein phosphatases, have emerged as in vivo candidates for negative regulators of MAPKs [18,19]. In mammals, 10 MKPs have been reported that exhibit precise regulation of their substrate specificity to avoid inappropriate inactivation of MAPKs [18,19]. MKP-7 has been identified as a JNK-specific phosphatase (JNK ≫ p38 > ERK) with the characteristics of a nucleocytoplasmic shuttle protein [20].

In the present study, we demonstrated for the first time that JNK negatively regulates VCAM-1 expression by TNF-α in endothelial cells and JNK down-regulation by MKP7 leads to up-regulation of IRF-1, which is important for activation of VCAM-1, but not ICAM-1.

2. Materials and methods

2.1. Materials

Tissue culture medium 199, fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), glutamine and collagenase were supplied by Gibco-BRL (Rockville, MD). MKP-7 small interfering RNA (siRNA), siRNA transfection kit, anti-ICAM-1, anti-VCAM-1, anti-IRF-1, anti-GATA6, and anti-PCNA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phosphor-SAPK/JNK (Thr183/Tyr185) and anti-SAPK/JNK (Thr183/Tyr185) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence (ECL) Western blotting detection reagent was obtained from Amersham (Buckinghamshire, UK). All other chemicals, including endothelial cell growth supplements (ECGS) and heparin, were supplied by Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA), grown in medium 199 supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 5 U/mL heparin, 100 IU/mL penicillin, 10 μg/mL streptomycin and 50 μg/mL ECGS and incubated in a humidified 5% CO_2 incubator. Cells were used between passages 3 and 6.

2.3. Western blot analysis

To isolate the total cell extracts, cells were lysed in PRO-PREP protein extract solution. Samples were centrifuged at 13,000 rpm × 20 min at 4 °C. Protein concentration was determined by the Bradford method. An equal volume of 2× SDS sample buffer (0.1 M Tris–Cl, 20% glycerol, 4% SDS, and 0.01% bromophenol blue) was added to each aliquot of the supernatant fraction from the lysates, and the samples were boiled for 5 min. Nuclear proteins were extracted as previously described [21]. Aliquots of 30 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis for 1 h 30 min at 110 V. The separated proteins were transferred to PVDF membrane for 2 h at 20 mA using an SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) for 2 h at room temperature. Then, membranes were incubated with primary antibodies in 5% skim milk in TBS-T overnight at 4 °C, and bound antibody was detected using horseradish peroxidase-conjugated anti-rabbit IgG. Membranes were washed and then developed using a Western blotting Luminol Reagent system (Amersham).

2.4. Plasmid construction

Plasmids containing wild-type VCAM-1-luciferase and plasmids containing VCAM-1-luciferase with mutated IRF-1 site (mIRF-1), with mutated GATA site (mGATA) and with both mutated sites (mIRF and mGATA) were constructed as previously described [21]. The dominant negative (DN)-JNK1 plasmid pcDNA3-DN-JNK1 and the control vector were kindly provided by Dr A.-K. Yi (University of Tennessee Health Science Center, Memphis, Tennessee). The full-length coding sequence of human JNK1 (GenBank accession no. L26318) was obtained by PCR using cDNA produced from HUVEC mRNA and was then inserted into a T&A Cloning Vector (RBC, Chung Ho, Taipei). In this subcloned vector, *Kpn* I/*BamH* I digested fragments were cloned into the *Kpn* I/*BamH* I site of the pcDNA3.1/V5-His mammalian cell expression vector (Invitrogen, Carlsbad, CA). The sequences were confirmed by automated DNA sequencing.

2.5. Transfection

Transient transfection was performed using Superfect® from QIAGEN (Valencia, CA) according to the manufacturer's protocol. Briefly, 5×10^5 cells were plated into 60-mm dishes the day before transfection and grown to about 70% confluence. Cells were transfected with empty vector (pGL3 and/or pcDNA3) or with 1 μg of reporter gene construct + 0.5 μg of p-RL-TK-luciferase. Transfections were allowed to proceed for 12 h. Transfected cells were washed with 4 mL of PBS and then stimulated with TNF-α (10 ng/mL). The cells were continually cultured in serum-free medium 199 until they were harvested. Luciferase activity was normalized using pRL-TK-luciferase activity (*Renilla* luciferase activity) in each sample.

2.6. Luciferase assay

After these treatments, 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 performed in triplicate. Data are presented as the ratio between Firefly and *Renilla* luciferase activities.

2.7. Chromatin immunoprecipitation (ChIP) assay

This procedure was performed with the ChIP assay kit from Cell Signaling Technology (Beverly, MA), according to the manufacturer's instructions. HUVECs were transfected with vectors, later stimulated with TNF-α for 2 h, and then fixed in 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by adding glycine. DNA was digested using Micrococcal Nuclease to a length of approximately 150–900 bp, and chromatin was incubated with 10 μg anti-IRF-1 antibody at 4 °C (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immune complexes were precipitated, washed, and eluted as