Histone Deacetylase-1 Is Enriched at the Platelet-derived Growth Factor-D Promoter in Response to Interleukin-1β and Forms a Cytokine-inducible Gene-silencing Complex with NF-κB p65 and Interferon Regulatory Factor-1*

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The platelet-derived growth factor (PDGF) family of ligands and receptors has been implicated in a wide variety of diseases involving aberrant migration and proliferation, including malignancy and atherogenesis (1–3). PDGF is synthesized by many different cell types, and its expression is broad. PDGF-D is one of two nonclassical PDGF ligand chains (other is PDGF-C) originally discovered in 2001 (4, 5). PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD act via two receptor tyrosine kinases, PDGF receptors α and β. PDGF-A and PDGF-B are activated during intracellular transport by exocytic secretion, whereas PDGF-C and PDGF-D are secreted as latent factors that require activation by extracellular proteases. PDGF-D has a two-domain structure similar to PDGF-C and is secreted as a disulfide-linked homodimer and is a specific agonistic ligand for PDGF receptor β (5). PDGF-D mRNA is widely expressed in organs such as the heart, pancreas, kidney, and ovary and has been linked to lung, prostate, and ovarian cancers. PDGF-D promotes tumor growth by accelerating tumor cell proliferation and inducing tumor neovascularization (6, 7). PDGF-D is also shown to induce Notch-1-dependent angiogenesis (8). Notch activation promotes the differentiation of blood vessels during development and has been shown to be depressed upon vascular injury or induced by nitric oxide (9). PDGF-D also mediates p65 interaction with IRF-1 and the accumulation of both transcriptional factors at the PDGF-D promoter. Mutation of DNA-binding elements relieved the promoter-mediated repression. PDGF-D repression involves histone deacetylation and interaction with HDAC-1 small interfering RNA (siRNA), induced by IL-1β and binds to a different element upstream in the promoter. Immunoprecipitation and chromatin immunoprecipitation experiments showed that IL-1β regulate PDGF-D promoter activity involves phosphorylation of multiple residues in the zinc finger of Sp1 (14). Whether histone modification in chromatin by enzymes such as histone deacetylases (HDACs) regulate PDGF-D transcription is poorly understood. We recently demonstrated that angiotensin II (12, 13). More recently, we demonstrated that Sp1 regulation of PDGF-D promoter activity involves phosphorylation of multiple residues in the zinc finger of Sp1 (14). Whether histone modification in chromatin by enzymes such as histone deacetylases (HDACs) regulate PDGF-D transcription is also unexplored. HDACs play a central role in the epigenetic regulation of gene expression. Recent findings from our group indicate that IL-1β facilitates HDAC dissociation from the PDGF receptor α promoter and that HDAC inhibition potentiates induction of PDGF receptor α transcription (15).

Proliferative cytokines activate a complex network of intracellular signaling pathways that alter gene expression and cellular phenotype, which include nuclear factor-κB (NF-κB) and interferon regulatory factor-1 (IRF-1). NF-κB is a ubiquitous transcription factor that can be activated by diverse array of proatherogenic stimuli such as inflammatory cytokines, oxidant stress, and hemodynamic forces (16, 17). Interferon regulatory factor-1 (IRF-1) was isolated by virtue of its affinity to specific DNA sequences in the interferon-β promoter that mediate virus responsiveness. The IRFs are a family of factors that regulate cytokine signaling, cellular growth regulation, hematopoietic development, and pathogen response (18). Our understanding of NF-κB regulation of PDGF ligand expression is limited. KLF5 transactivation of PDGF-A involves cooperative interactions with NF-κB p50 but not Egr-1 (19, 20) in endothelial cells exposed to phorbol ester (21). We showed that...
IRF-1, p65, and HDAC-1 Regulate IL-1β Repression of PDGF-D

NF-κB binds to the PDGF-B promoter in vascular endothelial cells exposed to fluid shear stress and activates the PDGF-B promoter (22, 23). Tumor necrosis factor-α activation of PDGF-B gene expression involves NF-κB (24). It is not known whether NF-κB controls PDGF-C or PDGF-D transcription in any cell type or whether IRF-1 is involved in transcription of any ligand chain. Here, we were surprised to find that IL-1β suppresses PDGF-D promoter activity, mRNA, and protein in SMCs, and this involves transcriptional repression by NF-κB p65 and IRF-1, both of which form a complex in response to the cytokine and interact with the PDGF-D promoter. This study provides the first direct link between NF-κB and the PDGF-D promoter and IRF-1 with any member of the PDGF family.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Primary rat and human aortic SMCs were obtained from Cell Applications (San Diego, CA) and cultured in Waymouth’s media containing 10% fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2/air. Cells were used between passages 3 and 8. Cells were incubated for 24 h in serum-free media to achieve quiescence before being stimulated with indicated concentrations of IL-1β. Human recombinant IL-1β and ondronin were purchased from Calbiochem. Trichostatin was bought from Sigma. Rabbit polyclonal antibodies to NF-κB p65, NF-κB p50, c-Rel, RelB, IRF-1, p65, NF-κB p50, c-Rel, RelB, IRF-1, and HDAC-1, and the ON-TARGETplus nonspecific siRNA and nonspecific siRNA were obtained from Santa Cruz Biotechnology.

RNA Extraction and Reverse Transcription—Total RNA was prepared using TriZOL reagent, and 2 μg was used for synthesis of cDNA in a 25 μl volume in the presence of 0.5 μg of oligo dT primer (Sigma), 1 μM of dNTP mix (Roche), 40 units of RNase inhibitor (Promega), 200 units of SuperScript™ II reverse transcriptase (Invitrogen), and 4 μl of 5× first strand buffer (Invitrogen) in diethyl pyrocarbonate-treated water. Reactions were allowed to proceed at 42 °C for 50 min and at 70 °C for 15 min. 1 μl of cDNA was amplified for PDGF-D fragment in a final volume of 20 μl. The primers for amplifying PDGF-D were: forward, 5’-ACC ACA GTC CAT GCC ATC AC-3’ and reverse, 5’-CTC ACC ACC CTG TTG CTG TA-3’. The amplification conditions for GAPDH were: 94 °C, 1 min; 94 °C, 30 s; 58 °C, 10 s; 72 °C, 1 min for 20 cycles; and 72 °C, 4 min.

Real-time Quantitative PCR—Quantitative PCR was carried out using Rotor-Gene 3000 (Corbett Life Science). The reaction was set in a final volume of 10 μl containing 1 μl of cDNA, 5 μl of 2× SYBR Green Master Mix (Applied Biosystems), 0.2 μl of 10 μM of forward and reverse primer (Sigma) and 3.6 μl of DNase-free water. Primers used were: rat PDGF-D: (forward) 5’-ATC GGG ACA CTT TGG CGA CT-3’, (reverse) 5’-GTG CCT GCC CGA ATG TT-3’; and rat GAPDH: (forward) 5’-ACA AGA TGG TGA AGG TCG GTG-3’, (reverse) 5’-AGA AGG CAG CCC TGG TAA CC-3’. The PCR conditions were: 95 °C for 10 min, followed by 40 cycles at 95 °C for 5 s, 60 °C for 45 s.

Transient Transfection Assays—SMCs in 100-mm petri dishes were transfected with 10 μl of the indicated siRNA at 1 μg of the indicated luciferase reporter construct. 1 μl of pRL-null was added to normalize transfection efficiencies. Transfections were performed using FuGENE6 reagent (Roche). After a 48-h incubation, cells were harvested, and cell lysates were prepared as described (12). The concentration of protein was determined with the BCA protein assay kit (Pierce). 20 μg total cell lysates or 50 μg nuclear extracts were resolved in 12% SDS-polyacrylamide gel and transferred onto Immobilon polyvinylidene difluoride transfer membranes (Millipore). Membranes were blocked for 1 h at 22 °C with 5% skim milk in 0.05% Tween 20 phosphate-buffered saline and then incubated overnight with primary antibody at 4 °C. Primary antibodies were used at a final dilution of 1:300–500. Membranes were washed three times with 0.05% Tween 20 phosphate-buffered saline followed by incubation with swine anti-rabbit or rabbit anti-goat IgG conjugated with horseradish peroxidase (diluted at 1:2000) for 1 h at room temperature. Membranes were then incubated with chemiluminescence (PerkinElmer Life Sciences) and then exposed to film for 1–15 min.

Electrophoretic Mobility Shift Assays (EMSA)—Typically, 15 μg of nuclear extracts from untreated or IL-1β-treated SMCs were incubated with 150,000 cpm of 32P-labeled double-stranded oligonucleotide bearing a NF-κB or IRF-1 binding site in binding buffer (10 mM Tris, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 mg/ml salmon sperm DNA, 1 mg/ml poly(dI-dC)) at 22 °C for 30 min. In supershift experiments,
nuclear extracts were incubated with 2 μl (4 μg) of the rabbit polyclonal antibody in binding buffer for 20 min at room temperature before adding probe and then incubated with probe for another 30 min. The reaction mixture was run on 8% non-denaturing polyacrylamide gels in Tris borate-EDTA (TBE) buffer at 120V for 3 h at room temperature; the gel was vacuum dried and exposed to x-ray film for autoradiography (25, 26).

For EMSA using recombinant human proteins, reactions were conducted in binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.2% Nonidet P-40, 1 mg/ml bovine serum albumin) for 30 min at 22 °C. Oligo sequences were as follows: Oligo D -481/-457, 5′-AAG AAG TGC TGG GAT TCT ACC CTC TC-3′ (putative NF-κB binding site underlined); mOligo D -481/-457, 5′-AGT AGG AGG AGG ATG AAA GGT AAA GCT ACC C-3′ (putative IRF-1 binding site underlined); and mOligo D -847/-823, 5′-AGT TCG CGA AAA GGT AAA GCT ACC C-3′ (mutations italicized). Oligo PD-κB, 5′-CAA CAG GGG AAT TCC CCT CTC CTT-3′ (NF-κB site underlined) (22).

**Immunoprecipitation**—500 μg of whole cell lysates or 200 μg of nuclear extracts from growth quiescent or IL-1β-stimulated SMCs were used in immunoprecipitation analysis. Protein G-Sepharose beads were precleaned with radioimmunoprecipitation assay buffer containing protease inhibitor mixture. Preincubated whole cell lysates were incubated with 5 μg of primary antibody overnight at 4 °C with gentle rotating followed by incubation with 30 μl of bead suspension. Sepharose bound immunoprecipitates were washed, boiled in SDS sample buffer, and separated by electrophoresis on 12% polyacrylamide gels, then transferred to nitrocellulose membranes, which were probed with rabbit polyclonal antibody in binding buffer for 20 min at room temperature (4°C), followed by washing in TBS containing 1% Tween 20 (TBST) and incubation with a mixture of alkaline phosphatase-conjugated secondary antibodies (1:2000 dilution in TBST) for 1 h. After cross-linking with 0.2% glutaraldehyde containing sonicator, the cells were washed twice with phosphate-buffered saline, pH 7.4 before ChIP (ChIP) using the appropriate antibody. PCR was carried out in presence of 1 μM of MgCl2, 0.1 μM of dNTPs, 0.1 μM primers, and 1 unit of Platinum Taq polymerase (Invitrogen) and 2 μl of cDNA. Amplification conditions were as follows: 94 °C, 5 min; 94 °C, 1 min; 55 °C, 45 s; and 72 °C, 45 s for 35 cycles; and 72 °C, 4 min. Primers for PDGF-D -663/-445: forward, 5′-CCC TGA CTC ATG GAC TT-3′; reverse: 5′-GGA GGA AGT GGG AGG TA-3′. Primers for PDGF-D -863/-445: forward, 5′-CCC TGA CTC ATG GAC TT-3′; reverse: 5′-GCC ATG GGG AGG CTT-3′. Primers used for PDGF-D -445: reverse, 5′-TCA CAC TGA TGG GAC CTC AA-3′; reverse, 5′-GGA GGA AGT GGG AGG TA-3′.

**RESULTS**

**Proinflammatory Cytokine IL-1β Reduces Levels of PDGF-D mRNA, Promoter Activity, and Protein Expression**—Because PDGF-D and IL-1β are both generated in response to vascular injury and regulate SMC proliferation (10, 11, 27–30) and because other members of the PDGF family are induced by IL-1β, we began this study with the hypothesis that PDGF-D expression would be controlled by IL-1β. Growth-quiescent rat aortic SMCs were incubated with IL-1β (10 ng/ml) for various times (0–24 h) and PDGF-D mRNA levels were measured by reverse-transcription PCR. Surprisingly, the cytokine caused a progressive decrease in levels of PDGF-D mRNA despite un inhibited loading (Fig. 1A). In contrast, using the same samples, we found that IL-1β stimulates VCAM-1 mRNA expression (Fig. 1A). VCAM-1 is a well recognized IL-1β-dependent gene, positively regulated by IL-1β in an NF-κB-dependent manner in multiple cell types (31, 32). Quantitative real-time PCR and transient transfection analysis with pGL3-basic confirmed IL-1β suppression of PDGF-D mRNA and promoter activity, respectively (Fig. 1B). Further confirmation of these findings was achieved by electrophoretic mobility shift assay (EMSA) using a nuclear extract from growth quiescent or IL-1β-stimulated SMCs, which revealed a decrease in NF-κB binding activity in IL-1β-stimulated SMCs (Fig. 1C). Western blot analysis demonstrated that PDGF-D mRNA levels (50 and 21 kDa) (12) were down-regulated by IL-1β.

**NF-κB promotes SMCs, Interacts with a Novel Element in the PDGF-D Promoter and Inhibits PDGF-D Proinflammatory Cytokine IL-1β**—Because other members of the PDGF family are induced by proinflammatory cytokines and regulate SMC proliferation (10, 11, 27–30) and inhibited PDGF-D mRNA levels (Fig. 2C) did not affect PDGF-D transcription, either alone or in conjunction with NF-κB binding element at -462 (Table 1). EMSA, using a double-stranded oligonucleotide spanning this element (32P-labeled Oligo D -481/-457) in NF-κB binding conditions (32), revealed that IL-1β induced the formation of two distinct nucleoprotein complexes within 1 h, A and B (Fig. 2A, left). Antibody elimination analysis revealed that Complex A contains NF-κB p65 and HDAC-1, whereas Complex B contains p65 but no HDAC-1 (Fig. 2A, right). Western blotting of cytoplasmic and nuclear extracts of untreated and IL-1β-treated cells confirmed the existence of immunoreactive p65, p50, c-Rel, RelB, or IRF-1 (Fig. 2A, left). No supershifts were obtained with antibodies to p50, c-Rel, RelB, or IRF-1 (Fig. 2A, left). Mutation of the GGAT-467 sequence to TTG (p65) disrupted complex formation in EMSA with control or IL-1β-treated nuclear extracts (Fig. 2A, upper right). Moreover, wild-type oligonucleotide bound recombinant p65-p65 homodimers in a dose-dependent manner, whereas the mutant probe failed to interact (Fig. 2A, lower right). Western blotting of cytoplasmic and nuclear extracts of untreated and IL-1β-treated cells confirmed the existence of immunoreactive p65, p50, c-Rel, RelB, or IRF-1 in the extracts (Fig. 2B, left). Of these, levels of p65 and IRF-1 were increased in the nucleus. EMSA performed with 32P-labeled Oligo PD-κB, which bears a high affinity consensus palindromic binding element for NF-κB (22), revealed that the nucleoprotein complex that formed with these IL-1β-treated extracts contained p65 but not p50, c-Rel, or RelB (Fig. 2B, right).

To provide functional evidence for the influence of p65 on PDGF-D promoter activity, we overexpressed p65 in a transient transfection system with the PDGF-D promoter-luciferase construct. p65 suppressed PDGF-D promoter activity (Fig. 2C) and inhibited PDGF-D mRNA levels (Fig. 2C). p50 has no effect on PDGF-D transcription, either alone or in conjunction with...
IRF-1, p65, and HDAC-1 Regulate IL-1β Repression of PDGF-D

A

IL-1beta, 10 ng/ml

No Addition

1 2 4 8 12 24 h

PDGF-D

-900 bp

VCAM-1

-700

GAPDH

-500

B

Fold Change in PDGF-D mRNA

No Addition

IL-1beta, 10 ng/ml

Fold Change in Luciferase Activity

No Addition

IL-1beta

p1168D-luc

C

Fold Change in PDGF-D mRNA

No Addition

0.1

Fold Change in Luciferase Activity

No Addition

p1168D-luc

D

INTERLEUKIN-1β reduces PDGF-D mRNA, promoter activity, and protein expression. A, time course of IL-1β suppression of PDGF-D mRNA expression. SMCs were treated with 10 ng/ml of IL-1β for the times indicated, mRNA was extracted, and semi-quantitative reverse transcription-PCR was performed for PDGF-D and VCAM-1. GAPDH was also amplified as a loading control. B, quantitative real-time PCR (left) and transient transfection analysis (right) confirms IL-1β suppression of PDGF-D mRNA levels and PDGF-D promoter activity, respectively. In the latter, SMCs were transfected with 10 ng/ml of p1168D-luciferase (luc) plasmid and 1 µg of pRL-null and then exposed to IL-1β for 8 h. Firefly luciferase activity was normalized to Renilla. C, dose-response of IL-1β suppression of PDGF-D mRNA levels. SMCs were treated with different amounts of IL-1β for 4 h prior to extraction of total RNA and assessment of PDGF-D mRNA levels by real-time quantitative PCR. D, IL-1β suppression of PDGF-D protein expression. SMCs were treated with IL-1β for various times, and 20 µg total protein was used in Western blot analysis with PDGF-D antibodies. No Addition refers to samples that did not receive IL-1β. Data are representative of at least three experiments. Error bars represent S.E. performed in triplicate. An asterisk denotes p < 0.05.

IRF-1 is induced by IL-1β in SMCs and interacts with a second novel binding element in the PDGF-D promoter—Previous studies in certain other genes, such as VCAM-1 (36), have demonstrated that p65 and IRF-1 cooperatively activate gene expression. To the best of our knowledge, this has not been shown in SMCs nor is there any report of negative cooperation between these factors. We hypothesize that these factors bind not only one another but also the PDGF-D promoter in response to SMC exposure to IL-1β. Immunoprecipitation analysis and Western blotting was performed with lysates of cells that had been exposed to IL-1β for various times (0–2 h). We pulled down with IRF-1 antibodies and then immunoblotted for p65. Alternatively, we immunoprecipitated with p65 antibodies and then probed for IRF-1. p65 and IRF-1 formed a complex within 1 h of IL-1β exposure using either of these conditions, and complex formation was transient (Fig. 4A, left). ChIP analysis was performed to demonstrate the inducible interaction of endogenous proteins with the 840 GAAAAGTGAAA −830 sequence to −840 GACAAAGTGAAC −830 in 32P-labeled oligo D −847/−823 abrogated an IL-1β-inducible complex formation (Fig. 3A). Antibody elimination experiments demonstrated the presence of IRF-1 in this IL-1β-inducible complex (Fig. 3B). This is the first association of IRF-1 with any PDGF promoter. The EMSA showed that this complex, with 32P-labeled oligo D −823 contains HDAC-1 (Fig. 3C). SMCs play a central role in the epigenetic regulation of gene expression. HDAC-1 is a predominant nuclear 482-amino acid isoform (34). Germline deletion of HDAC-1 results in embryonic lethality, which cannot be sufficiently compensated by HDAC2 (35), and like several other HDACs, is a drug target in cancer (34). HDAC-1 is also a component of the p65/32P-labeled oligo D −481/−457 complex in Fig. 2A (left).

IL-1β Induces Interaction of p65 and IRF-1 Together and with the PDGF-D Promoter—Previous studies have shown that p65 and IRF-1 cooperatively activate gene expression. To the best of our knowledge, this has not been shown in SMCs nor is there any report of negative cooperation between these factors. We hypothesize that these factors bind not only one another but also the PDGF-D promoter in response to SMC exposure to IL-1β. Immunoprecipitation analysis and Western blotting was performed with lysates of cells that had been exposed to IL-1β for various times (0–2 h). We pulled down with IRF-1 antibodies and then immunoblotted for p65. Alternatively, we immunoprecipitated with p65 antibodies and then probed for IRF-1. p65 and IRF-1 formed a complex within 1 h of IL-1β exposure using either of these conditions, and complex formation was transient (Fig. 4A, left). ChIP analysis was performed to demonstrate the inducible interaction of endogenous proteins with
IRF-1, p65, and HDAC-1 Regulate IL-1β Repression of PDGF-D

TABLE 1
Proximal region of the human PDGF-D promoter
The NF-κB and IRF-1 binding sites are indicated in blue. ChIP amplicon (−863 to −445) is indicated in red. Four putative Ets sites described previously (12) (Ets-D1, -D2, -D3, and -D4) are in bold and underlined. The transcriptional start site is italicized and bolded (ccAGGCAG). The primers originally used for primer extension analysis (12) are bolded/underlined and labeled as primer A and B. Capital letters represent 5′-untranslated region (UTR). *atg* denotes the predicted translational start site. Putative binding sites for other factors are indicated.

| −1168  | gagaatcccaaaaagctctcaacggaccatcgctttctctattggaactgtgaaggcaagtgaagcagcttctgc | | | |
| −1093  | cttctctcccttctcggctgctcctgctcaacccacctctctctctggccctttctctgtggaaggggctgga | Ets-D1 |
| −1018  | ctatgtgcataaaacgctctgaagactctcttggctattgtgatacagcctgcacattgccctacgtctca | |
| −943   | agcctcgttgatagctggttcctcatctgctcaacccttgaagaaggcatctttggtgttcacatactcctggttacca | p53 |
| −868   | ggtgaagcgctgactacactctggagttgctggaagaagttaagctacggccatcctctcactctgactctgc | IRF-1 |
| −793   | ggccattttttgatagctgaggacctcaagagctgatttctcttggtgtatatactttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt...
IRF-1, p65, and HDAC-1 Regulate IL-1β Repression of PDGF-D

A

32P-Oligo D-481-457

|          | No Addition | IL-1beta, 10ng/ml, 1h |
|----------|-------------|----------------------|
| No Ab    |             |                      |
| cap65    |             |                      |
| cap50    |             |                      |
| oc-Rel   |             |                      |
| ocRelB   |             |                      |
| nIRF-1   |             |                      |
| nHDAC-1  |             |                      |

B

Cytosolic Nuclear

- p65
- p50
- c-Rel
- RelB
- IRF-1
- HDAC-1
- Coomassie

C

Fold Change in Luciferase Activity

Fold Change in Luciferase Activity

D

Fold Change in PDGF-D mRNA (32P-Oligo)

E

Fold Change in PDGF-D mRNA (32P-Oligo)

F

Fold Change in Luciferase Activity

IL-1beta, 10ng/ml

- p65
- beta-actin

WITHDRAWN

June 1, 2010
Repression of PDGF-D Transcription by IL-1β Involves Histone Deacetylation and Interaction of HDAC-1 with IRF-1 and p65—The role of the chromatin environment in regulation of IL-1β PDGF-D expression was next investigated. Rat aortic SMCs were pretreated for 18 h with trichostatin A, a HDAC inhibitor, and then treated with IL-1β for another 4 h. Repression of PDGF-D mRNA expression by IL-1β was completely blocked by trichostatin A (Fig. 6A, left). In contrast, trichostatin A had no effect on PDGF-D levels in the absence of IL-1β (Fig. 6A, left). This data implicates HDACs in the negative control of PDGF-D in SMCs exposed to the cytokine. Co-immunoprecipitation experiments revealed that IL-1β indeed triggers the inducible interaction between IRF-1, p65, and HDAC-1 (Fig. 6A, right). Co-immunoprecipitation experiments revealed that IL-1β clearly an anti-inflammatory phenomenon for this cytokine, which is well known for its inhibitory roles. The present study demonstrates the requirement of HDAC-1 in this process. IL-1β induces HDAC-1 complex formation with IRF-1 and HDAC-p65 (Fig. 6A, right) and the enrichment of HDAC-1, p65, and IRF-1 at the PDGF-D promoter (Fig. 4B).

To provide further insight on the role of HDAC-1 in IL-1β-dependent transcriptional repression, we performed additional ChIP experiments, this time using two separate PDGF-D promoter amplicons. One amplicon (−689PDGF-D−445) spans the NF-κB recognition motif...
IRF-1, p65, and HDAC-1 Regulate IL-1β Repression of PDGF-D

**Figure 4.** IL-1β induces physical interaction of p65 and IRF-1 with each other and with the PDGF-D promoter. A, left, immunoprecipitation (IP) and immunoblotting (IB) or immunoblotting alone were performed with total cell lysates or nuclear extracts of SMCs treated with IL-1β for various times. Right, HDAC-1 siRNA was used at 100 nM. SMCs were treated with 100 nM HDAC-1 siRNA for 24 h then incubated with 10 ng/ml IL-1β for 1 h. No Addition refers to samples that did not receive IL-1β. B, ChIP analysis was performed using primers amplifying the −863/-445 region of the human PDGF-D promoter containing NF-κB and IRF-1 elements, with antibodies against p65, IRF-1, and HDAC-1. Total input (TI) and IgG serve as positive and negative controls. C, EMSA with 32P-labeled Oligo D−481/-457 and 32P-labeled mOligo D−847/-823 with recombinant human (rh) p65, HDAC-1, and IRF-1. Data are representative of at least two independent experiments. siRNAs, nonspecific siRNA. Arrow denotes nucleoprotein complex.

(−471GGGATTCTAC−662), and the other amplicon (−863PDGF-D−674) spans the IRF-1 binding site further upstream (−840GAAAGAGTGAAA−830) (Table 1). Our findings demonstrate that IL-1β stimulates the accumulation of p65, IRF-1, and HDAC-1 to both of these regions of the PDGF-D promoter (Fig. 6B). HDAC-1 associates with the promoter within 15 min (Fig. 6C). HDAC-1 levels are unchanged in cells exposed to IL-1β for 1 h (Fig. 6D, lower). Yet, HDAC-1 siRNA, which reduces basal HDAC-1 expression (Fig. 6D, lower), abrogates cytokine-inducible occupancy of HDAC-1, and that of
IRF-1, p65, and HDAC-1 Regulate IL-1β Repression of PDGF-D

p65 and IRF-1, at both regions of the promoter (Fig. 6B). HDAC-1 siRNA rescues the PDGF-D promoter from IL-1β repression (Fig. 6D, upper). Importantly, HDAC-1 siRNA also blocks the IL-1β-inducible interaction of IRF-1 with p65, as demonstrated by either pulling down with IRF-1 antibodies and immunoblotting with p65 antibodies or pulling down with p65 antibodies and immunoblotting for IRF-1 (Fig. 4A, right). These results indicate that HDAC-1 is enriched at the PDGF-D promoter in cells exposed to IL-1β and forms a cytokine-inducible gene silencing complex with p65 and IRF-1 (Fig. 6E).

DISCUSSION

In this study, we have shown that proinflammatory cytokine IL-1β down-regulates PDGF-D expression in a time- and dose-dependent manner. This contrasts with levels of VCAM-1, which, as expected, are induced by the cytokine. p65, induced by IL-1β, interacts with a novel element in the PDGF-D promoter and inhibits PDGF-D transcription. IRF-1 is also induced by IL-1β and binds to a second novel element in the PDGF-D promoter. ChIP experiments with two separate sets of primers, one amplicon (PDGF-D [471GGGATTCTAC]) spanning the NF-κB recognition motif (PDGF-D [462]), with the other (PDGF-D [840GAAAAGTGAAA]) spanning the IRF-1 binding site further upstream (PDGF-D [830]). p65, IRF-1, and HDAC-1 are enriched at each region of the PDGF-D promoter in cells exposed to IL-1β (Fig. 6E). Co-immunoprecipitation studies demonstrate that IRF-1 and p65 form a complex containing HDAC-1 within 1 h of exposure to IL-1β (Figs. 4A, left and 6A, right). Moreover, HDAC-1 siRNA ablates complex formation with IRF-1 and p65 (Fig. 4A, right) and in ChIP analysis, abrogates IRF-1 and p65 occupancy of the PDGF-D promoter (Fig. 6B). NF-κB and IRF-1 are transcription factors activated by a variety of proinflammatory...
cytokines including IL-1β and play a positive regulatory role in many genes implicated in the pathogenesis of vascular disease. These include VEGF (37), VCAM-1 (36), and iNOS (38) in SMCs. Cooperativity between NF-κB and IRF-1 has been reported previously. For example, recent studies demonstrate that IRF-1 and NF-κB form a functional complex at the LTR-B sites in the human immunodeficiency virus type 1 enhancer, which is abrogated by specific mutations in NF-κB sites in the enhancer region (39). Similarly, cooperation between IRF-1 and NF-κB p65 positively regulates IL-6 (40) and iNOS (41) transcription. In the present study, IL-1β simulates p65 IRF-1 complex formation, but rather than activating PDGF-D transcription, p65 and IRF-1 bind to and repress the PDGF-D promoter. HDAC-1 is enriched at the PDGF-D promoter in cells exposed...
to IL-1β and forms a cytokine-inducible gene silencing complex with p65 and IRF-1 (Fig. 6E). We used IRF-1 antibodies in the EMSA shown in Fig. 2A (left) (with 32P-labeled Oligo D381–457, containing the p65 binding site); however, this did not perturb formation of either IL-1β-inducible Complex A or B. Conversely, p65 antibodies used in the EMSA (with 32P-labeled Oligo D843–827, containing the IRF-1 binding site) shown in Fig. 3B did not affect formation of the IL-1β-inducible complex. Although p65 and IRF-1 each bound their respective sites (Figs. 2A, left, and 3B), HDAC-1 was the only nuclear protein bound by either factor (Figs. 2A, left, and 3B). However, it is unreasonable to expect that more than two proteins could be successfully supershifted/eliminated when short oligonucleotides such as those employed in this study are used. The split ChiP nonetheless revealed that p65, IRF-1, and HDAC-1 were inductively enriched at the PDGF-D promoter (Fig. 6B). p65 and IRF-1 form a complex containing HDAC-1 within 1 h of exposure to IL-1β (Figs. 4A, left, and 6A, right). Moreover, HDAC-1 siRNA ablates complex formation with IRF-1 and p65 (Fig. 4A, right) and abrogates IRF-1 and p65 occupancy of the PDGF-D promoter (Fig. 6B). These data, in combination, indicate that HDAC-1 is enriched at the PDGF-D promoter in cells exposed to IL-1β and forms a cytokine-inducible gene silencing complex with p65 and IRF-1 (Fig. 6E). This is consistent with the emerging role of HDAC-1 as a "transcriptional switch," shifting from active gene expression to repression as a consequence of its interactions with other proteins. For example, HDAC1, which can either activate or repress gene expression, is a repressor of vimentin expression by binding to a vimentin promoter (42). HDAC1-mediated repression of an IL-1-inducible gene silencing transcription factors before PDGF-D expression.

The literature points toward PDGF-D and IL-1β suppressing a regulatory role in growth factor gene expression upon vascular injury and stabilization (10, 11). Balloon injury of porcine coronary arteries induces IL-1β production in the vessel wall (27). Administration of IL-1β to porcine coronary arteries causes neointima formation (28). The absence of IL-1 receptor antagonist in mice results in a 250% increase in neointima formation after injury (29), while IL-1 receptor antagonist infusion after balloon injury reduces intimal thickening (30). The irony here is our present demonstration of IL-1β repression PDGF-D expression.

Cytokine or growth factor suppression of growth factor expression is an interesting but not well understood phenomenon. TNF-α represses insulin-like growth factor-1 expression (43). TNF-α and IL-1β down-regulate the dipeptidyl carboxypeptidase angiotensin-converting enzyme, generates angiotensin II from AT1 (44). Angiotensin II and transforming growth factor-β (TGF-β) negatively regulate hepatocyte growth factor (45). IL-1β inhibits VEGF-D expression (46). We showed that FGF-2 inhibits expression of PDGF receptor α transduction (47). Antagonism between PDGF and IL-1β has been observed. Li and colleagues (48) have demonstrated that migration and proliferation of retinal pigment epithelial cells induced by PDGF-CC and PDGF-DD are abolished by the presence of IL-1β, TNF-α, and interferon-γ. Moreover, IL-1β can block baboon aortic SMC migration and proliferation induced by PDGF (49, 50). These reports, combined with the present findings, suggest that IL-1β suppression of PDGF-D expression through IRF-1/p65/HDAC-1 may represent a negative regulatory mechanism in the vessel wall. Our data provides new insights into the mechanisms governing cytokine transcriptional control of growth factor gene expression. In the biological context, PDGF-D by IL-1β, or other factors, proinflammatory cytokine may help constrain the extent of intimal hyperplasia. This need not be confined to SMCs. For example, PDGF-D regulates numerous cellular processes, including proliferation, transformation, invasion, and angiogenesis. PDGF-D deregulation has been implicated in tumorigenesis and progression to metastatic disease (51). Down-regulation of PDGF-D results in inhibition of cell growth and angiogenesis through inactivation of Notch-1 and NF-κB signaling (8). Thus, IL-1β control of PDGF-D expression may represent an autoregulatory mechanism whereby PDGF-D-dependent biological processes are restrained by the cytokine.

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Mary Y. Liu and Levon M. Khachigian

PAGE 35108:

The published version of the left panel of Fig. 4C inadvertently duplicated the first five lanes from the right panel of Fig. 2A. The correct version of Fig. 4C is shown below.

PAGE 35110:

In the published version of Fig. 6B, the fourth panel was inadvertently duplicated as the sixth panel. The correct version of Fig. 6B is shown below.

These additions and corrections do not result in any change in the conclusions of the article.