Transcriptional Regulation of the Platelet-derived Growth Factor α Receptor Gene via CCAAT/Enhancer-binding Protein-δ in Vascular Smooth Muscle Cells*

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Inflammatory cytokines stimulate the proliferation of vascular smooth muscle cells (VSMC) and play a pivotal role in the pathogenesis of vascular diseases including atherosclerosis and restenosis. Mitogenic response of interleukin-1β (IL-1β) on VSMC is thought to be mediated by induction of endogenous platelet-derived growth factor (PDGF), especially PDGF-AA. Although the action of PDGF-AA is mediated by its specific receptor, PDGFα-receptor (PDGFαR), very little is known about the regulatory mechanism of PDGFαR gene expression in VSMC. To understand the mechanism, we studied the transcriptional control of the PDGFαR gene in VSMC after treatment with IL-1β. IL-1β (10 ng/ml) drastically increased both PDGFαR and CCAAT/enhancer-binding protein δ (C/EBPδ) mRNA levels in a time dependent manner. A rapid induction of C/EBPδ mRNA within 30 min was followed by slower emergence of PDGFαR mRNA, which reached the maximum level in 12 h, whereas C/EBPδ mRNA was detectable at 30 min and reached the maximum level at 3 h. Electromobility shift and supershift assays revealed that IL-1β markedly increased DNA-protein complex, which was mainly composed of C/EBPβ and/or -δ. Both Western blotting and immunohistochemistry demonstrated that either C/EBPβ or -δ expression was induced by IL-1β exclusively in nuclei of VSMC. On the other hand, overexpression of C/EBPδ specifically transactivated the promoter activity of the PDGFαR gene and significantly enhanced VSMC proliferation in PDGF-treated cells. We conclude that induction of PDGFαR expression is mainly mediated by C/EBPδ expression in VSMC, and a high level of C/EBPδ expression may be involved in the pathogenesis of atherosclerosis and restenosis.

Excessive or uncontrolled replication and migration of vascular smooth muscle cells (VSMC) are critical events involved in a number of vascular diseases including atherosclerosis, hypertension, and restenosis that often occurs after balloon angioplasty (1–3). Morphologic studies of the sequenc- ing events in the arterial wall of animals with artificially induced hypercholesterolemia showed that macrophages are present in all processes of the formation of atherosclerotic lesions (4–7). The normal function of the macrophage is to act not only as an antigen-presenting cell to T lymphocytes but also as a source of several growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor, tumor necrosis factor α, and transforming growth factor β1, which are generally not expressed in the normal artery, whereas they are up-regulated in the lesions of atherosclerosis (3). Thus, the macrophage is thought to be a principal inflammatory mediator of cells in the atheromatous plaque microenvironment.

Interleukin (IL)-1β is one of the major secretory products of activated macrophage and can induce proliferation of cultured fibroblasts and VSMC (8–11). Previous studies (12–14) have demonstrated that mitogenic activity of IL-1β for fibroblasts and VSMC is mediated indirectly via an autocrine loop by causing the release of PDGF-AA, which then specifically binds to the PDGF α-receptor (PDGFαR) subtype on cell surface. Furthermore, recent studies (15, 16) have also demonstrated that IL-1β can up-regulate PDGFαR expression in rat lung fibroblasts, thereby enhancing PDGF-mediated mitogenesis and chemotaxis of lung fibroblasts. Although the pathophysiological implications of IL-1β-induced PDGFαR expression are beginning to be recognized, little is known about the molecular mechanism involved. Therefore, we have investigated the molecular mechanism of PDGFαR gene transcription in VSMC and obtained results indicating that IL-1β induces PDGFαR gene expression via a trans-acting nuclear factor, CCAAT/enhancer-binding protein δ (C/EBPδ).

EXPERIMENTAL PROCEDURES

Materials—Actinomycin D and cycloheximide (CHX) were purchased from Sigma, and recombinant mouse IL-1β was from Roche Molecular Biochemicals (Tokyo, Japan). [α-32P]dCTP (110 TBq/mmol) and [γ-32P]ATP (220 TBq/mmol) were obtained from Amersham Pharmacia Biotech (Tokyo, Japan). Affinity-purified rabbit polyclonal antibodies for PDGFαR and C/EBPα, -β, and -δ raised against peptidic epitopes corresponding to amino acid residues of human PDGFαR (residues 951–1,089), rat C/EBPs (residues 253–265), rat C/EBPβ (residues 258–276), and rat C/EBPδ (residues 247–268) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Expression vectors of C/EBPs, -β, and -δ (designated EBPα, -β, and -δ, respectively) were generous gifts of Dr. Steven L. McKnight.

Cell Culture—VSMC were isolated from the thoracic aorta of male Harlan Sprague-Dawley rats (Charles River Japan Inc., Kanagawa, Japan), weighing 280–320 g, by the method described previously (17). Cells were seeded onto 100-mm dishes at a density of 1 × 105 per dish and maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere of 95% air, 5% CO2. VSMC were passaged every 4–7 days, and experiments

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The abbreviations used are: VSMC, vascular smooth muscle cell(s); PDGF, platelet-derived growth factor; IL, interleukin; PDGFαR, platelet-derived growth factor α-receptor; PDGFβR, platelet-derived growth factor β-receptor; C/EBP, CCAAT/enhancer-binding protein; CHX, cycloheximide.
were performed on cells at 3–10 passages from primary culture. In preparation for experiments, confluent cells, which exhibited a typical hill and valley pattern of smooth muscle cells in culture, were made quiescent by placing them in a defined serum-free medium containing insulin (10 μg/ml), transferrin (10 μg/ml), and sodium selenite (10 ng/ml each). Each cell culture medium has been optimized to maintain VSMC in a quiescent and noncatabolic state for an extended period of time (18).

Preparation of cDNA Probes and Northern Blotting—A 0.6-kilobase (kb) pair fragment of rat PDGFαR cDNA (19), 1.1-kilobase (kb) pair Neol fragment of βEβPα or β1.0-kilobase pair EcoRI–BamHI fragment of βEβPα was used as a probe for Northern blotting. Each fragment was labeled with [α-32P]dCTP using the random primer method. Total cellular RNA extraction from VSMC and Northern blot analysis were carried out by the methods described previously (19, 20).

Electromobility Shift and Supershift Assays—Nuclear extracts were prepared from VSMC according to the method described by Dignam et al. (21). After protein concentrations were determined using Bio-Rad Protein Assay Reagent, nuclear extracts were divided into small aliquots, quickly frozen in liquid nitrogen, and stored at −80 °C. For electrophoretic mobility shift assay and supershift assay, a double-stranded oligodeoxynucleotide probe for the consensus sequence of C/EBP was generated by annealing two complementary oligodeoxynucleotides corresponding to the nucleotide sequence spanning −165 to −32P]dCTP using T4-polynucleotide kinase. Nuclear extracts (2 μg) were incubated with 2.0 × 10^6 cpm of the labeled C/EBP probe for 30 min at room temperature in a 10-μl binding buffer containing 12 mM Hepes-KOH, pH 7.9, 60 mM KCl, 4 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 50 μg/ml of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech). For competition experiments or supershift assay, a 100-fold molar excess of unlabeled probe or 1–2 μl of antibodies against each subtype of the C/EBP family was added to nuclear extracts, respectively, and was incubated for 30 min at room temperature before addition of the labeled C/EBP probe. Then all reaction mixtures were analyzed by 5% polyacrylamide gel electrophoresis under non-denaturing conditions, and the gel was dried and processed as described previously (19).

Plasmid Construction and DNA Transfection—PDGFαR promoter/firefly luciferase fusion vector, which was designated as −1,381/+68 WT, was prepared by insertion of the basal promoter region (−1,381 to +68) of the rat PDGFαR gene (19) onto pGL2-Basic vector (Promega, Madison, WI). Mock vector, which was designated as MSV, was prepared by deletion of the coding region of C/EBPα Basic vector (Promega, Madison, WI). EBPα, −8 and −1.2-kilobase pair Neol fragment of βEβPα or MSV (0.2 kb) were used as an unrelated competitor. The C/EBP probe was end-labeled with [γ-32P]ATP using T4-polynucleotide kinase. Nuclear extracts (2 μg) were incubated with 2.0 × 10^6 cpm of the labeled C/EBP probe for 30 min at room temperature in a 10-μl binding buffer containing 12 mM Hepes-KOH, pH 7.9, 60 mM KCl, 4 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 50 μg/ml of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech). For competition experiments or supershift assay, a 100-fold molar excess of unlabeled probe or 1–2 μl of antibodies against each subtype of the C/EBP family was added to nuclear extracts, respectively, and was incubated for 30 min at room temperature before addition of the labeled C/EBP probe. Then all reaction mixtures were analyzed by 5% polyacrylamide gel electrophoresis under non-denaturing conditions, and the gel was dried and processed as described previously (19).

Luciferase Construction and Cell Proliferation Assays—Promoter activity was determined by the Dual-Luciferase Reporter Assay System (Promega) as described previously (22, 23). After normalization for transfection efficiency in reference to sequentially determined Renilla luciferase activity, each promoter activity was presented as a relative luciferase activity in reference to the activity of −1,381/+68 WT cotransfected with MSV that was set to unity. Cell proliferation reagent WST-1 (Roche Molecular Biochemicals) was used for VSMC proliferation assay according to the manufacturer’s specifications. One day after transfection, PDGF-AA or -BB (50 ng/ml each) was directly added to the culture medium, and cells were incubated for an additional 24-h. The culture medium was changed with fresh medium twice a day, and cells were incubated for 30 min at 37 °C. Finally, the absorbance at 490 nm of each well was measured by an enzyme-linked immunosorbent assay reader, and cell proliferation activity was presented as a relative activity in reference to the activity of MSV-transfected VSMC after treatment with PDGF-AA that was set to unity.

Western Blotting—Western blotting was performed by the method described previously (17, 19). Briefly, nuclear extracts (2.5 μg) prepared from VSMC were directly subjected to Western blotting for C/EBPα, −β, and −δ. After boiling with sample buffer, SDS-polyacrylamide gel electrophoresis was performed using a 12.5% gel according to Laemmli (24), and proteins in the gel were transferred to a polyvinylidene difluoride membrane. The membrane was stained with Coomassie Brilliant Blue for 1 h at 100 V. The membrane was treated with diluted primary antibodies against each C/EBP member, and immunoreactive proteins were detected by autoradiography using a chemiluminescence detection system (the ECL Western blotting analysis system; Amersham Pharmacia Biotech).

Immunohistochemistry—VSMC were cultured for 24 h in wells of chamber slides (Lab-Tek II Chamber Slide; Nalege Nunc International, Naperville, IL) at a density of 1 × 10^4 cells/well and then fixed with 100% cold acetone for 30 min. All slides were treated with PBS containing 0.3% H2O2 for 10 min at 37 °C, and the following steps were performed according to the manufacturer’s specifications for the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA). After blocking for 30 min at 37 °C with diluted normal goat serum, slides were covered with diluted primary antibodies for 1 h at room temperature. After exposure to a solution containing diluted biotinylated secondary antibodies, the slides were treated with a Vectastain Elite ABC Reagent. Positive staining cells were visualized with a solution of 3,3'-diaminobenzidine tetrahydrochloride substrate kit (Vector Laboratories, Inc.). The slides were counterstained with hematoxylin, dehydrated with ethanol gradient and with 100% xylene, and then mounted in mounting medium (Mount-Quick; Daido Sangyo Co., Ltd., Tokyo, Japan).

Statistical Analysis—Analysis of variance with Bonferroni-Dunn post hoc analysis was used to analyze differences between two experimental groups. All data are expressed as mean ± S.E., and statistical significance is defined as p < 0.05.

RESULTS

Messenger RNA Induction of PDGFαR and C/EBPβ by IL-1β—VSMC derived from Harlan Sprague-Dawley rats were incubated for 6 h in the presence of IL-1β at various concentrations (0–40 ng/ml), and mRNA levels of both PDGFαR and C/EBPβ were determined by Northern blotting. Although baseline levels of both mRNA expression were very low or almost negligible in quiescent VSMC, they were markedly increased by the treatment with IL-1β at doses up to 10 ng/ml in a dose-dependent manner (data not shown). Therefore, we used the dose of IL-1β at 10 ng/ml hereafter. To identify the kinetic relationship between C/EBP and PDGFαR expression during treatment with IL-1β, mRNA levels of C/EBP members and PDGFαR were monitored for 48 h. As shown in Fig. 1, A and B, a high level of PDGFαR mRNA expression was accompanied by a similarly marked induction of C/EBPα mRNA in VSMC following the addition of IL-1β. A rapid induction of C/EBPα mRNA within 30 min was followed by slower emergence of PDGFαR mRNA, which reached the maximum level (12.2-fold higher than the zero time level) in 12 h, whereas C/EBPα mRNA reached the maximum level (10.5-fold higher than the zero time level) at 3 h, continued at least for 12 h, and then decreased gradually to a basal level within 48 h. A significant induction of PDGFαR mRNA expression began to increase at 3 h and continued at least for 24 h. This time course indicates a causal relationship in which C/EBPα induces PDGFαR gene expression. In contrast, in high level of IL-1β, C/EBPβ mRNA expression was observed even in a quiescent state, and IL-1β did not significantly alter it. Although the induction of C/EBPβ mRNA expression was also detectable at 30 min, it continued for an extended period up to 48 h.

Effect of IL-1β on a Half-life Time of PDGFαR mRNA—To determine whether the induction of PDGFαR mRNA expression after treatment with IL-1β is due to the effect of IL-1β on the mRNA stability, the half-life time of PDGFαR mRNA was determined in the presence of actinomycin D (Fig. 2). VSMC were pretreated with IL-1β for 12 h, washed with PBS, and then exposed to a freshly prepared medium with or without
IL-1β in the presence of 5 mg/ml actinomycin D. A half-life time of the PDGFαR mRNA seen in cells incubated in the medium with IL-1β was 8.6 h, and that without IL-1β was 10.0 h, indicating that IL-1β does not significantly affect the PDGFαR mRNA stability.

Characterization of C/EBP Members That Interact with PDGFαR Gene Promoter—Electrophoretic mobility shift assay was performed by using a labeled C/EBP probe containing the consensus sequence of C/EBP recognition site of rat PDGFαR promoter region (Fig. 3A). Nuclear extracts were prepared from either quiescent or IL-1β-treated VSMC. Although the C/EBP probe was not shifted by nuclear extracts from quiescent VSMC, it was clearly shifted by nuclear extracts from VSMC treated with IL-1β for 12 h, generating a band of the DNA-protein complex. The DNA-protein complex was markedly competed out by a 100-fold molar excess of unlabeled C/EBP probe but not by a 100-fold molar excess of unlabeled nuclear factor-1 probe. To determine the specific subtype of C/EBP that is bound by the probe and actually involved in the transcriptional activation of the PDGFαR gene, supershift assay was performed using antibodies against three major members of C/EBP family, C/EBPα, -β, and -δ (Fig. 3B). In IL-1β-treated VSMC, the band was clearly supershifted by antibodies against either C/EBPβ or -δ but not C/EBPα.

Effects of C/EBP Overexpression on PDGFαR Gene Promoter and Cell Proliferation Activities—To further clarify the direct evidence for C/EBP family in regulating the transcription of the rat PDGFαR gene, we evaluated the ability of the C/EBP family
tein levels of PDGF were very low or almost negligible in quiescent VSMC, IL-1β drastically induced immunoreactive PDGFαR expression exclusively in the cytoplasm of cells. A high level of C/EBPα protein expression was observed in both quiescent and IL-1β-treated VSMC. Although protein levels of PDGFαR were very low or almost negligible in quiescent VSMC, IL-1β drastically induced immunoreactive C/EBPα protein was not detected in quiescent VSMC, whereas that of immunoreactive C/EBPβ protein was identified specifically in the peripheral portion of the nuclei.

Effect of CHX against C/EBPβ Induction by IL-1β—To see if C/EBPβ gene expression is induced by IL-1β without any other de novo protein synthesis, the ability of IL-1β to induce C/EBPβ gene expression was determined in the presence of CHX (10 µg/ml) (Fig. 5). Although induction of PDGFαR mRNA expression by IL-1β was markedly reduced in the presence of CHX, that of C/EBPα mRNA expression was even greater than in the absence of CHX. On the other hand, CHX alone did not cause the superinduction of C/EBPα mRNA expression.

Immunohistochemistry of Cultured VSMC—In Fig. 6, protein levels of PDGFαR and C/EBPα, -β, and -δ expression were evaluated by immunohistochemistry in quiescent or IL-1β-treated VSMC. Although protein levels of PDGFαR were very low or almost negligible in quiescent VSMC, IL-1β-treated VSMC was localized mainly in the cytoplasm. Positive staining of immunoreactive C/EBPβ protein was not detected in quiescent VSMC, whereas that of immunoreactive C/EBPβ protein was identified specifically in the peripheral portion of the nuclei.
or IL-1β-treated VSMC were directly subjected to Western blotting using specific antibodies against C/EBPα, -β, and -δ (Fig. 7). Although nuclear extracts from quiescent VSMC contained only a recognizable level of C/EBPβ protein but not C/EBPα or -δ protein, either C/EBPβ (36 kDa) or C/EBPδ (33 kDa) protein was markedly induced in the nuclear extracts from IL-1β-treated VSMC for 12 h. The expression level of C/EBPα (42 kDa) protein was almost negligible in the nuclear extracts from either quiescent or IL-1β-treated VSMC.

**DISCUSSION**

C/EBPδ has been originally identified in the liver as one of the closely related members of C/EBP family that belongs to the basic leucine zipper transcriptional factors (25–27). Previous studies revealed that C/EBPδ expression was usually at an undetectable or minor level in normal cells or tissues and was rapidly induced by lipopolysaccharide and inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor α (28–30). Therefore, C/EBPδ is thought to be an important factor to regulate the gene transcription of acute phase reactive proteins (28, 31). Both PDGF-A and its specific receptor, PDGFαR, are also known to be up-regulated by the treatment with IL-1β in several cells including VSMC and pulmonary fibroblasts, causing the cell migration and/or proliferation in the pathologic conditions (12, 15, 16). However, little has been known about detailed molecular mechanisms of its gene up-regulation. Recently, Khachigian et al. (32) have demonstrated that major vascular growth-related genes such as PDGF-A chain, PDGF-B chain, transforming growth factor β1, and tissue factor are transactivated by the interaction of two specific regulatory nuclear factors, Sp-1 and Egr-1, suggesting that a common mechanism may exist in the transcriptional regulation of these genes. Interestingly, we have recently demonstrated that the PDGF β-receptor (PDGFβR) gene is mainly regulated by the CCAAT box located at position −67 of its promoter region in
of the Na+/H+ exchanger gene in hepatocytes. Hohaus et al. (35) have reported that the c-fms gene, which belongs to the class III receptor tyrosine kinase family together with PDGFαR and PDGFβR, also has a C/EBP binding site in the promoter region, and either PU.1 (Spi-1) or C/EBPα mainly regulates the cell type-specific gene expression in hematopoietic cells. Taken together, these data strongly support the hypothesis that the C/EBP family, especially C/EBPβ, is a major determinant of PDGFαR gene transcription in VSMC. Supershift assay and Western blotting indicated that IL-1β markedly induced specific DNA-binding proteins, which are identified as C/EBP family, and inducible C/EBP isoforms, interacting with the C/EBP binding site of PDGFαR promoter region, are C/EBPβ and -δ (Figs. 3B and 7). Although C/EBPδ was induced by IL-1β, it was also detected even in quiescent VSMC (Figs. 6 and 7). On the other hand, C/EBPβ was identified exclusively in the nuclei after treatment with IL-1β and was actually capable of interacting with the C/EBP binding site of the PDGFαR gene. Furthermore, overexpression studies have demonstrated that C/EBPδ but not C/EBPα or -β specifically transactivated PDGFαR promoter activity in VSMC (Fig. 4A), and that cell proliferation activity following treatment with PDGF-AA or -BB was significantly enhanced in the transfected VSMC with C/EBPδ compared with those with MSV (Fig. 4B). Since PDGF-BB can bind to not only PDGFβR but also PDGFαR, enhanced effect on the cell proliferation following treatment with PDGF-BB is mediated by the action through the PDGFαR (but not PDGFβR) up-regulated by C/EBPδ overexpression. Moreover, -fold enhancement of cell proliferation was significantly higher in the VSMC after treatment with PDGF-BB compared with those with PDGF-AA. This result was in agreement with our previous study (17) in which we evaluated the mitogenic activity after treatment with PDGF-AA or -BB by measuring radioactive incorporation of [methyl-3H]thymidine and found that it was significantly higher in the cells after treatment with PDGF-BB compared with those with PDGF-AA.

Previously, we have isolated and characterized the promoter region of the rat C/EBPδ gene to understand the regulatory mechanism of C/EBPδ gene transcription by IL-1β in VSMC (36). A similar study with respect to the molecular mechanism of rat C/EBPδ gene transcription in human hepatoma cell lines, HepG2, has demonstrated that the C/EBPδ gene is activated by IL-6 through the regulatory domain, which is recognized by acute phase response factor/signal transducers and activators of transcription 3 (37). Especially, phosphorylation of acute phase response factor/signal transducers and activators of transcription 3 by IL-6 increased its DNA binding activity and caused an induction of C/EBPδ gene transcription, suggesting that a similar mechanism may exist on the transactivation of the C/EBPδ gene by IL-1β and giving support to the hypothesis that de novo synthesis of other proteins is not necessary for its action.

In conclusion, the present study is aimed at delineating the molecular mechanism in the tissue-specific gene expression of PDGFαR in VSMC. The results obtained herein show a direct evidence for new significant roles of the C/EBP family, especially C/EBPδ, on vascular growth and development and also provide important information to understand the mechanism underlying pathogenesis of vascular remodeling and ensuing atherosclerosis or restenosis.

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