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The transcriptional factor PREB mediates MCP-1 transcription induced by cytokines in human vascular endothelial cells

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ABSTRACT

Objective: The prolactin regulatory element binding (PREB) protein is a transcriptional factor that regulates prolactin promoter activity in rat anterior pituitary. It is expressed not only in the anterior pituitary but also in the cardiovascular system, including in human umbilical vascular endothelial cells (HUVECs). Monocyte chemoattractant protein-1 (MCP-1) is a major chemotactic factor for monocytes and a key factor initiating the inflammatory process of atherogenesis. MCP-1 is expressed in HUVECs in response to several different stimuli, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α.
Methods and results: MCP-1 expression was regulated by IL-1β and TNF-α and cytokine-induced PREB expression. Conversely, over-expression of PREB using a PREB-expressing adenovirus increased MCP-1 expression in HUVECs. In addition, PREB induced the expression of the luciferase reporter protein under the MCP-1 promoter control. EMSA showed that the transcriptional effect of PREB was mediated by its binding to the PREB-responsive cis-element of the MCP-1 promoter. Finally, we used siRNA to inhibit PREB expression in HUVECs and demonstrated that knockdown of PREB expression attenuated the effects of IL-1β and TNF-α on MCP-1 expression.
Conclusions: In summary, our findings show that PREB can function as a transcriptional regulator of the MCP-1 promoter in response to cytokines.

1. Introduction

The PREB gene encodes a 1.9-kb mRNA that translates into a transcriptional factor, which binds to and activates the basal prolactin promoter [1,2]. The primary sequence of the PREB protein contains 2 potential trans-regulatory PQ-rich domains and 3 regions of high similarity to the WD-repeat, thus making it a member of a eukaryotic family of WD-repeat proteins. Members of this ever-growing family are involved in multiple cellular functions that include signal transduction, RNA processing, cytoskeletal assembly, and vesicle trafficking [3]. Although PREB is ubiquitously expressed, the levels of expression vary greatly among tissues, with very high levels detected in human pituitary gland, pancreas, and cardiovascular systems [1,2,4]. However, the role of PREB in vascular endothelial cells has not been clarified yet.

Chemokines are small molecular mass proteins (8–16 kD) that were originally classified as a family on the basis of the presence of a conserved 4-cysteine motif and their ability to cause the directed migration of leukocytes in vitro [5]. Monocytes have been shown to be selectively attracted to specific chemokines that predominantly belong to the C–C family of chemoattractants, which includes human monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a 76-amino-acid chemokine thought to be the major chemotactic factor for monocytes [6]. MCP-1 is expressed by a variety of cell types including monocytes, smooth muscle cells, and vascular endothelial cells in response to several different stimuli such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α [7,8]. The expression of MCP-1 is strongly dependent on activation of the transcriptional factor nuclear factor (NF)-κB [9]. Previously, we reported that the MCP-1 proximal promoter deleted the binding sites for NF-κB still had been activated by TNF-α or IL-1β-stimulation [10,11].

In this study, we examined the effect of PREB on the transcription of the MCP-1 gene. These results suggest that PREB is an important transcriptional factor that regulates the MCP-1 gene in human umbilical vein endothelial cells.

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2. Methods

2.1. Cell culture

HUVECs were purchased from Clonetics (San Diego, CA) and used between passages 1 and 6. They were maintained in M199 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical Co., Tokyo, Japan), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.1.1. Plasmid construction

The reporter contains the human MCP-1 gene sequence spanning the region from −515 to +44, which was amplified by PCR and cloned into the luciferase reporter gene (pMCP-LUC) as previously described [10]. To generate the mutant construct (pMCP-mt-LUC) of the PREB response sequence (PRS) within the vector, pMCP-LUC was mutated from −195–5′-TTAACAGCCCACTT*ATCA*CTCATGGAAGAT-CCCT-3′ −161 to 5′-TTAACAGCCCACTT*GGTA*CTCATGGAAGATCCCT-3′ (mutated nucleotides are italicized) by site-directed mutagenesis.

2.1.2. Real-time reverse transcriptase polymerase chain reaction

Polymerase chain reactions (PCRs) were performed in a final volume of 20 μl in LightCycler glass capillaries (Roche, Mannheim, Germany) as previously described [12]. The sequences of the forward and reverse human MCP-1 primers were 5′-AATAGGAAGATCTCAGTGCA-3′ and 5′-TCAAGTCTTCGGAGTTTGGG-3′, respectively. The cycling program consisted of initial denaturation for 600 s at 95 °C followed by 55 cycles of 95 °C for 5 s, 62 °C for 5 s, and 72 °C for 15 s, with a 20 °C/s slope. Known amounts of DNA were then diluted to provide standards and a regression curve of crossing points versus concentration generated with the LightCycler. GAPDH was used as housekeeping standard.

2.2. Transfection of HUVECs and luciferase reporter gene assay

To confirm the transcriptional regulation of MCP-1 expression by PREB, we used a promoter construct of the MCP-1 gene [10,11]. The purified reporter plasmid was transfected into HUVECs (at 60% confluence) by conventional cationic liposome transfection methods (Lipofectamine; Life Technologies, Gaithersburg, MD). Rous sarcoma virus-β-galactosidase was added to all transfection mixtures to monitor the efficiency of DNA uptake by HUVECs as described previously [10]. We used 20-μl aliquots for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

2.3. MCP-1 enzyme-linked immunosorbent assay

The levels of immunoreactive MCP-1 were quantified using a commercially available sandwich-type enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN).

2.3.1. Transfection of siRNA

The following siRNAs were designed to target cDNA sequences: scrambled, 5′-CCGTTCTGTACAGGGAGTACT-3′; and PREB-siRNA, 5′-AATGGCGTGCACTTTCTGCAG-3′ [4]. PREB-siRNA was transfected using siPORT Amine (Ambion, CA). MCP-1 promoter activity was examined by performing a transient transfection analysis 3 days after transfection.

2.3.2. Electrophoretic mobility shift analysis

Nuclear extracts from HUVECs were prepared according to a technique described previously [13]. We used a synthetic DNA duplex spanning the MCP-1 promoter (5′-TTAACAGCCCACTTATC-ACTCATGGAAGATCCCT-3′), and mutant MCP-1 promoter probe (mt-MCP) (5′-TTAACAGCCCACTTGGTACTCATGGAAGATCCCT-3′) (Nihon Bioservice, Asagiri, Japan) in these studies; they were radiolabeled at the 5′-end by incubating each strand separately with [γ^{32}P]-ATP and polynucleotide kinase prior to annealing.

2.3.3. Western blot analysis

Cells were washed, scraped in PBS, and lysed as described previously [4]. The membranes were incubated for 1 h at 4 °C with 0.2% Tween 20 in PBS (PBS-T) containing anti-PREB antiserum (dilution 1:250) as described previously [4].

2.3.4. In vitro transcription and translation

The pcDNA3.1 (+) vector carrying the PREB cDNA or vector only was transcribed in vitro with T7 RNA polymerase (Gibco-BRL, Tokyo, Japan) as previously described [4]. The RNA product was translated using a rabbit reticulocyte lysate system (Promega, San Luis Obispo, CA).

2.3.5. Generation of adenovirus and adenovirus treatment

Full-length rat PREB cDNA was inserted into the pShuttle vector plasmid as previously described [4]. Adenovirus expressing the PREB (Ad-PREB) was constructed using the Adeno-X Expression System kit (CLONTECH Laboratories, Inc., Palo Alto, CA) according to the instructions of the manufacturer. Adeno-X-lacZ adenovirus (Ad-LacZ) was used as a control. Adenoviruses were amplified in HEK 293 cells, and purified and concentrated to 10^{12} plaque-forming units per milliliter (pfu/ml) by CsCl ultracentrifugation. Expression of PREB was induced by incubation with the Ad-PREB adenoviruses for 3 h at a multiplicity of infection of 1000 pfu/cells.

2.3.6. Chromatin immunoprecipitation (ChIP) assays

After cross-linking for 10 min with 1% formaldehyde in serum-free medium, phosphate–glycine buffer was added to a final concentration of 0.125 M and cells were washed twice with ice-cold

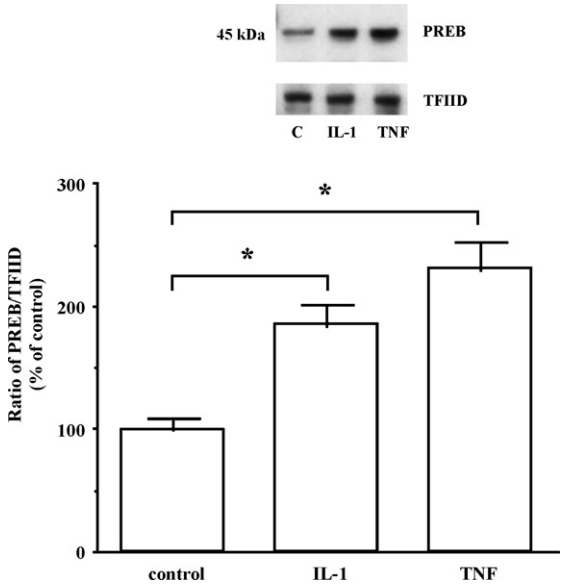

Fig. 1. Effects of cytokines on PREB expression in HUVECs. Nuclear extract from HUVECs treated with IL-1β (1 ng/ml) and TNF-α (10 ng/ml) (shown on top of each of lanes) and probed for PREB using western blot analysis. Abundance of TFIID served as control is shown on the bottom of each lane and the ratio of PREB/TFIID is shown as % of control in the figure. A graph showing the mean ± SEM of 3 experiments for each treatment group is shown. The asterisk and N.S. denote a significant difference ($P < 0.01$).