Regulation of CD44 Gene Expression by the Proinflammatory Cytokine Interleukin-1β in Vascular Smooth Muscle Cells*

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The CD44 gene codes for a family of alternatively spliced, multifunctional adhesion molecules that participate in extracellular matrix binding, lymphocyte activation, cell migration, and tumor metastasis. In a mouse model of transplant-associated arteriosclerosis, CD44 protein was induced in the neointima of allografted vessels and colocalized with a subset of proliferating vascular smooth muscle cells (SMC). To elucidate the molecular mechanisms regulating CD44 expression in this model, we investigated the regulation of CD44 gene expression by interleukin (IL)-1β. Treatment of rat aortic SMC with IL-1β resulted in a 5.3-fold increase in cell surface CD44 expression. Northern analysis showed that IL-1β promoted a dose- and time-dependent induction of CD44 mRNA which reached 6.6-fold after 48 h, and nuclear run-on analysis showed that IL-1β increased the rate of CD44 gene transcription within 8 h of stimulation. In transient reporter gene transfection experiments in rat aortic SMC, a 1.4-kilobase fragment of the mouse CD44 5′-flanking sequence mediated this response to IL-1β. Regulation of CD44 gene expression by the proinflammatory cytokine IL-1β may contribute to SMC phenotypic modulation in the pathogenesis of arteriosclerosis.

Vascular smooth muscle and the associated connective tissue matrix are central to blood vessel integrity and function, and activation of vascular smooth muscle cells is characteristic of arteriosclerosis and hypertension. After vessel wall injury, smooth muscle cells are transformed from a contractile, quiescent phenotype to a proliferative, migratory phenotype that secretes abundant extracellular matrix (1). Vascular smooth muscle cells are subject to complex regulation by soluble extracellular signals provided by growth factors, cytokines, and vasoactive agents as well as cell-cell and cell-matrix interactions (1). Therefore, factors controlling smooth muscle cell behavior, including migration, proliferation, and lipid metabolism, are critical to the pathogenesis of cardiovascular disease. Cell surface signal transduction and adhesion molecules such as vascular cell adhesion molecule-1 (2) and the β1 and β3 integrins (3, 4) have been implicated in the modulation of smooth muscle cell function. Previous studies from our laboratory indicate that the proteoglycan CD44 may also mediate vascular smooth muscle cell activation during vascular remodeling (5).

The CD44 transmembrane glycoprotein exists in a variety of isoforms generated by alternative splicing of one (or more) of 10 variable exons in the extracellular domain (6, 7). CD44 is the principal cell receptor for hyaluronic acid (8) and interacts with other extracellular matrix molecules including osteopontin, collagen, and fibronectin (9, 10). Present on many cell types, CD44 has been correlated with cell proliferation (11, 12) and oncogenic transformation (13). Ligation of CD44 stimulates cytokine release by monocytes/macrophages (14, 15), and it may modulate T lymphocyte activation signals (16, 17). In a variety of cell systems CD44 imparts a novel cellular adhesive and/or migratory phenotype to transfected cells (9, 18, 19), and an isoform containing the sixth variable exon (v6) confers metastatic potential to rat pancreatic carcinoma cells (20). The importance of CD44 in vivo has also been demonstrated in a mouse model in which an antisense CD44 transgene is expressed selectively under the control of a keratinocyte-specific promoter (21). Suppression of CD44 expression inhibits keratinocyte proliferation and results in abnormal hyaluronate metabolism in the skin. Moreover, CD44 is induced on smooth muscle cells after vascular injury, and it may mediate the proliferative effects of hyaluronate (5).

Our laboratory has developed a mouse model of transplant-associated arteriosclerosis in which a carotid artery loop is transplanted between inbred strains in syngeneic and alloge neic combinations (22). Lesion development depends on an acquired immune response and begins with infiltration of inflammatory cells, after which follows accumulation of smooth muscle cells in the neointima (23). In the present study we evaluated CD44 cell surface protein expression in vivo during the pathogenesis of transplant arteriosclerosis in order to understand the role of CD44 in modulating vascular smooth muscle cell phenotype. To elucidate the molecular mechanisms regulating CD44 expression in vascular smooth muscle cells after injury, we studied the effect of interleukin (IL)1β on CD44 gene expression in cultured rat aortic smooth muscle cells (RASMC).

EXPERIMENTAL PROCEDURES

Immunocytochemistry—Carotid artery transplantation was performed as described (22). In brief, a carotid artery loop was transplanted between two strains of inbred mice incompatible in the H-2 region. B10.A(2R) (H-2b) mice were used as donors in the allograft group, and C57BL/6J (H-2b) mice were used as donors in the isograft group. C57BL/6J (H-2b) mice were used as recipients in both groups. Transplant samples were prepared and immunostained as described by

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§ The abbreviations used are: II, interleukin; RASMC, rat aortic smooth muscle cells; FCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; kb, kilobase(s).
Induction of CD44 by Interleukin-1β

Shi et al. (22). Grafs were harvested and processed in methyl Carnoy’s fixative, embedded in paraffin, and cut in a microtome in 4-μm sections. After removal of the paraffin, tissue sections were incubated with 10% normal serum for 20 min at room temperature.

Immunohistochemical analysis for CD44 was carried out with a rat anti-human monoclonal antibody (IM7, rat IgG2b, 1 μg/ml; Pharmingen, San Diego, CA). Proliferating cells were detected with a proliferating cell nuclear antigen (PCNA) antibody (clone PC10, 3 μg/ml; Oncogene Research Products, Cambridge, MA), and vascular smooth muscle cells with an anti-smooth muscle actin monoclonal antibody (anti-NH₂-terminal decapeptide, clone 1A4, 1:400, Sigma). Tissue sections were incubated with the antibodies at room temperature for 1 h and then at 4 °C overnight. Biotinylated rabbit anti-IgG (1:100, Vector Laboratories, Burlingame, CA) was used as a secondary antibody to detect CD44 staining, and biotinylated goat anti-mouse IgG2a antibody (RPN 1181, 1:100, Amersham Pharmacia Biotech) was used as a secondary antibody to detect PCNA and α-actin staining. Sections stained for CD44 and PCNA were labeled with avidin-biotin enzyme complex peroxidase (Vector Laboratories) and developed in 3,3′-diaminobenzidine. Sections stained for α-actin were labeled with avidin-biotin enzyme complex alkaline phosphatase and developed with VectorRed (Vector Laboratories). Sections treated for CD44 and PCNA were counterstained with 1% methyl green, and sections treated for α-actin were counterstained with Verhoeff’s stain for elastic tissue.

RASMC were harvested from the thoracic aorta of adult male Sprague-Dawley rats (200-250 g) by enzymatic dissociation according to the method of Gunther et al. (24). The cells were cultured in Dulbecco’s modified Eagle’s medium (JRH Biosciences, Lenexa, KS) and supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 μM Hepes (pH 7.4). RASMC were passaged every 4–7 days, and experiments were performed on cells four to seven passages from primary culture. Reconstitual IL-1β and platelet-derived growth factor-BB were obtained from Collaborative Biomed-2 Life Science Products). The samples were then extracted with phenol/chloroform, precipitated, and resuspended at equal counts/min/ml in hybridization buffer (1.4 × 10⁶ cpm/ml). Denatured probes (1 μg) dot-blotted on nitrocellulose filters were hybridized at 40 °C for 4 days in the presence of formamide. cDNAs for the CD44 and β-actin genes were used as probes. The filters were scanned, and radioactive signal was measured on a PhosphorImager running the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RNA Blot Hybridization—Total RNA was obtained from cultured cells by guanidinium isothiocyanate extraction and centrifuged through cesium chloride (25). The RNA was sonicated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose filters. The filters were prehybridized overnight at 42 °C, and hybridization was performed on an Ortho 2150 cytofluorograph (CytoTrends, Sunnyvale, CA) equipped with a Cyclops data acquisition and analysis system (Cyclops Software, Cytomation, Fort Collins, CO).

Nuclear Run-on Analysis—Confluent RASMC were either treated with PBS (control) or stimulated with IL-1β for 8 h. The cells were then lysed, and nuclei were isolated as described (26). The nuclear suspension (200 μl) was incubated with a 0.5 mM concentration each of CTP, ATP, and GTP with 125 μCi of [α-³²P]UTP (3,000 Ci/mmol; NEN Life Science Products). The samples were then extracted with phenol/chloroform, precipitated, and resuspended at equal counts/min/ml in hybridization buffer (1.4 × 10⁶ cpm/ml). Denatured probes (1 μg) dot-blotted on nitrocellulose filters were hybridized at 40 °C for 4 days in the presence of formamide. cDNAs for the CD44 and β-actin genes were used as probes. The filters were scanned, and radioactive signal was measured on a PhosphorImager running the ImageQuant software. The amount of sample hybridizing the CD44 probe was divided by that hybridizing the β-actin probe, and the corrected densitometry was recorded as a percentage increase from the control density.

Isolation of the CD44 5′-Flanking Sequence—A fragment of the 5′-flanking region of the mouse gene encoding CD44 was amplified by using the PromoterFinder Kit (CLONTECH, Palo Alto, CA). Primers were designed according to the published mouse CD44 sequence (27, 28). A CD44-specific primer (5′-CCAAGAGGCAAAATGCCCACATTTG-T-3′) and a nested primer (5′-CCAGGCTTTGCCCACAAACTTG-3′) were used in the primary and secondary polymerase chain reactions, respectively, to amplify 1,400-base pair fragment from mouse genomic DNA. The polymerase chain reaction fragment was subcloned and sequenced by the dyeodeoxy chain termination method (25) with Sequenase 2.0 DNA polymerase (US Biochemical Corp.). A fragment of the mouse CD44 5′-flanking sequence was radiolabeled with [α-³²P]dCTP and used to screen a phage library of mouse genomic DNA in the AFinII vector (Stratagene, La Jolla, CA) as described (29). Hybridizing clones were isolated and purified, and phage DNA was prepared according to standard procedures (25). Restriction fragments derived from the mouse CD44 gene were subcloned into pSP72 (Promega, Madison, WI) and sequenced with Thermo Sequenase DNA polymerase (Amersham Pharmacia Biotech). Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI).

Plasmids—Plasmid pGL2-Basic contained the firefly luciferase gene with no promoter (Promega), and phagemid pOPRSVI-CAT (Stratagene) contained the prokaryotic chloramphenicol acetyltransferase (CAT) gene driven by the RSV-LTR (Rous sarcoma virus-long terminal repeat) promoter. Reporter constructs containing fragments of the mouse CD44 5′-flanking sequence were named according to the location of the fragment from the transcription start site in the 5′ and 3′ directions. A gene fragment amplified from mouse genomic DNA containing 1,262 base pairs of the CD44 5′-flanking sequence upstream and 109 base pairs downstream of the transcription initiation site was cloned into pGL2-Basic and named CD44 (−1,334 to +0.1 kb). A larger fragment containing 3,900 base pairs 5′ to fetal calf serum and stimulated with 10 ng/ml of IL-1β (PBS) or IL-1β (10 ng/ml) for 48 h. Cell extracts were prepared by a detergent lysis method (Promega), and luciferase activity was measured in duplicate for all samples by using the Promega luciferase assay system and an EG&G Autolumat LB953 luminometer (Gaithersburg, MD). The CAT assay was performed by a modified two-phase flow diffusion method as described (30). The ratio of luciferase activity to CAT activity in each sample served as a measure of normalized luciferase activity. Each construct was transfected at least five times, and data for each construct are presented as the mean ± S.E. Relative luciferase activity in groups treated with vehicle was compared with that in groups treated with IL-1β by analysis of variance. Statistical significance was accepted at p < 0.05.

RESULTS

Expression of CD44 in Transplant Arteriosclerosis—To understand how CD44 may modulate vascular smooth muscle cell phenotype during vascular remodeling, we studied CD44 expression in a mouse model of transplant arteriosclerosis. Immunohistochemical analysis was performed on histological sections from allografted (Fig. 1, left) and isografted (Fig. 1, right) carotid arteries harvested at day 15 after transplantation, a time at which neointimal thickening is substantial (22). Allograft sections displayed robust CD44 protein expression in the neointima (Fig. 1A, brown), whereas isografted
sections showed minimal CD44 expression and little neointima formation (Fig. 1B). The level of proliferation among neointimal cells, as evidenced by staining for PCNA (Fig. 1C, brown), was significant in allograft sections. PCNA staining was absent in the isograft control (Fig. 1D). Note that CD44 and PCNA exhibited a similar pattern of induction in the neointima in day 15 allografted arteries. Vascular smooth muscle cells predominated in the neointima, as evidenced by staining for smooth muscle α-actin (Fig. 1E, pink). Taken together, these data suggest that proliferating vascular smooth muscle cells account for the majority of CD44 expression in the neointima during the development of transplant-associated arteriosclerosis.

**Induction of CD44 Protein and mRNA by IL-1β in RASMC**—To investigate signals regulating CD44 expression in vascular smooth muscle cells, we tested the effect of the proinflammatory cytokine IL-1β on CD44 protein and mRNA levels. RASMC were cultured in 0.4% fetal calf serum and treated with IL-1β (10 ng/ml) for 48 h. Cell surface CD44 expression was then assessed by antibody staining and flow cytometry. CD44 surface antigen was present on RASMC under basal conditions (Fig. 2, Control) and increased by 5.3-fold after IL-1β stimulation.

We then performed Northern blot analysis with total RNA from RASMC treated with vehicle (PBS) or IL-1β and hybridized with a rat CD44 cDNA probe. The CD44 mRNA hybridization pattern (Fig. 3, A and B) showed four distinct bands at 1.6 kilobases (kb), 2.0 kb, 3.3 kb, and 4.5 kb, sizes similar to those reported previously for CD44 (5). These isoforms are thought to arise from alternative splicing of variant exons or from the use of multiple polyadenylation signals. In the representative Northern blot of the time course of CD44 mRNA induction (Fig. 3A), an IL-1β dose of 10 ng/ml increased CD44 mRNA as early as 6 h after stimulation. CD44 mRNA induction reached 6.6-fold after 48 h. In addition, a dose-dependent induction of CD44 mRNA was observed in RASMC stimulated for 24 h with various doses of IL-1β (Fig. 3B). An increase in CD44 mRNA levels was evident at an IL-1β dose of as little as 1 ng/ml, and maximal up-regulation occurred at a dose of 10 ng/ml. On the basis of these experiments, an IL-1β dose of 10 ng/ml was used in all subsequent experiments.) The ability of IL-1β to increase the level of CD44 mRNA was retained in the presence of the protein synthesis inhibitors cyclohexamide and anisomycin, indicating that up-regulation was independent of de novo protein synthesis (data not shown). Taken together, these data indicate that the cytokine IL-1β acts as a potent stimulus to induce CD44 protein and mRNA expression in RASMC.

**Regulation of CD44 mRNA Expression by Other Inflammatory Cytokines**—To assess the specificity of the effect of IL-1β on CD44 gene regulation, we tested the ability of other cytokines and growth factors implicated in the development of arteriosclerotic lesions to modulate CD44 mRNA expression. Serum-starved RASMC were treated with tumor necrosis factor-α, interferon-γ, and platelet-derived growth factor-BB for various lengths of time, and the CD44 message was measured by Northern analysis (Fig. 4). Tumor necrosis factor-α, an inflammatory cytokine whose effects are often similar to those of IL-1β, promoted a gradual increase in CD44 mRNA which reached 3.7-fold at 48 h. Interferon-γ, which inhibits vascular smooth muscle cell growth (31, 32) yet stimulates macrophage (33) and lymphocyte (34, 35) activation, had only a minimal effect on CD44 message levels. Platelet-derived growth factor-BB, a potent mitogenic and chemotactic agent for smooth muscle cells, produced a 3.5-fold induction of CD44 mRNA at 4 h which returned to base line by 12 h. In light of this experiment, we concentrated our efforts on IL-1β, which produced a dramatic and sustained induction of CD44 in vascular smooth muscle cells.

**IL-1β Increases CD44 Gene Transcription**—To elucidate the mechanism by which IL-1β elevates CD44 message levels, we performed nuclear run-on experiments to assess the effect of IL-1β on the rate of CD44 gene transcription. CD44 mRNA transcription in RASMC increased by 3.7-fold within 8 h of IL-1β stimulation compared with control (Fig. 5). Exposure of
The transcription was allowed to resume in the presence of IL-1β

Northern blot analyses were performed with 10 μg of total RNA/lane. In panel A, the corrected CD44 mRNA values (CD44/18s) comprising the sum of all four isoforms are plotted as a percentage of the control value at each time point (standardized to 100%). In panel B, the corrected mRNA values are plotted as a percentage of the 0-dose control value.

To investigate further the transcriptional mechanisms regulating the expression of CD44 in vascular smooth muscle cells, we cloned the 5′-flanking sequence of the mouse CD44 gene by primer extension analysis. A complementary oligonucleotide probe corresponding to CD44 positions +36 to +56 (Fig. 6A, underlined with arrow) was used to prime gene transcription on total RNA from C2C12 skeletal myoblasts and the CD44-negative cell line S49. Transcription was initiated only in C2C12 cells, from a single predominant transcription start site (Fig. 6A, bent arrow) corresponding to a C nucleotide located 112 base pairs upstream of the site of translation initiation (Fig. 6A, double underline). Identical primer extension results were obtained with total RNA from mouse aortic smooth muscle cells (data not shown) and were confirmed with an alternate CD44-specific primer (positions +115 to +139). This transcription initiation site identified by primer extension studies was consistent with results obtained by ribonuclease protection analysis (data not shown).

Although the CD44 upstream sequences lack a TATA box, a binding site for transcription factor IID (Fig. 6A, position −51) sufficient for activation of transcription through an initiator element is present (36). A comparison of the mouse CD44 5′-regulatory sequences with sequences in the Transcription Factor Data base revealed a variety of cis-acting elements, including multiple SP1 and PEA3 sites as well as a CACCC motif, a GATA element, and a cyclic AMP response element (Fig. 6A). Sequence analysis also identified an AP1 site at position −110 which is spatially conserved in the human CD44 promoter (13).

IL-1β Induces CD44 Promoter Activity in RASMC—We constructed luciferase reporter plasmids driven by 1.4-kb and 4.0-kb fragments of the CD44 5′-flanking sequence to analyze CD44 promoter function in RASMC. Promoter constructs in pGL2-Basic were cotransfected into RASMC with pOPRSVIII-CAT, and corrected luciferase activity was expressed as a percent of the 0-dose control value.

The nucleotide sequence of the mouse CD44 promoter.

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percentage of activity in unstimulated controls. The promoter activity of plasmid CD44 (−1.3/+0.1 kb) increased by 3.5-fold upon stimulation with IL-1β (Fig. 7). Similarly, IL-1β treatment produced a 2.8-fold increase in the activity of plasmid CD44 (−3.9/+0.1 kb), which contains additional upstream regulatory sequences. These data suggest that the most important IL-1β regulatory elements are contained in the 21.3 to 10.1 kb fragment of the CD44 5′-flanking sequence. The 21.3 to +0.1 kb fragment cloned into pGL2-Basic in the antisense orientation had only minimal luciferase activity that was not altered by treatment with IL-1β (data not shown). In conjunction with the nuclear run-on experiments, these experiments demonstrate that the induction of CD44 mRNA by IL-1β is due to a transcriptional regulatory mechanism.

**DISCUSSION**

In a variety of cell systems, functions have been proposed for CD44 in cellular activation and movement, processes that are central to the smooth muscle cell response to arterial wall injury. In our mouse model of transplant-associated atherosclerosis, CD44 is expressed minimally in the medial layer of the normal vascular wall in vivo and is induced markedly on smooth muscle cells in the neointima of allografted carotid arteries. In addition, extracellular matrix ligands for CD44,
including hyaluronate and osteopontin, are elaborated in developing atherosclerotic lesions and may be important in modulating vascular smooth muscle cell function (5, 37, 38). The coincident expression of the CD44 adhesion receptor and its ligands may facilitate biological processes such as smooth muscle cell replication and migration during vascular remodeling.

Although CD44 expression has been documented in a variety of cell types, little is known about the molecular regulation of the CD44 gene. CD44 transcription is up-regulated in ras-transformed rat embryonic fibroblasts in an AP-1-dependent fashion, and expression correlates with cellular metastatic potential (13). Epidermal growth factor acts through a novel cis-acting element to induce CD44 expression in mouse fibroblasts, which is accompanied by enhanced cell attachment to hyaluronic acid (39). In the context of lymphocyte activation during humoral immune responses, the CD44 promoter is activated by the EGR1 transcription factor after B cell antigen receptor stimulation (40). Our present studies give new insight into the mechanisms by which cytokines regulate CD44 gene expression in vascular smooth muscle cells.

Cytokines and growth factors regulate cellular functions that are central to atherogenesis, including proliferation, chemotaxis, lipid metabolism, and synthesis of extracellular matrix components (1, 41). The proinflammatory cytokine IL-1β is present in atherosclerotic lesions and mediates changes in cellular gene expression which correlate with the development of pathological smooth muscle cell behavior (42). IL-1β is mitogenic for vascular smooth muscle cells in vitro (43, 44), and it contributes to the regulation of cytokine production, extracellular matrix deposition, adhesion molecule expression, and matrix metalloproteinase secretion by these cells (45–48). We demonstrate here that stimulation of primary cultured RASMC with IL-1β induced CD44 protein and mRNA, which was attributable to an increase in the rate of CD44 gene transcription. Transient transfection experiments in RASMC showed that induction by IL-1β was mediated by regulatory sequences that reside within a 1.4-kb fragment of the mouse CD44 promoter. We propose that IL-1β induction of CD44 gene expression contributes to a coordinated response of smooth muscle cells to arterial wall injury. Thus, further insight into the transcriptional control of the CD44 promoter may elucidate regulatory mechanisms that modulate smooth muscle cell phenotype in the pathogenesis of vascular disease.

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