Binding of upstream stimulatory factor 1 to the E-Box regulates the 4G/5G polymorphism–dependent plasminogen activator inhibitor 1 expression in mast cells

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Background: Plasminogen activator inhibitor (PAI)–1 is a key regulator of the fibrinolytic system. PAI-1 levels are markedly elevated in the asthmatic airways. The 4G/5G polymorphism of the PAI-1 gene is associated with allergic asthma.
Objective: To characterize the mechanisms of the 4G/5G-dependent PAI-1 expression in mast cells (MCs), a major source of PAI-1 and key effector cells in asthma.
Methods: Transcription of PAI-1 was assessed by transiently transfecting human MC line (HMC-1) cells with the luciferase-tagged PAI-1 promoters containing the 4G or 5G allele (4G–PAI-1 or 5G–PAI-1 promoter). Upstream stimulatory factor (USF)–1 and the E-box interactions were studied by electrophoretic mobility shift assays and supershift assays. Expression of USF-1 was determined by Western blot analysis.
Results: The 4G–PAI-1 promoter has higher promoter activity than the 5G–PAI-1 promoter in stimulated HMC-1 cells, and the E-box adjacent to the 4G/5G site (E-4G/5G) regulates the genotype-specific PAI-1 transcription. USF-1 binds to the E-4G with greater affinity than to the E-5G. USF-1 level is increased in HMC-1 cells after stimulation, and elevated USF-1 enhances PAI-1 transcription. Overexpression of wild-type USF-1 or dominant-negative USF remedies the 4G/5G-dependent PAI-1 transcription.
Conclusion: Binding of USF-1 to the E-4G/5G regulates the 4G/5G polymorphism–dependent PAI-1 expression in MCs.
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Key words: *Plasminogen activator inhibitor 1, 4G/5G polymorphism, transcription, human mast cells, upstream stimulatory factor 1, E-box*

Abbreviations used
bHLH: Basic helix-loop-helix
DN: Dominant-negative
E-4G/5G: E-box adjacent to the 4G/5G allele
EMSA: Electrophoretic mobility shift assay
hMC: Human mast cell
MC: Mast cell
PAI: Plasminogen activator inhibitor
TFE3: Transcription factor E3
USF: Upstream stimulatory factor
WT: Wild-type
Zic2: Zinc finger transcription factor member 2

Plasminogen activator inhibitor (PAI)–1 is a key regulator of the fibrinolytic system.[1] We previously demonstrated that the PAI-1 levels are markedly elevated in the airways of human and murine allergic asthma.[2,3] Others also demonstrated that PAI-1 expression is increased in nasal tissues of human and murine allergic rhinitis.[4,5] Depletion of PAI-1 reduces the infiltration of inflammatory cells and collagen deposition in these models. These findings strongly suggest that PAI-1 promotes the development of allergic diseases by inducing allergic inflammation and tissue remodeling.

Mast cells (MCs) are key effector cells in asthma and other allergic diseases. We discovered that the PAI-1 gene is induced at the highest level among the inducible human MC (hMC) genes analyzed with DNA microarray.[2] Nakajima et al[6] also demonstrated PAI-1 is one of the most highly upregulated genes in IgE-stimulated hMCs. We previously showed that MCs secrete abundant amounts of PAI-1 in the airways of the ovalbumin-challenged murine model of allergic asthma.[3] PAI-1 production is 2-fold lower in the airways of MC-deficient W/W^v mice after ovalbumin challenge compared with that in wild-type (WT) mice (S.H. Cho and C.K. Oh, unpublished data, December 2003). These findings suggest that MCs are a major source of PAI-1 in asthma.

Molecular regulations of PAI-1 expression have been studied in various cell types. A single guanosine nucleotide insertion/deletion polymorphism (4G/5G) at −675 bp of the PAI-1 gene has been extensively studied. The plasma level of PAI-1 is higher in patients with the 4G/4G genotype than in those with the 5G/5G genotype, whereas the 4G/5G has intermediate values.[7-13] The 4G allele frequency is higher than the 5G (0.63-0.71 vs 0.29-0.37) in Asian people,[14] whereas in healthy white people, they are similar.[7,10-13] However, the frequency of the 4G is significantly higher than the 5G in patients with asthma. We reported that in Nottingham families of United Kingdom, the 4G frequency of the children with asthma is 0.67, but the 5G frequency is as low as 0.33.[15] In Czech population, the 4G and 5G frequency is similar, but in patients with asthma or other allergic diseases, the 4G frequency is significantly higher than the 5G (0.6 vs 0.4).[16] In house dust mite–sensitive patients with allergic asthma, the 4G frequency is 0.7, whereas in healthy controls, it is 0.55.[12] In patients with the 4G/4G, the serum total IgE and PAI-1 levels during allergen challenge are significantly higher than in patients with the 5G/5G.[12,17] These findings suggest that genetically elevated PAI-1 levels are linked to a propensity to develop asthma and other allergic diseases. However, the molecular mechanisms involved in the

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genotype-specific PAI-1 expression in asthma or other allergic diseases are unknown. Angiotensin II,[18] very-low-density lipoproteins,[19] IL-1,[8,14] and circadian clock proteins BMAL/CLOCK[9] are known to induce higher levels of PAI-1 expression with the 4G allele compared with the 5G allele in human hepatocellular carcinoma (HepG2) cells, endothelial cells, or monkey kidney fibroblast-like (COS)-7 cells. In the current study, we report the first evidence that the 4G allele renders higher PAI-1 promoter activity in stimulated hMCs by binding to upstream stimulatory factor (USF)–1 with greater affinity than the 5G allele.

METHODS

Reagents and materials

DMRIE-C trransfection reagent and 6% DNA Retardation Gels were purchased from Invitrogen (Carlsbad, Calif). Dual-Luciferase Assay Kit and luciferase report vectors pGL3-Basic and pRL-CMV were from Promega (Madison, Wis). Ionomycin and poly (dI-dC) were from Sigma (St Louis, Mo). Antibodies against human USF-1 (C-20), USF-2 (C-100), transcription factor E3 (TFE3; H-300), Wilms tumor suppressor (C-19), Zinc finger transcription factor member 2 (Zic2; N-13), and histone H1 (FL-219), and Western Blotting Luminol Reagent were from Santa Cruz Biotechnology (Santa Cruz, Calif).

hMC culture and stimulation

Human mast cell 1 (HMC-1) cells (a kind gift from J. H. Butterfield, Mayo Clinic, Rochester, Minn) were maintained as described[2] and were stimulated with 2 μmol/L ionomycin for the indicated times.

Plasmids constructs and site-directed mutagenesis

Plasminogen activator inhibitor 1 promoter luciferase construct containing 5′ flanking −800 bp to +71 bp of human PAI-1 promoter inserted in pGL3-Basic vector (pGL3-Basic-800),which contains the 4G allele, was a kind gift from Dr Anh D. Le, University of Southern California (Los Angeles, Calif). To generate pGL3-Basic-800 with the 5G allele, site-directed mutagenesis was performed by using QuikChange Site-Directed Mutagenesis Kit (Stratagene Inc, San Diego, Calif). To engineer 5′-deletion PAI-1 promoter luciferase reporter constructs containing 5′ flanking −698 bp and −41 bp, PCR products with 4G–pGL3-Basic-800 or 5G–pGL3-Basic-800 as template were inserted into pGL3-Basic at *Sac* I and *Bgl* II sites. Site-directed mutagenesis was also performed to introduce dinucleotide substitution (CACGTG→CAATTG) into the E-box at −682 bp to −677 bp of PAI-1 promoter in 4G–pGL3-Basic-800 and 5G–pGL3-Basic-800. The primers used for these constructions are summarized in this article's Table E1 in the Online Repository at www.jacionline.org. Each construct was sequenced for verification and orientation.

Transient transfection of HMC-1 cells and luciferase assay

Human mast cell 1 cells (2×10^6 cells per well) were transfected in 6-well tissue culture plates by using DMRIE-C according to the manufacturer's protocol. Briefly, cells were cotransfected with 4 μg pGL3-Basic containing different PAI-1 promoter fragments and 20 ng *Renilla* luciferase reporter vector pRL-CMV as internal control. Transfected cells were stimulated with 2 μmol/L ionomycin or an equal volume of ethanol 24 hours after transfection, and the luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wis) 12 hours after stimulation. Final firefly luciferase activities were normalized to corresponding *Renilla* luciferase activities, and the relative transcriptional activity was expressed as the ratio of the normalized luciferase activities between the constructs assayed and pGL3-Basic-41 (minimal PAI-1 promoter construct). To study the effects of USF-1 on PAI-1 transcription, HMC-1 cells were cotransfected with 4G–pGL-3 Basic-800 or 5G–pGL-3 Basic-800 PAI-1 promoter constructs and human USF-1 expression vector pSG5-hUSF-1 or dominant-negative (DN) USF construct p566-A-USF, the kind gifts of Dr Nanyue Chen (MD Anderson Cancer Center, Houston, Tex). Experiments were performed at least 3 times independently.

Nuclear extracts purification and radiolabeled probe preparation

Briefly, 2×10^7 HMC-1 cells were stimulated with 2 μmol/L ionomycin or equal ethanol for 2 hours. Nuclear extracts were prepared using Qproteome Nuclear Protein Kit (QIAGEN, Valencia, Calif). Native PAI-1 promoter fragments −687/−664 (4G) or the −688/−664 (5G) segment [GTCTG GACA CGTGGGG(G)AGTCAGCC] and 4T-substituted 5G segment GTCTGG ACACGTGTTTTAGTCAGCC (1G) were synthesized and labeled with [α-^{32}P]CTP by using a Rediprime II Labelling System (Amersham Biosciences, Piscataway, NJ).

Electrophoretic mobility shift assays

Nuclear extracts 10 μg were incubated with ^{32}P-labeled 24-bp or 25-bp PAI-1 promoter probes in 20 μL binding buffer (10 mmol/L TRIS-HCl, pH 7.5, 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA) containing 1 μg poly (dI-dC) for 20 minutes at room temperature, and the resulting complexes were then separated on 6% DNA Retardation Gels. For competition assay and supershift assay, 50-fold molar excess of unlabeled oligonucleotides or 2.0 μg antibodies against USF-1, USF-2, TFE3, Wilms tumor suppressor 1, and Zic2 were incubated with nuclear proteins for 20 minutes before adding radiolabeled probes. Intensities of protein-DNA complexes were quantified with densitometric analysis.

Western blot analysis

Nuclear proteins (10 μg) from HMC-1 cells stimulated with 2 μmol/L ionomycin for 0, 2, 4, or 8 hours were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were incubated for 1 hour at room temperature with the primary rabbit antibodies against human USF-1 (1:1000), followed by incubation with the secondary antibody of goat antirabbit IgG. The Western Blotting Luminol Reagent was used for detection. Histone H1 was used as the internal control to show similar amounts of samples were loaded at each condition. Signal intensities of USF-1 proteins were quantified with densitometric analysis.

Statistical analysis

Results were expressed as means ± SEs. Differences between 2 groups were identified with a paired Student *t* test. Significance was defined as $P < .05$.

RESULTS

4G–PAI-1 promoter has higher promoter activity than 5G–PAI-1 promoter in stimulated HMC-1 cells

On the basis of the kinetics assay that showed PAI-1 promoter activity reaches its peak 12 hours after ionomycin stimulation (see this article's Fig E1 in the Online Repository at www.jacionline.org), transfected HMC-1 cells were stimulated for 12 hours for promoter assays in the current study.

To determine the 4G/5G polymorphism–dependent regulation of PAI-1 expression, HMC-1 cells were transiently transfected with 4G–pGL3-Basic-800 or 5G–pGL3-Basic-800. Promoter activity was 32% higher in 4G–pGL3-Basic-800 than 5G–pGL3-Basic-800 in ionomycin-stimulated HMC-1 cells ($P < .001$), whereas promoter activities of these constructs were comparable in unstimulated HMC-1 cells (Fig 1). Further deletion of sequences from –800 bp to –699 bp had no effect on promoter activity of these constructs, suggesting that the sequences from