Y-Box Binding Protein-1 Controls CC Chemokine Ligand-5 (CCL5) Expression in Smooth Muscle Cells and Contributes to Neointima Formation in Atherosclerosis-Prone Mice

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Background—The CC chemokine CCL5/Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) is upregulated in mononuclear cells or deposited by activated platelets during inflammation and has been implicated in atherosclerosis and neointimal hyperplasia. We investigated the influence of the transcriptional regulator Y-box binding protein (YB)-1 on CCL5 expression and wire-induced neointimal hyperplasia.

Methods and Results—Analysis of the CCL5 promoter revealed potential binding sites for YB-1, and interaction of YB-1 with a sequence at position −204/−173 was confirmed by DNA binding assays. Both YB-1 expression and CC chemokine ligand-5 (CCL5) mRNA expression were increased in neointimal versus medial smooth muscle cells, as analyzed by real-time polymerase chain reaction. Overexpression of YB-1 in smooth muscle cells (but not macrophages) enhanced CCL5 transcriptional activity in reporter assays, mRNA and protein expression, and CCL5-mediated monocyte arrest. Carotid arteries of hyperlipidemic apolipoprotein E–deficient mice were subjected to intraluminal transfection with a lentivirus encoding YB-1 short hairpin RNA or empty vector directly after wire injury. Double immunofluorescence revealed YB-1 expression in neointimal smooth muscle cells but not macrophages and colocalization with neointimal CCL5, which was downregulated by YB-1 short hairpin RNA. Neointima formation was decreased significantly after YB-1 knockdown compared with controls and was associated with a diminished content of lesional macrophages. A reduction of lesion formation by YB-1 knockdown was not observed in apolipoprotein E–deficient mice deficient in the CCL5 receptor CCR5 or after treatment with the CCL5 antagonist Met-RANTES, which indicates that YB-1 effects were dependent on CCL5.

Conclusions—The transcriptional regulator YB-1 mediates CCL5 expression in smooth muscle cells and thereby contributes to neointimal hyperplasia, thus representing a novel target with which to limit vascular remodeling.

Key Words: muscle, smooth remodeling chemokines restenosis

Inflammatory chemokines recruit effector cells to sites of inflammation and play a key role in wound healing; however, the sustained expression of these inducible chemokines can cause or aggravate chronic inflammation. Thus, chemokines have been implicated as promising immunotherapeutic targets in the treatment of vascular diseases.1–3

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Expression of the CC (adjacent N-terminal cysteines) chemokine CCL5 (CC chemokine ligand-5)/RANTES (regulated on activation, normal T cell expressed and secreted) has been detected in atherosclerotic and neointimal lesions after vascular injury, and it has been reported to trigger leukocyte arrest and transmigration across endothelial cells.4–6 In addition, upregulation of CCL5 expression has been demonstrated in activated vascular smooth muscle cells (SMCs),7 which play an important role in atherosclerotic lesion formation. In fact, a pronounced reduction in injury-induced neointimal hyperplasia and diet-induced atherosclerotic lesion formation in mice treated with the CCL5 receptor antagonist Met-RANTES or with genetic deficiency in the CCL5 receptor CCR5 corroborated a major role for CCL5 in atherogenesis.8–11 Through the identification of a large number of potential transcriptional consensus elements in the immediate

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upstream region of the CCL5 gene,12 some of the mechanisms regulating the expression of CCL5 have been determined. For instance, promoter studies have revealed the constitutive and interferon (IFN)-γ- or tumor necrosis factor-α-inducible transcription factors, IFN-regulatory factor 1, and nuclear factor-κB (NF-κB), which synergistically activate the CCL5 promoter.13,14 Apart from consensus elements for IFN-regulatory factor 1, NF-κB, activating protein-1, and other transcription factors, DNA footprinting showed that the CCL5 promoter also contains possible binding sites for the cold shock protein YB-1 (Y-box binding protein-1),12 which implies a regulatory role for YB-1 in CCL5 expression; however, this has not been verified biochemically or functionally to date.

YB-1 belongs to the evolutionarily ancient and highly conserved group of Y-box binding proteins that control the expression of a large number of gene products. An increased expression of YB-1 in certain inflammatory diseases has been described (eg, by activated eosinophils in allergic asthma,15 in expression of a large number of gene products. An increased expression, here showing a binding preference for single-stranded DNA.18–20 The 2 possible binding sites for YB-1 in the proximal CCL5 promoter region suggest a role for YB-1 in regulating the expression of CCL5. Here, we examined the role of YB-1 in CCL5 gene transcription in vascular SMCs and macrophages and its effects on neointimal hyperplasia after arterial injury in apolipoprotein E (ApoE)−/− mice, ApoE−/−CCR5−/− mice, or ApoE−/− mice treated with the CCL5 receptor antagonist Met-RANTES.

Methods

Cells

The macrophage cell line RAW264.7 (ATCC, Manassas, Va) and MonoMac6 cells (from Dr H.W.L. Ziegler-Heitbrock, Institute for Immunology, Munich, Germany) were maintained as described previously.21–23 Human coronary artery SMCs (hCASMCs; Promocell, Heidelberg, Germany) were cultured in SMC growth medium 2 (PromoCell). SMCs from media and neointima of adult Sprague-Dawley rats (rSMCs) were isolated and cultured as described previously.24

Plasmids, Transfections, and Luciferase Reporter Assay

Full-length YB-1 cDNA inserted into expression plasmid pSG5 (Stratagene, La Jolla, Calif)25 and reporter plasmids that harbor regulatory sequences of the human CCL5 gene extending up to −1014 bp26 have been described previously. Knockdown of endogenous YB-1 was achieved by means of short hairpin RNA (shRNA) generated by pSuperduper plasmid (OligoEngine, Seattle, Wash) with the sequence 5′-GGTCATCGCAGCGAGATTTT-3′ introduced as a tail-to-tail tandem repeat corresponding to base pairs 285 to 305 of the human YB-1 coding sequence (GenBank accession No. J03827). For transfection of SMCs, adherent cells were trypsinized and resuspended in RPMI media supplemented with 20% fetal calf serum at 7 × 103 cells/mL. Cells (1.4 × 105) were incubated with a total of 12 μg of plasmid DNA in electroporation cuvettes (0.4-cm gap, Bio-Rad, Hercules, Calif) for 30 minutes on ice and electroporated at 300 V and a capacitance of 500 μF in a Gene Pulser II electroporation system (Bio-Rad). Cells were suspended for 15 minutes on ice and thereafter resuspended in RPMI 1640/20% fetal calf serum medium and transferred to 6-well tissue-culture plates. As indicated, human IFN-γ (100 U/mL) with or without interleukin-1β (100 U/mL) was added to some cells. Luciferase measurements were performed 24 hours after transfection with the Promega luciferase assay system (Mannheim, Germany) with measurements in a single-tube luminometer (Berthold Detection Systems, Pforzheim, Germany). Results were calculated as fold-change relative to luciferase activity measured with pGL3 plasmid without promoter or relative to pGL3basic plus pSG5 plasmid–transfected cells. The pGL3−/−168 CCL5 promoter-reporter construct was generated by polymerase chain reaction of a −974 nucleotide CCL5 5′-upstream fragment based on pGL3-based plasmid constructs (pGL3; Promega, Madison, Wis), as described previously.27 The transient expression vectors pRRI-cPp-H1.PreSIN (H1.Empty) and pRRI-cPp-H1.shYB-1-PreSIN (H1.shYB-1) were constructed by exchanging the cytomegalovirus (CMV) promoter from the expression vector pRRI-cPp-CMV-PreSIN28 with the H1 promoter or the complete H1.shYB-1 construct via the Clal and SpeI restriction sites. Virus was produced as described previously29 with transient calcium phosphate transfection of 293T cells.

Electrophoretic Mobility Shift Analysis

For preparation of nuclear extracts, hCASMCs were lysed in ice-cold hypotonic buffer (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L MgCl2, 10 mmol/L KCl) supplemented with a proteinase inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany) and 0.5 mmol/L DTT. Sequential centrifugation at 15 000 g EDTA, and 25% glycerol for 20 minutes on ice. After final centrifugation at 15 000g, supernatant containing nuclear proteins was collected, and concentrations were determined by Bio-Rad protein assay with bovine serum albumin as standard. Extracts were stored at −80°C until performance of electrophoretic mobility shift analysis.

Recombinant YB-1 was prepared from a pRSET vector (Invitrogen, Carlsbad, Calif) containing an insert encoding for a hexahistidine T7 epitope−YB-1 fusion protein, as described previously.18 Synthetic DNA probes corresponding to the sense and antisense strands of the CCL5 promoter sequences −204/−173 were end-labeled by means of the biotin 3′ end DNA labeling kit (Pierce, Rockford, Ill). Double-stranded DNA was generated by mixing equal amounts of labeled complementary oligonucleotides and incubating the mixture for 1 hour at room temperature. The nucleotide sequences were as follows: 5′-CCGGTACCCATTGGTGCTTGGTC-3′ (sense), 5′-GGCAATTCGATGAGGTCTCTTCTTGGACCAACATTGGTCGCG-3′ (antisense).

Biotin-labeled DNA was incubated with affinity-purified recombinant YB-1 protein or nuclear cell extract for 20 minutes on ice, subjected to gel electrophoresis on native 6% polyacrylamide gels, and transferred to nylon membranes. Bands were visualized by streptavidin−horseradish peroxidase conjugate and chemiluminescent substrate (LightShift chemiluminescent electrophoretic mobility shift analysis kit, Pierce). For supershift analyses, peptide-derived, affinity-purified rabbit anti-YB-1 antibody (peptide antibody against amino acids 185 to 206) was incubated with
nuclear proteins 12 hours before addition of probes. The binding reaction was as above, and samples were subjected to electrophoresis in 6% polyacrylamide gels.

Quantitative Real-Time Polymerase Chain Reaction and ELISA

Human SMCs were transiently transfected with the overexpression vector pSG5/YB-1 or the control vector with the human aortic SMC nucleoexchange kit (Axama, Cologne, Germany) according to the manufacturer’s instructions and incubated for 48 hours. Total messenger RNA from hCASMCs or rSMCs was isolated (RNeasy Mini Kit, Qiagen, Hilden, Germany) and reverse-transcribed with oligo-dT primers (OmniScript, Qiagen). A real-time polymerase chain reaction with up to 100 ng of cDNA was performed with the QuantiTect Kit with SYBRGreen (Qiagen) and specific primers for YB-1, CCL5, and GAPDH according to the manufacturer’s protocol, as follows: 5'-CACCTTACTACATCGCGAGACCT-3', 5'-TTG-TCAAGCACCCTCCATCACT-3' (YB-1); 5'-TTGCTGTTCGCTGTTGC-TGTTCCT-3', 5'-TGTAACTGCCTGCGGTGT-3' (CCL5); and 5'-GCCCTAACAGTACGACGTTGAC-3' (GAPDH). Amplification (40 cycles, with annealing at 55°C) was performed via the MJ Research Opticon 2 (Biozym, Oldendorf, Germany). Experiments were performed in triplicate. CCL5 ELISA was performed according to the manufacturer’s protocol (R&D Systems, Minneapolis, Minn), and optical density was detected with the 3,3,5,5-tetramethylbenzidine (TMB) peroxidase substrate kit (Vector Labs, Burlingame, Calif).

Parallel-Plate Flow-Chamber Adhesion Assay

MonoMac6 adhesion to YB-1–overexpressing hCASMC monolayers was measured in a parallel-plate flow chamber that creates a laminar flow field, as described previously. Briefly, hCASMCs were transfected with the plasmid pSG5/YB-1 or control vector and grown in 35-mm Petri dishes for 24 hours. MonoMac6 cells (5 × 10^5/mL) were stained with 0.3 μg/mL calcein AM (Molecular Probes, Carlsbad, Calif) and perfused over the hCASMC monolayers in assay buffer (1× HBSS, 10 mmol/L HEPES, 1 mmol/L CaCl2/7 M Mg2+, and 0.5% bovine serum albumin) at 1.5 dyne/cm2. Some monocytes were pretreated with Met-RANTES (kindly provided by Dr Amanda Proudfoot, Serono, Geneva, Switzerland) at 1 μg/mL for 15 minutes before the flow experiment. After 2 minutes of perfusion, firmly adherent cells were determined with the Cell AnalySIS System (Olympus, Hamburg, Germany).

Mouse Model of Arterial Wire Injury and Lentiviral Transduction

ApoE−/− and CCR5−/−/ApoE−/− mice obtained from the local animal breeding facility at the RWTH Aachen University Hospital, were fed a Western-type diet (containing 0.25% cholesterol and 15% cacao butter, SDS, Sussex, United Kingdom) from 1 week before surgery and continuing throughout the experiment. All animal work was approved by regulatory authorities of Leiden and complied with Dutch government guidelines. Transluminal wire injury of the left common carotid artery via the left external carotid artery, and euthanized at 4 weeks after injury. Starting at the bifurcation, mice (n = 6 each) were treated with Met-RANTES (40 μg 3 times per week) after wire injury and lentiviral transduction. Animals were euthanized at 4 weeks after injury. Statistic analyses were performed with 4 to 6 animals per group.

Immunofluorescence

Tissue sections of the left common carotid artery were stained for YB-1 (peptide-derived polyclonal antibody raised against an oligopeptide located within the N-terminal domains; P.R.M.), SMCs (α-smooth muscle actin, clone 1A4, Dako, Glostrup, Denmark), macrophages (Mac-2, clone M3/38, Cedarlane, Ontario, Canada), or CCL5 (clone C19, Santa Cruz, Calif). For detection, cyanine 3– or fluorescein isothiocyanate–conjugated secondary antibodies were used. The relative macrophage content was determined by quantifying the area that was positive for Mac-2. Images were recorded with a Leica DM LB fluorescence microscope and charge-coupled device camera (Leica, Wetzlar, Germany).

Statistical Analysis

In vivo data, luciferase reporter assay data, and arrest data were compared by 1-way ANOVA and Newman-Keuls post hoc test or Student unpaired t test with Welch’s correction using GraphPad Prism version 4.00 software for Windows (GraphPad Software, San Diego, Calif).

Results

YB-1 Binds to a Y-Box in the CCL5 Promoter

to detect additional regulatory elements involving YB-1 within the CCL5 promoter, we compared its sequence with Y-boxes of other genes. A Y-box with homologies with known YB-1 binding motifs was identified within the proximal CCL5 sequence between −204 and −173 bp from the start codon; it contained several matching nucleotides but also an inverted repeat motif (Figure 1A). To test YB-1 binding to this Y-box DNA sequence, DNA binding studies were performed. Whereas recombinant YB-1 readily bound to the single-stranded proximal sense (Figure 1B, lane 4) and antisense (Figure 1B, lane 6) promoter region, no binding to a double-stranded DNA probe was observed (Figure 1B, lane 2). Specificity of binding was confirmed by inhibition of YB-1 binding to the single-stranded antisense probe in the presence of homologous competitor DNA at 500-fold molar excess (Figure 1B, lanes 7 to 9). Of note, complex formation of the same mobility with the single-stranded antisense probe was observed when we tested nuclear extracts derived from SMCs (NEsmc; Figure 1B, lane 10). Furthermore, the antisense-NEsmc complex mobility was supershifted with a YB-1–specific antibody (Figure 1C, lane 2), which supports the notion that YB-1 has a function in the regulation of CCL5 promoter activity.

Increased Expression of YB-1 and CCL5 Correlates in Inflammatory SMCs

Because an upregulation of chemokines has been identified in neointimal SMCs with a proinflammatory phenotype, we determined the expression of YB-1 and CCL5 in rSMCs isolated from the media and neointima of balloon-injured aortas. Indeed, CCL5 mRNA expression was increased markedly in neointimal SMCs compared with medial cells, as determined by quantitative real-time polymerase chain reaction analysis. In parallel, the expression of YB-1 mRNA was increased 3- to 4-fold in neointimal versus medial rSMCs (Figure 2A), which suggests a role for YB-1 in the regulation of CCL5 in injured or proinflammatory SMCs.

YB-1 Controls the Transcriptional Activity and Protein Expression of CCL5

Next, we used a luciferase reporter assay to study regulatory effects of YB-1 on CCL5 expression in hCASMCs. Whereas
transfection with empty vector pGL3 alone had no effect, transfection with a reporter construct harboring the proximal 1014 bp (full length) of the CCL5 promoter (pGL3/CCL5) caused a moderate increase in luciferase activity (Figure 2B). Cotransfection with YB-1 (pSG5/YB-1) and pGL3/CCL5 led to a significant induction of luciferase activity in hCASMCs (Figure 2B). The induction by YB-1 was almost completely attenuated in cells cotransfected with a plasmid that coded for the double-stranded probe (DS; compare lane 2 with lanes 4 and 6, complex indicated by *). Specificity of binding was confirmed by diminished bands after inclusion of homologous competitor DNA (homC; lane 9). With SMC nuclear extract (NE_SMC), a complex of the same mobility formed with the antisense strand (SS2) probe (lane 10). Binding of a YB-1–specific antibody resulted in a supershift of the SS2/NE_SMC complex (lane 2, indicated by *), whereas isotype control did not bind to the complex (lane 3).

![Figure 1](image)

**Figure 1.** Recombinant and endogenous YB-1 binds to a Y-box in the CCL5/RANTES promoter. A, Sequence alignment of the proximal CCL5/RANTES gene promoter with known YB-1 response elements (RE) in the matrix metalloproteinase-2 (MMP-2) and DNA polymerase A (DPA) genes revealed similarities of the sequence –204/–173 bp with known YB-1 binding motifs, which are matching nucleotides within the Y-box, and the presence of a 3’ localized inverted repeat motif (indicated by arrows). B, Conventional DNA binding studies (electrophoretic mobility shift analysis) demonstrated preferential binding of rat YB-1 (rYB-1) to sense (SS1) and antisense (SS2) strands of the –204/–173 element compared with the double-stranded probe (DS; compare lane 2 with lanes 4 and 6, complex indicated by *). Specificity of binding was confirmed by diminished bands after inclusion of homologous competitor DNA (homC; lane 9). With SMC nuclear extract (NE_SMC), a complex of the same mobility formed with the antisense strand (SS2) probe (lane 10). C, Binding of a YB-1–specific antibody resulted in a supershift of the SS2/NE_SMC complex (lane 2, indicated by *), whereas isotype control did not bind to the complex (lane 3).

**YB-1 Induces Monocyte Adhesion by Regulating CCL5 Expression**

Because CCL5 has been established as being able to control monocyte recruitment, we studied the role of YB-1–mediated CCL5 expression in MonoMac6 cell arrest on hCASMCs under flow conditions. Compared with vector-transfected controls, transfection of hCASMC monolayers with pSG5/YB-1 to overexpress YB-1 significantly increased monocyte arrest. This increase was mediated by YB-1–induced CCL5 protein expression, as evidenced by the inhibition after pretreatment of MonoMac6 cells with the CCL5 antagonist Met-RANTES (Figure 2I). Thus, YB-1–mediated CCL5 expression supports increased monocyte recruitment.

**Knockdown of YB-1 Protects Against Neointima Formation**

Because YB-1 and CCL5 mRNA levels are upregulated in inflammatory neointimal SMCs (Figure 2A), we investigated the role of YB-1 in neointimal lesion formation after arterial injury in atherosclerosis-prone mice in vivo. To this end, carotid arteries of CCR5 


c5+/ApoE–/– mice, CCR5 


c5+/ApoE–/– mice, or Met-RANTES–treated ApoE–/– mice were lumina transversed with lentiviral shRNA targeting YB-1 (shYB-1) or empty vector immediately after wire-induced endothelial denudation injury. Lentiviral shYB-1 transfer led to a decrease in YB-1 expression by ≈70% in CCR5 


c5+/ApoE–/–
mice, as evidenced by immunofluorescence and subsequent quantification (Figure 3A through 3C and Figure 4A). Analysis of lesional areas in the carotid artery after 4 weeks revealed a significant reduction in the neointima and media in YB-1 shRNA (H1.shYB-1) compared with control (H1.Empty)-treated CCR5+/ApoE−/− mice (representative images in Figure 4B, quantification in Figure 4C and 4D).

This inhibition of vascular remodeling was associated with a significant decrease in the medial and neointimal content of Mac-2+ macrophages in H1.shYB-1–transduced compared with H1.Empty-transduced carotid arteries of CCR5+/ApoE−/− mice (Figure 4E). To address the specific importance of CCL5 for the effects of YB-1, YB-1 knockdown was also performed in CCR5+/ApoE−/− mice, which are genetically...
deficient in the CCL5 receptor CCR5, which is crucial for neointima formation,\textsuperscript{11} or in ApoE\textsuperscript{−/−} mice treated with Met-RANTES to achieve complete blockade of CCL5 receptors.\textsuperscript{8} In line with previous reports,\textsuperscript{8,11} neointimal areas after wire injury in ApoE\textsuperscript{−/−} mice were reduced by 60% in CCR5\textsuperscript{−/−} mice and by 40% after Met-RANTES treatment, whereas medial areas were not altered (Figure 4C and 4D). Notably, the significant reduction of lesion formation by YB-1 knockdown was no longer observed in CCR5\textsuperscript{−/−} ApoE\textsuperscript{−/−} or Met-RANTES–treated mice (Figure 4C and 4D), which indicates that the contribution of YB-1 to neointima formation is mediated through CCL5.

**Figure 3.** YB-1 is expressed in lesional SMCs and colocalized with neointimal CCL5. After transluminal wire injury, carotid arteries of hyperlipidemic ApoE\textsuperscript{−/−} mice were treated for 15 minutes with lentivirus carrying either empty vector (H1.Empty) or plasmid encoding YB-1 shRNA (H1.shYB-1) or were mock treated (control) and analyzed after 4 weeks of atherogenic diet. Double-immunofluorescence staining of H1.Empty–treated carotid arteries revealed colocalization of YB-1 with CCL5 in the neointima (A, arrows) and with α-smooth muscle actin (α-SMA)–positive lesional SMCs in the media (B, upper arrow) or neointima (B, lower arrow) of H1.Empty– or mock-treated carotid arteries. No relevant colocalization of YB-1 with the macrophage marker Mac-2 was found in the plaque area (C, arrow indicates neointima). YB-1 expression and CCL5 expression were markedly reduced in H1.shYB-1–treated arteries (A through G).
After wire injury, YB-1 was increased in the empty vector– and mock-transduced carotid arteries. Conversely, introduction of shYB-1 prevented induction of YB-1 protein in these lesions. The expression of YB-1 and CCL5 was colocalized in neointimal cells but also in medial cells of carotid arteries after injury (Figure 3A, arrows), and lesional CCL5 expression was almost completely abolished after knockdown of YB-1 in H1.shYB-1–treated arteries (Figure 3A). This strongly indicates that YB-1 mediates CCL5 expression after arterial injury. Of note, YB-1 in vascular lesions was expressed primarily in colocalization with α-smooth muscle actin–positive SMCs (Figure 3B, arrows); however, it was not colocalized with Mac-2+ macrophages (see arrow indicating neointima without containing in Figure 3C). This supports a predominant role of YB-1 in CCL5 expression by lesional SMCs after arterial injury.

Discussion
In the present study, we have demonstrated that YB-1 is a novel regulator of CCL5 expression. An increased activity of YB-1 in inflammatory SMCs and after arterial injury upregulates the expression of CCL5 and thereby controls CCL5-mediated monocyte adhesion and contributes to neointima formation in atherosclerosis-prone ApoE–/– mice. This provides the first evidence for the in vivo relevance of YB-1–dependent gene regulation in a model of vascular disease, namely, lesion formation after atherosclerotic conditions.

Whereas several synergistic activators of the CCL5 promoter have been described, including NF-κB, IRF (IFN regulatory factor)-3, and IRF-7, we have here for the first time identified YB-1 binding sites in the proximal CCL5 promoter region as a critical regulator of CCL5 transcription and protein expression in SMCs. Because YB-1 has been shown to synergistically interact with other transcription factors, including AP-2 and p65, a subunit of NF-κB, it is conceivable that beyond a direct activation, YB-1 might also transactivate and/or cooperate with other transcription factors in the regulation of CCL5 (eg, AP-1, NF-κB, and CCAAT enhancer binding protein, which are known regulators of CCL5 expression). It has been reported that YB-1 affects the expression of various proliferative and inflammatory proteins, such as granulocyte-macrophage colony–stimulating factor and matrix metalloproteinase-2, at both transcriptional and translational level.

Vascular expression and deposition of the chemokine CCL5 is a key force that drives atherosclerotic and neointimal lesion formation. The present data for the first time provide in vivo evidence that expression of YB-1 is upregulated after injury and that YB-1 serves as a strong activator of CCL5 expression in SMCs. YB-1 knockdown with shRNA led to a significant decrease in neointimal and medial hyperplasia after injury but also reduced macrophage infiltration.
Interestingly, these in vivo effects of YB-1 knockdown during the process of remodeling resemble findings obtained with injury-induced neointima formation in Met-RANTES–treated mice or in mice deficient in its receptor, CCR5.\textsuperscript{4,9,11} Moreover, in addition to SMCs, platelets, macrophages, and other cell types not affected by YB-1 knockdown but involved in the pathogenesis of vascular remodeling may be an additional source of CCL5.\textsuperscript{6,8} Because YB-1 can activate or repress CCL5 expression and actually inhibited the inflammatory secretion of CCL5 in macrophages, the effects of YB-1 on other cell types and cell functions involved in atherogenesis clearly need to be addressed. Initial experiments point toward an upregulation of CCL5 by YB-1 in T lymphocytes (U.R. and P.R.M., unpublished data, 2007).

Expression of YB-1 was found to be upregulated in infarcted areas of the heart\textsuperscript{39} and during mesangio proliferative renal disease, the latter being induced by platelet-derived growth factor (PDGF)-BB via a mitogen-activated protein kinase signal pathway in mesangial cells.\textsuperscript{16} Furthermore, \(\alpha\)-smooth muscle actin expression in lung myofibroblasts is increased by thrombin or transforming growth factor in a YB-1–dependent manner.\textsuperscript{40} Thrombin can also induce cleavage-related “activation” of YB-1 protein and its nuclear translocation in endothelial cells, which can subsequently upregulate the expression of PDGF-B.\textsuperscript{41} Triggered by exposure of subendothelial matrix after endothelial denudation injury, thrombotic events and thrombin formation might also contribute to the activation of YB-1 in the present model. Because PDGF-B is also expressed after arterial injury, and its inhibition is known to reduce neointima formation,\textsuperscript{42–44} it is not inconceivable that an autostimulatory loop of PDGF-B–mediated YB-1 activation may amplify expression of PDGF-B, in addition to its effects on CCL5 expression, and may thus contribute to enhanced neointimal hyperplasia after injury. In addition, because several chemokines, such as CCL2 and CXCL8, are nonredundantly involved in lesion formation,\textsuperscript{3} and taking into account that the YB-1–induced monocyte arrest on SMCs was not completely blocked by Met-RANTES, we hypothesized that YB-1 effects were not mediated exclusively through regulation of CCL5. However, experiments in CCR5\textsuperscript{−/−} or Met-RANTES–treated ApoE\textsuperscript{−/−} mice clearly revealed that YB-1 knockdown was ineffective in the absence of CCL5, so that the contribution of YB-1 to neointima formation appears to require CCL5 as a transcriptionally regulated mediator.

The capability of Met-RANTES to diminish diet-induced neointima formation after arterial injury in ApoE\textsuperscript{−/−} mice and the protective effects of genetic deficiency in the CCL5 receptor CCR5 during injury-induced neointimal hyperplasia, as well as in native and diet-induced atherosclerosis,\textsuperscript{8,10} have validated this chemokine as being of paramount importance in vascular pathophysiology and lesion formation. The present results identify a new and suitable target in the treatment of vascular disease and extend previous findings that suggested an interference with mechanisms that regulate the expression of CCL5.\textsuperscript{45} Given the unexpectedly selective dependence of YB-1 effects on CCL5, targeted downregulation of CCL5 might be achieved by a regional (ie, vascular) blockade of YB-1 in the context of neointimal lesion formation. shRNA-mediated knockdown of YB-1, as well as antibodies, small-molecule antagonists, and inactivation by proteases, may harbor feasible options for this approach, provided that suitable vehicles for local delivery are available that avoid systemic side effects.

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**Disclosures**

None.

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Atherosclerosis is an inflammatory process and the principal cause of cardiovascular disease. The progression of atherosclerosis and neointimal hyperplasia involves many mediators, such as cytokines and chemokines, which promote plaque growth through accumulation of macrophages and other immune cells at the inflamed vessel wall. One chemokine that has also been implicated, by genetic studies in humans, as playing a major role in this process is CCL5/Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES). By means of promoter studies, we identified Y-box binding protein (YB-1) as a new transcriptional regulator of CCL5. YB-1 clearly induced CCL5 expression in vascular smooth muscle cells, which entailed an increased monocyte arrest under flow conditions. We next used a mouse model of accelerated atherosclerosis to investigate the role of YB-1-regulated CCL5 in neointima formation. Our findings demonstrate that YB-1 is a potent mediator of neointimal plaque growth via upregulation of CCL5 expression. Immunofluorescence revealed a high YB-1 expression in neointimal and medial smooth muscle cells, which was associated with a high CCL5 expression. Furthermore, lentiviral transduction of YB-1 small interfering RNA after arterial wire injury significantly reduced neointima formation, which was primarily due to the reduced expression of CCL5. By identifying YB-1 as a novel target, our results might extend the therapeutic options for preventing postinterventional neointima formation at the molecular level. Thus, limiting the local expression of inflammatory mediators by RNA interference, antibodies, or small molecular antagonists directed against YB-1 may represent a suitable strategy to prevent restenosis after arterial angioplasty and stenting.
Y-Box Binding Protein-1 Controls CC Chemokine Ligand-5 (CCL5) Expression in Smooth Muscle Cells and Contributes to Neointima Formation in Atherosclerosis-Prone Mice

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