Induction of the MMP-14 Gene in Macrophages of the Atherosclerotic Plaque

Role of SAF-1 in the Induction Process

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Abstract—Based on epidemiological and pathological studies, it is becoming increasingly clear that matrix metalloproteinases (MMPs) play an important role in the pathogenesis of atherosclerosis by participating in vascular remodeling, smooth muscle cell migration, and plaque disruption. MMP-14, because of its unique ability to cause pericellular degradation, its broad substrate specificity, its synthesis in an active form, and its ability to activate other matrix metalloproteinases, is recognized as a prominent member of this family. MMP-14 is detected at high levels in the atherosclerotic plaque. To understand the induction mechanism of MMP-14 under atherogenic conditions, we examined its expression pattern in response to oxidized low-density lipoproteins (ox-LDLs) that are believed to play an important role in atherogenesis. We report that in macrophages, ox-LDLs markedly elevate the levels of MMP-14 mRNA and protein. The *cis*-acting elements supporting this increase were identified to be present within −213 and −1 nucleotides of the MMP-14 promoter. DNase I protection assay revealed, within this region, two major elements, of which one serves as the DNA-binding site for SAF-1 transcription factor. Increased binding of SAF-1 to the MMP-14 promoter correlated with the transcriptional upregulation of MMP-14 gene. Furthermore, induction of endogenous MMP-14 gene, MMP-14 promoter driven reporter gene expression and MMP-2 processing activity during overexpression of SAF-1 and coexpression of SAF-1 and MMP-14 in the macrophages present in the atherosclerotic plaque implicate SAF-1 as a key regulator of MMP-14 gene induction in macrophage cells. **(*Circ Res*. 2004;95:1082-1090.)**

Key Words: MMP-14 ■ SAF-1 ■ atherosclerotic plaques ■ oxidized LDL ■ macrophages

Pathology associated with acute coronary syndrome begins with the rupture of atherosclerotic plaques in which plaque composition, rather than the degree of stenosis, is a key factor.[1,2] Vulnerable plaques characteristically have a thin fibrous cap structure, a larger lipid-rich core, and an abundance of macrophage cells. The cap of the atherosclerotic plaque is composed of fibrous extracellular matrix, primarily consisting of collagen particles. Although it is debatable whether the thin fibrous cap structure is attributable to impaired synthesis or focal degradation of collagen, accumulating evidences appear to support the latter possibility. Matrix metalloproteinases (MMPs), a family of proteases are capable of degrading all components of extracellular matrix (ECM), as well as nonmatrix, components.[3] MMPs, usually undetectable in cells under normal conditions, are prominently expressed during many biological processes, including embryonic development and growth, tissue remodeling, bone development, wound healing, and the menstrual cycle.[3–5] However, excessive synthesis of MMPs is linked to many disorders, including atherosclerotic plaque formation and rupture, arthritis, tumor progression, periodontitis, and multiple sclerosis.[3–5]

Among the MMPs, MMP-14, also known as membrane-type matrix metalloproteinase-1 (MT1-MMP), plays an important role in localized degradation of ECM associated with plaque rupture because of its anchorage in the cell membrane,[6] broad substrate specificity,[7] ability to be secreted in an active form, and its involvement in the processing and activation of MMP-2[8] and MMP-13,[9] other members of the MMP family. MMP-2 has broad substrate specificity and is an effective gelatinase, whereas MMP-13 has collagenase activity. Recent evidence from MMP-14–null mice indicates that it is a major physiological collagenase.[10,11] MMP-14 was identified at high levels in atherosclerotic plaques[12] and at sites of vascular injury in balloon injured rat carotid arteries.[13]

ox-LDLs have been implicated in the pathogenesis of atherosclerosis because of their ability to change expression of many genes that are linked to cardiovascular diseases.[14,15] Although ox-LDLs and cytokines have been shown to induce MMP-14 expression in vascular endothelial cells,[16] the mech-

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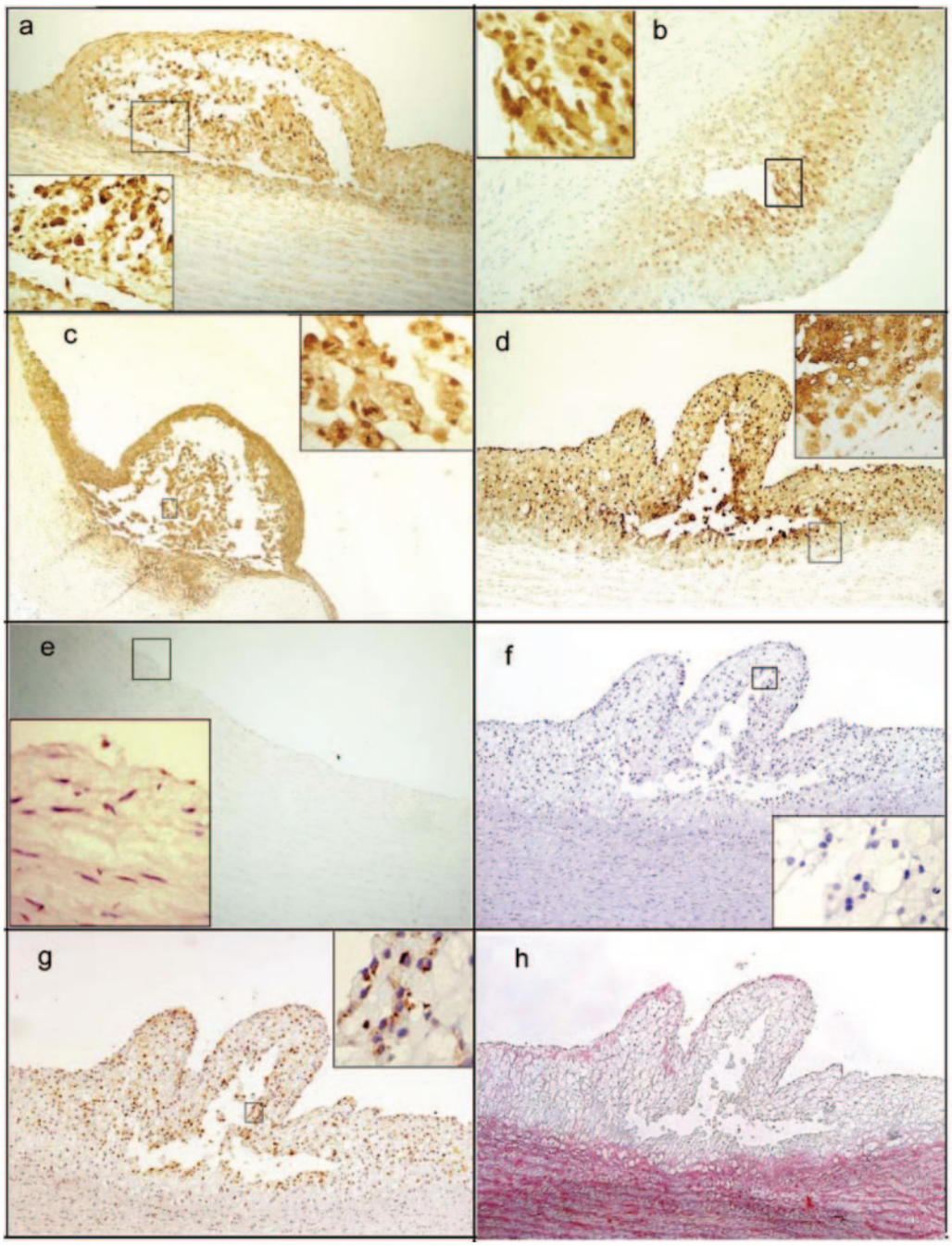

Figure 1. Increase of MMP-14 in the atherosclerotic plaques. a through e, Immunohistochemistry of atherosclerotic (a through d) and normal (e) porcine arteries with anti–MMP-14 antibody. f, Immunohistochemistry of a serial section of atherosclerotic artery shown in d with mouse nonspecific IgG (negative control). g, Immunohistochemistry of a serial section of atherosclerotic artery used in d with anti-SRA antibody to identify macrophage cells. h, Serial section of atherosclerotic artery used in d is stained with picosirius red to demonstrate the collagen level. Magnifications are ×20 and ×100 (inset).

anism of this process has not been elucidated. In this study, we examined whether monocyte/macrophage cells modify MMP-14 gene expression by responding to ox-LDLs, and, if so, how this process is regulated. We provide evidence that the SAF-1 transcription factor is a regulator of MMP-14 gene induction in monocyte/macrophage cells and SAF-1 and MMP-14 coexpress in the cells of atherosclerotic plaques. Together, these results identify a novel mechanism for the induction of MMP-14 under atherogenic conditions.

Materials and Methods

Cell Culture and Transfection

The human monocyte cell line THP-1 and smooth muscle cell HIVS-125 were obtained from American Type Culture Collection and maintained according to the protocol of the supplier. For transient transfection, THP-1 cells were incubated with reporter plasmid DNA and varying concentrations of pcD-SAF1, pcDNA3, pcDSAF-1(mt), or pCMV-revSAF-1 expression plasmids, as indicated, and then exposed to ox-LDL (100 μg/mL). To measure transfection efficiency, 1 μg of pSV-β-gal DNA (Promega) was cotransfected in each assay. Cell

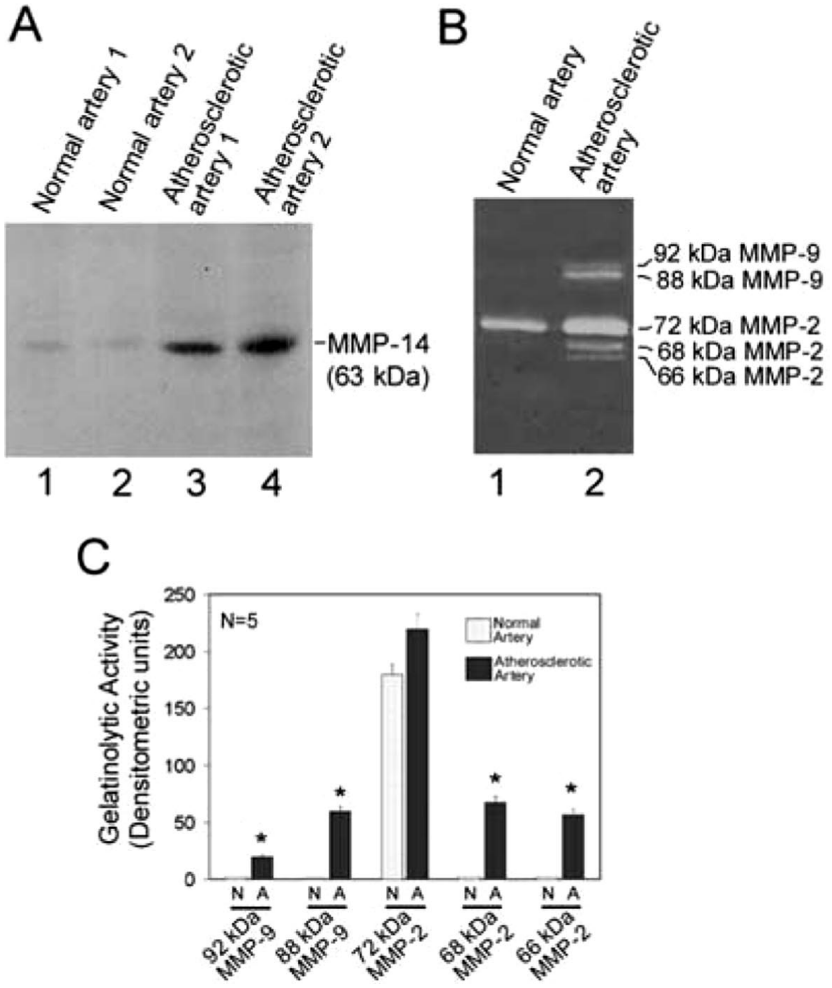

Figure 2. Atherosclerotic tissues express higher levels of MMP-14. A, Equal amount of tissue extracts (50 μg of protein/lane) from normal (lanes 1 and 2) and atherosclerotic (lanes 3 and 4) arteries of pigs were immunoblotted using anti–MMP-14 antibody. B, Increased gelatinolytic activity in atherosclerotic tissues. Fifty micrograms of protein/lane from normal (lane 1) and atherosclerotic (lane 2) pig arteries were subjected to gelatin zymography. C, Densitometric analysis of the zymogram. Results represent mean±SE of five separate experiments. *$P<0.05$ compared with normal tissue.

extracts containing equivalent amounts of β-galactosidase activity were used for the CAT assay as described earlier.[17,18] Serum-free conditioned medium of HIVS-125 cells was centrifuged to remove floating cells and used as the source of 72-kDa MMP-2 protein.

Preparation of Ox-LDL

ox-LDL was prepared essentially as previously described.[19] Native human LDL (Sigma Chemical Co) was incubated with $CuSO_4$ (5 μmol/L) at 37°C for 24 hours, and the conjugated diene content was determined by measuring absorbance at 234 nm.[20] Oxidation of LDL was stopped by the addition of EDTA and ox-LDL was concentrated by ultracentrifugation, dialyzed against 0.15 mol/L NaCl/0.5 mmol/L EDTA, pH 8.0, and filter sterilized before use. Endotoxin level in all preparations of LDL and ox-LDL was measured using a BioWhittaker QCL-1000 colorimetric assay kit.

Cell Viability

Viability of THP-1 cells after exposure to native LDL and ox-LDL was determined by trypan blue exclusion assay. THP-1 cells in a 6-well plate (5×10^5 cells/well) were grown in the absence or presence of native LDL and ox-LDL (50 and 100 μg/mL) in RPMI-1640 containing 10% fetal calf serum. After 48 hours, viable cell number was counted using trypan blue exclusion method.

Plasmid Construction

CAT reporter plasmids containing progressive deletions of the mouse *MMP-14*[21] promoter were constructed by PCR amplification and cloning these segments in pBLCAT3 vector.[22] 0.2MMP-14CAT reporter plasmid contains −213 to +149 of MMP-14 promoter ligated into pBLCAT3 vector. The 0.2mutMMP-14CAT reporter contains altered SAF-1 element at −122 to −77 position of MMP-14 promoter. The sequence of this region is GTCTCTACCGAACAACTAGGTCATTGTGTCGCGTTCGGACGATAGG. Underline base represents altered sequence. The pcDSAF-1 and pcDSAF-1(mutant) plasmids were prepared by cloning a full-length SAF-1 cDNA[17] and out of frame SAF-1 cDNA in pcDNA3 vector (Invitrogen), respectively. The pCMV-RevSAF-1 plasmid contains a full-length SAF-1 cDNA in reverse orientation, which expresses an antisense RNA to SAF-1 mRNA, resulting reduction of SAF-1 protein synthesis.[23]

Nuclear Extracts, DNase I Footprinting, and Electromobility Shift Assay

Nuclear extracts from THP-1 cells and were prepared as described previously,[17,24] and protein concentration was measured by the Bradford method.[25] For DNase I footprint assay, MMP-14 DNA (−213/+1) was radiolabeled at one end and incubated with nuclear extracts, and DNase I–protected regions were determined as described earlier.[17] Radiolabeled DNAs containing MMP-14 promoter, −140 to +1 and −213 to −140, were used in the electromobility shift assay, performed as described previously.[17] Competitor oligonucleotides were added at 100-fold molar excess of the probe DNA. Homologous oligonucleotide contained the same sequence as the probe. In some assays, anti–SAF-1 antibody[23] (1 μL of a 10-fold diluted antibody) was added during a 30-minute preincubation on ice.

RNA Isolation and Northern Blot and Western Blot Analysis

THP-1 cells incubated with different concentrations of native LDL or ox-LDL for either 48 hours or different lengths of time were used for

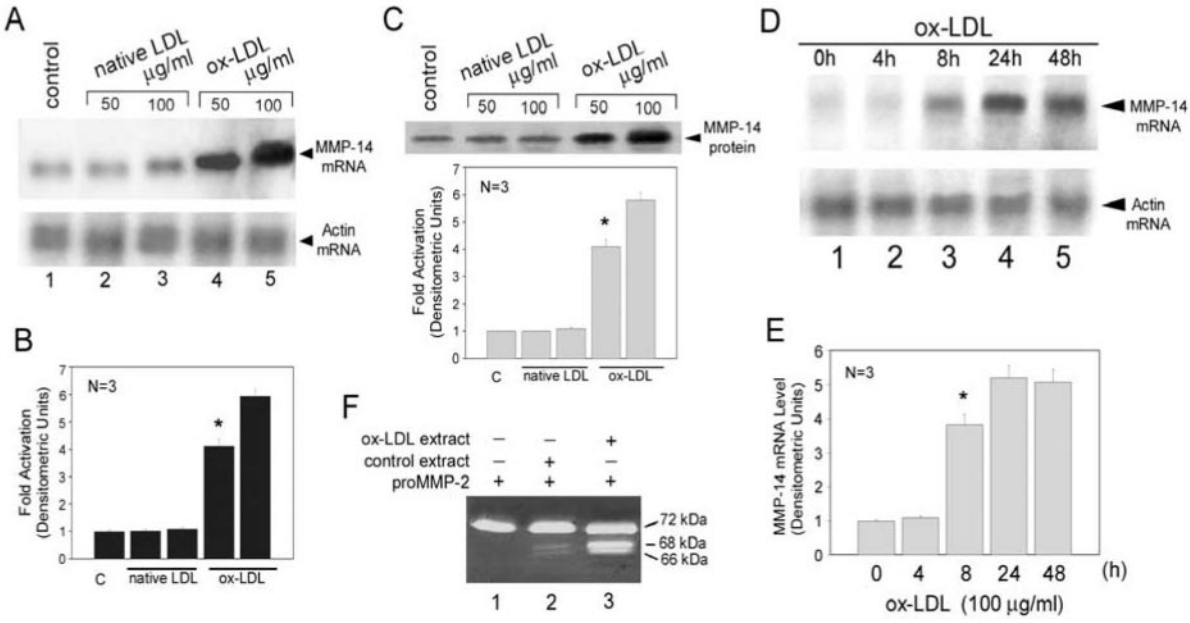

Figure 3. Ox-LDL induces MMP-14 mRNA level. A, THP-1 cells were incubated in the absence (lane 1) or presence of different concentrations of native LDL (lanes 2 and 3) or ox-LDL (lanes 4 and 5) for 48 hours, as indicated. RNA (50 μg/lane) was subjected to Northern analysis using MMP-14 cDNA probe. The same membrane was stripped and rehybridized with actin cDNA probe. B, Densitometric analysis of Northern blots showing the effects of native LDL and ox-LDL for 48 hours. Results represent mean±SE of three separate experiments. *$P<0.05$ compared with untreated cells. C, Densitometric analysis of Western blots showing the effects of native LDL and ox-LDL on MMP-14 protein level. The blot on top represents three separate experiments in which 50 μg of proteins/lane were immunoblotted using anti–MMP-14 antibody. Results represent mean±SE of three separate experiments. *$P<0.05$ compared with normal tissue. D, RNA (50 μg/lane) isolated from 100 μg/mL of ox-LDL–treated THP-1 cells for different periods of time was subjected to Northern blot analysis using MMP-14 cDNA probe. The same membrane was rehybridized with actin cDNA probe. E, Densitometric analysis of the Northern blots as shown in D. Results represent mean±SE of three separate experiments. F, Equal protein amount of THP-1 cell membrane fractions of untreated (lane 2) and 48-hour ox-LDL–treated (lane 3) cells were incubated for 60 minutes at 37°C with conditioned medium of HIVS-125 cells, which was the source of 72-kDa MMP-2. Lane 1 contains conditioned medium of HIVS-125 cells only. The products were subjected to zymography.

preparing total RNA by as described.[26] Fifty micrograms of RNA was loaded in each lane and the blot was hybridized to a radiolabeled MMP-14 cDNA probe. Equal amounts of proteins prepared from THP-1 cells or tissues were separated by 11% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted using anti–MMP-14 antibody.

Membrane Preparation, MMP-2 Processing, and Gelatin Zymography

Plasma membrane from control and ox-LDL–treated THP-1 cells was prepared as described.[27] Equal protein of membrane fraction was incubated with serum-free conditioned medium of HIVS-125 cells (source of 72 kDa MMP-2 protein) at 37°C for 60 minutes before gelatin zymography. Samples normalized for protein content were applied to 11% SDS-PAGE containing 0.1% gelatin. After electrophoresis, SDS was removed by dialysis with 2.5% Triton X-100 buffer (2 changes, 30 minutes each) and incubated in 50 mmol/L Tris (pH 7.5), 10 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, 1.25% Triton X-100 buffer overnight at 37°C. Gels were stained with coomassie blue.

Atherosclerotic Tissue Sections

Yucatan pigs (n=16) (Charles River Laboratories Inc, Me, and Sinclair Research Center, Inc, Columbia, Mo) between 9 and 12 months, divided in two groups, were fed either Purina mini-pig chow or high-fat diet, consisting of mini-pig chow supplemented with 2% cholesterol, 17.1% coconut oil, 2.3% corn oil, and 0.7% sodium cholate.[28] After 20 weeks, animals were euthanized and samples of the carotid artery were fixed in neutral buffered 10% formalin and embedded in paraffin. All animals were handled according to the protocols and guidelines approved by the Animal Care and Use Committee at the University of Missouri.

Immunohistochemical Analysis

Serial sections of carotid arteries were subjected to immunohistochemical staining for MMP-14, SAF-1, and SRA proteins. Tissue sections (5 μmol/L) were deparaffinized with xylene, followed by immersion in graded ethanol. Slides were incubated in sodium citrate buffer at 100°C for 30 minutes to unmask the antigens and blocked with 4% goat serum at 37°C for 1 hour. The slides were incubated with anti–MMP-14 antibody (1:250 dilution; Santa Cruz Biotechnology), anti–SAF-1 antibody[23] (1:750 dilution), anti-SRA antibody (1:100 dilution; SRA-E5, Cosmo Bio) or preimmune serum as the negative control at 4°C for 16 hours. Next, slides were washed twice in PBS plus 0.05% Tween 20 and incubated with secondary antibody followed by detection using the horseradish peroxidase method and counterstained with hematoxylin solution.

Picosirius Red Staining for Collagen Content

Arterial sections were incubated with 0.1% sirius red in saturated picric acid for 90 minutes to identify collagen types I and III. Sections were rinsed twice with 0.1 N HCl for 1 minute, immersed in distilled water, dehydrated with 70% ethanol for 1 minute, and viewed under polarized light or without polarization.

Results

MMP-14 Is Present in the Lesions of Atherosclerotic Plaques

Typical advanced atherosclerotic lesions were selected from carotid arteries (n=16) of Yucatan pigs fed a high-fat diet, rich in cholesterol for 20 consecutive weeks (Figure 1). As seen in the Figure 1a through 1d, high level of MMP-14 protein is present in the core of the plaques and adjacent

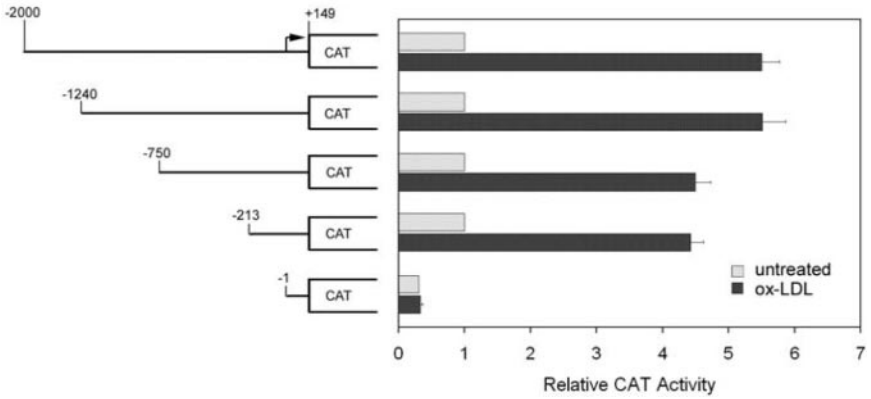

Figure 4. Mapping of the ox-LDL response elements in mouse *MMP-14* promoter. THP-1 cells transfected with 1 μg of reporter plasmids containing progressively deleted *MMP-14* promoter were incubated with or without 100 μg/mL of ox-LDL for additional 48 hours. CAT activity was determined as described in Materials and Methods. The result represents an average of three independent experiments.

surrounding areas. In contrast, MMP-14 immunostaining is negligible outside the lesion area (Figure 1a through 1d) and also in the normal artery (Figure 1e). Nonspecific IgG used in place of primary antibody showed no background or nonspecific staining (Figure 1f). Immunostaining with anti-SRA antibody, a macrophage-specific marker, showed that MMP-14 protein is most abundant in the macrophages (Figure 1g), but endothelial cells and smooth muscle cells also exhibited increased MMP-14 expression. Picosirius red staining of collagen indicated the presence of a thin fibrous cap (Figure 1h).

In correlation with immunohistochemistry, MMP-14 protein level was much higher in atherosclerotic arteries of high-fat diet fed pigs (Figure 2A). Gelatin zymography showed that atherosclerotic tissue extract contains higher levels of 68 kDa and 66 kDa, two processed forms of MMP-2 protein, which could result from increased MMP-14 proteinase activity (Figure 2B, lane 2). Furthermore, gelatinolytic activity migrating at 92 kDa, and 72 kDa positions representing MMP-9 and MMP-2 proteins also were much higher in atherosclerotic tissue (Figure 2B and 2C).

MMP-14 Expression in Monocyte/Macrophage Cells

Abundance of MMP-14 in the macrophages of atherosclerotic lesions suggested increased synthesis of this protease. During hyperlipidemia, oxidative modification of the entrapped LDL in the artery wall is critical in inducing all components of atherosclerotic reaction because of their unique ability to modulate and alter gene expression.[29] To understand the induction mechanism of MMP-14 under atherogenic conditions, THP-1 monocyte/macrophage cells that are well characterized and retained the ability to form foam cells were used.[30] Neither native LDL nor ox-LDL had any adverse effect on cell growth and viability. Incubation of THP-1 cells with 50 and 100 μg/mL concentrations of ox-LDL showed more than 98% and 94% viable cells, respectively. The quality of all preparations of native LDL and ox-LDL was also assessed by measuring endotoxin level, which ranged from 0.000 to 0.005 pg/μg, that was considered to be negligible. Both MMP-14 mRNA (Figure 3A) and protein levels (Figure 3C) were considerably induced by ox-LDL in a dose-dependent manner. Native LDL had no effect on MMP-14 induction (Figure 3A, lanes 2 and 3; Figure 3C, lanes 2 and 3). The level of increase was nearly 6-fold in response to 100 μg/mL ox-LDL (Figure 3B and 3C). MMP-14 mRNA was induced within 8 hours of ox-LDL addition, which reached a peak level at around 24 hours (Figure 3D and 3E). To determine whether increase in the

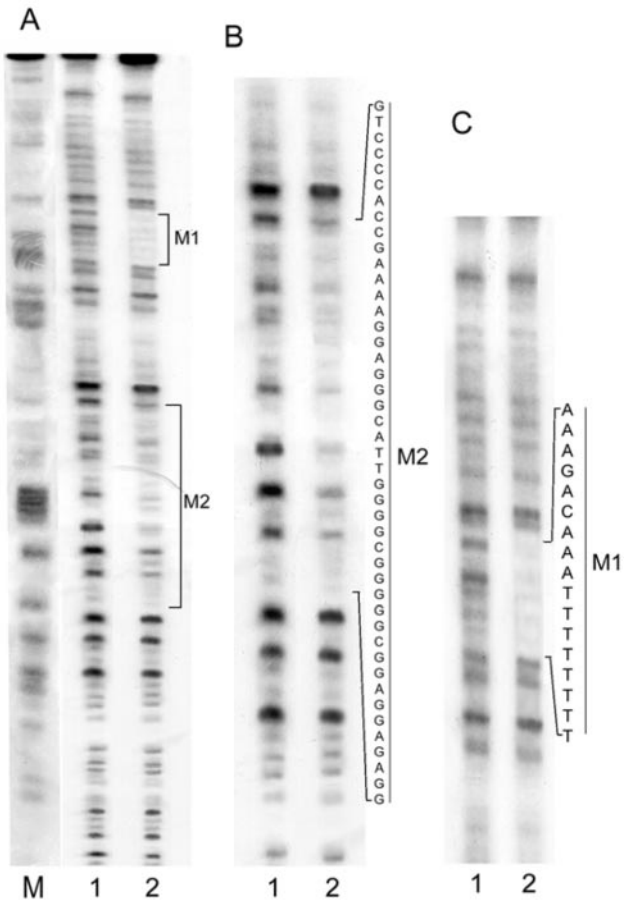

Figure 5. Identification of protein-binding domain in *MMP-14* promoter. A, Single 5′-end–labeled MMP-14 DNA fragment (−213 to +1) was incubated under conditions for DNA-binding assay without (lane 1) or with (lane 2) ox-LDL–treated THP-1 cell nuclear extract. DNA-protein complexes were incubated with DNase I, and the resultant fragments were fractionated in a 10% sequencing gel containing 8 mol/L urea. Two protected regions, M1 and M2, are identified. Lane M contains nucleotide size markers that determine the nucleotide positions of the protected region. B, Protected sequences in the M2 region is identified. C, Protected sequences in the M1 region is identified.

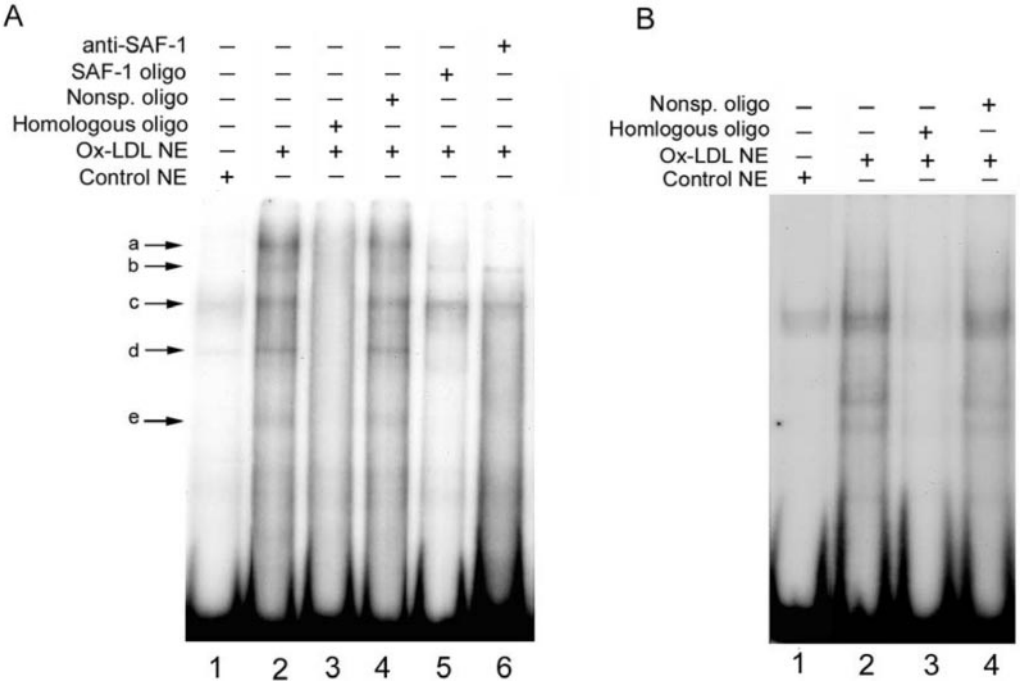

Figure 6. A, SAF-1 transcription factor interacts with M2 element of MMP-14 promoter. Ten micrograms of nuclear extracts from untreated (lane 1) or ox-LDL–treated (lanes 2 through 6) THP-1 cells were incubated with a ^{32}P-labeled *MMP-14* (−140/+1) DNA. In addition, lanes 3, 4, and 5 contained 100-fold molar excess of homologous probe, nonspecific oligonucleotide, or SAF-1 oligonucleotide, respectively. Lane 6 contains anti–SAF-1 antibody. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel. DNA-protein complexes are designated as a through e. B, Interaction of transcription factors with the M1 element of *MMP-14* promoter. A ^{32}P-labeled *MMP-14* DNA (−213/−140) was incubated with10 μg of nuclear extracts prepared from untreated cells (lane 1) or ox-LDL–treated THP-1 cells (lanes 2 to 4). In addition, lanes 3 and 4 contain 100-fold molar excess of homologous probe or nonspecific oligonucleotide, as indicated. Three DNA-protein complexes are designated as a, b, and c. Nonsp. indicates nonspecific, NE, nuclear extract.

MMP-14 protein level corresponds to an increase in the MMP-14 enzymatic activity, we sought direct evidence by assaying the conversion of 72 kDa MMP-2 protein. Equal protein amount of membrane fractions of untreated and ox-LDL–treated THP-1 cells were incubated with conditioned medium obtained from HIVS-125 cells, which are known to constitutively produce and release 72 kDa MMP-2 in the culture medium. Ox-LDL–treated THP-1 cells produced higher levels of processed MMP-2 proteins compared with the untreated cell membrane extract (Figure 3F, compare between lanes 2 and 3).

Mapping of Ox-LDL Responsive Regulatory Elements of MMP-14 Promoter

The above studies indicated that ox-LDL induces *MMP-14* expression in the macrophage cells. To understand the induction mechanism, we sought to identify the ox-LDL responsive regulatory elements of *MMP-14* promoter. We used a series of constructs containing progressively deleted promoter regions of mouse *MMP-14*[21] cloned in the pBLCAT3 vector[22] and examined their response to ox-LDL. Expression of 2.0MMP-14 CAT reporter that contained 2.0-kb sequences of *MMP-14* was induced more than 5-fold by ox-LDL (Figure 4). Although deletion of upstream sequences up to −213 nucleotides had virtually no adverse effect on ox-LDL–mediated induction, a significant loss of activity was observed when further deletion up to −1 nucleotide was made. These results suggested that ox-LDL responsive promoter is likely to be present within −213 to −1 nucleotides of *MMP-14*.

Identification of Nuclear Factor–Interacting Elements in MMP-14 Promoter

To locate the DNA elements within −213 to −1 region of *MMP-14* where nuclear factor(s) might interact, DNase I protection assays using ox-LDL–treated nuclear extracts were performed (Figure 5A). Two DNase I–protected regions, designated as M1 (−183 to −166) and M2 (−122 to −77), were identified (Figure 5B and 5C) as the promoter elements of *MMP-14* involved in regulating ox-LDL–mediated induction of *MMP-14*.

Identification of Inducible Nuclear Factors in Ox-LDL–Treated THP-1 cells

To identify the transcription factor(s) interacting at M1 and M2 elements, we performed electromobility shift assay. Using the M2 element (−122/−77) as probe, two faint DNA-protein complexes, c and d, were seen to be formed by nuclear extracts of untreated THP-1 cells (Figure 6A, lane 1). Equal amount of ox-LDL–treated nuclear extracts, however, formed several prominent complexes, designated as a through e (Figure 6A, lane 2). Moreover, the levels of c and d complexes were much higher in lane 2 as compared with those in lane 1. These DNA-protein complexes were efficiently eliminated by homologous oligonucleotide (Figure 6A, lane 3) but not by a nonhomologous oligonucleotide

(Figure 6A, lane 4), indicating specific interaction of nuclear factors. These results indicated that multiple nuclear factors are able to interact with the M2 element of MMP-14 promoter and, most importantly, the factors present in ox-LDL–activated cells are either distinctly different or present at a different level in untreated THP-1 cells. The database search showed that M2 element, containing GGGCGGGGGCG-GAGGAGAGG sequences, is quite similar to an extended DNA-binding element of SAF-1 transcription factor,[17] which is known to be activated by minimally modified LDL.[31] Thus, a consensus SAF-binding oligonucleotide was added as a competitor, which inhibited formation of complexes a, d, and e, but not complexes b, and c (Figure 6A, lane 5), indicating that complexes a, d, and e contain SAF. Similar result was obtained when anti–SAF-1 antibody was used (Figure 6A, lane 6) indicating that SAF-1 can specifically interact with the M2 element of *MMP-14* promoter.

To evaluate interaction with the M1 element, ^{32}P-labeled MMP-14 DNA (−213 to −140) was incubated with equal amounts of untreated and ox-LDL–treated THP-1 nuclear extracts. Three major DNA-protein complexes appeared with ox-LDL–treated nuclear extract (Figure 6B, lane 2), of which only one, although at a much lower level, was formed by untreated cell nuclear proteins (Figure 6B, lane 1). These complexes were inhibited by homologous oligonucleotide (Figure 6B, lane 3) but not by a nonhomologous oligonucleotide (Figure 6B, lane 4). Anti–SAF-1 antibody had no effect on these complexes, indicating absence of its involvement (data not shown).

Overexpression of SAF-1 Stimulates MMP-14 Expression

The above results suggested that at least part of the ox-LDL–mediated induction of MMP-14 may be mediated by SAF-1. To test this possibility, THP-1 cells were transfected with a SAF-1 expression plasmid, which increased transcription from *MMP-14* promoter in a dose-dependent manner (Figure 7A). Empty vector pcDNA3 or pcDSAF-1(mt), which does not express any SAF-1 protein, showed no stimulatory effect. Consistent with our hypothesis, overexpression of SAF-1 increased the level of endogenous MMP-14 protein (Figure 7B). Furthermore, conditioned medium of SAF-1–overexpressing THP-1 cells exhibited higher levels of gelatinolytic activities migrating at 92 kDa, 72 kDa, and 68 kDa positions than vector-transfected cells (Figure 7C). The *MMP-14* promoter was also induced by interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Figure 7D). However, stimulatory effect of ox-LDL, IL-1β, or TNF-α on *MMP-14* promoter was markedly reduced but not completely inhibited when an antisense SAF-1 was present (Figure 7D). For further verification, we specifically mutated SAF-1 DNA-binding element in *MMP-14* promoter. The 0.2mutMMP-14CAT was induced albeit at a much lower level with ox-LDL, IL-1β or TNF-α, but this induction was not eliminated by antisense SAF-1 plasmid (Figure 7D). These results indicated that although SAF-1 is a potential mediator of ox-LDL, IL-1β, or TNF-α action, other elements are also involved in this process.

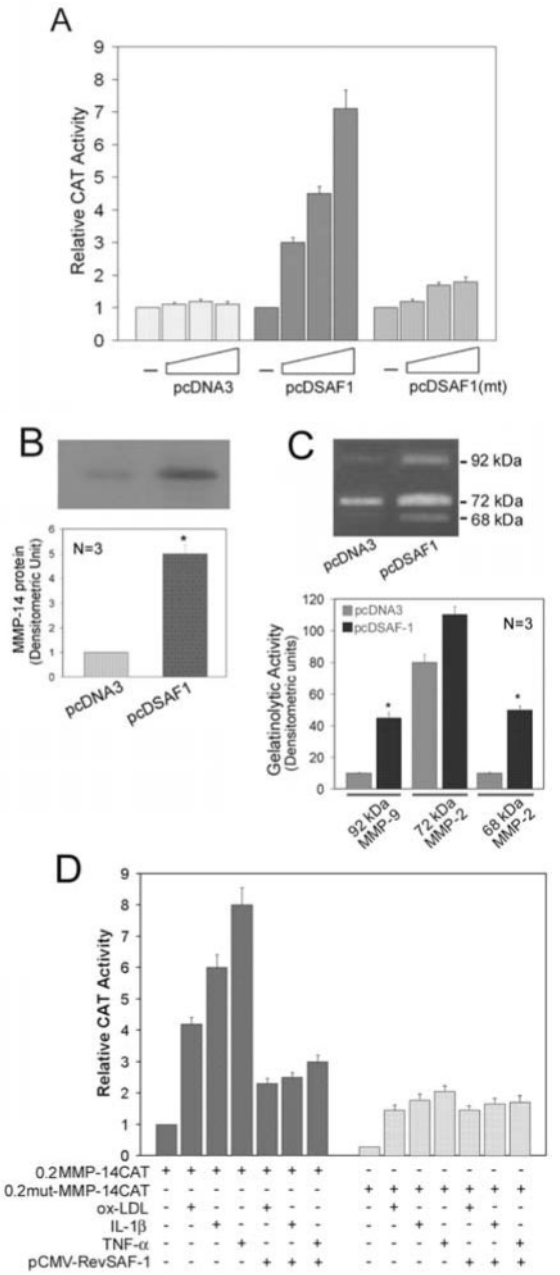

Figure 7. Stimulation of MMP-14 by ectopic expression of SAF-1. A, THP-1 cells were transfected with 1.0 μg of 0.2MMP-14CAT reporter plasmid alone (first column) or together with increasing concentrations of pcDNA3 (empty vector), pcDSAF-1, or pcDSAF-1(mt) expression plasmid DNAs. CAT activity was determined as described in Materials and Methods. The result represents an average of three independent experiments. B, Densitometric analysis of Western blots from THP-1 cells transfected with pcDNA3 or pcDSAF-1 plasmid. The blot on top represents an experiment in which 50 μg protein/lane were fractionated and immunoblotted using anti–MMP-14 antibody. Results represent mean±SE of three separate experiments; *$P<0.05$. C, Densitometric analysis of zymogram using 20 μL of serum-free conditioned medium of pcDNA3 or pcDSAF-1 transfected THP-1 cells. Results represent mean±SE of three separate experiments; *$P<0.05$. D, THP-1 cells were transfected with 1.0 μg of 0.2MMP-14CAT or 0.2mutMMP-14CAT reporter plasmids either alone or with 1.0 μg of pCMV-RevSAF-1 plasmid DNA. In some assays, ox-LDL (100 μg/mL), IL-1β (200 U/mL), or TNF-α (1000 U/mL) were added. The result represents an average of three independent experiments.

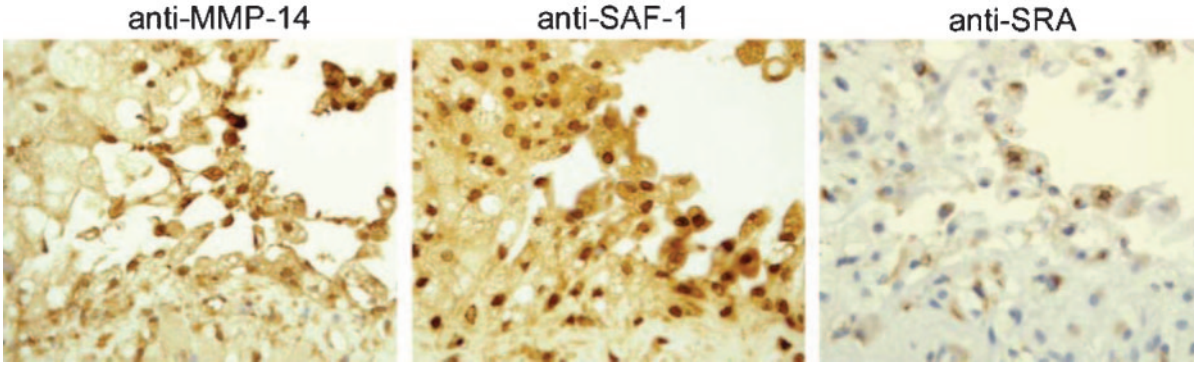

Figure 8. Coexpression of SAF-1 and MMP-14 in atherosclerotic carotid artery. Serial sections of pig carotid artery were immunostained with anti–MMP-14 antibody (a; magnification ×100), anti–SAF-1 antibody (b; magnification ×100), and anti-SRA antibody (c; magnification ×100).

Coexistence of MMP-14 and SAF-1 in Atherosclerotic Plaques

Regulation of MMP-14 by SAF-1 raised the possibility that these proteins are coexpressed in the atherosclerotic plaques. Immunohistochemistry of serial sections of atherosclerotic tissues showed that the same group of cells express high level of both proteins (Figure 8). Using anti-SRA antibody, the MMP-14– and SAF-1–expressing cells were identified predominantly as macrophages.

Discussion

We report a novel mechanism that delineates induction of *MMP-14* in response to oxidatively modified LDLs, an atherogenic factor involved in the development of atherosclerotic lesions. Our study shows that SAF-1 transcription factor plays a functional role in this process. This conclusion was supported by the evidence of (1) increase of MMP-14 promoter activity, increased endogenous MMP-14 protein level and increased processing of 72 kDa MMP-2 during ectopic expression SAF-1 in the monocyte/macrophage cells; (2) increased interaction of SAF-1 with *MMP-14* promoter in response to ox-LDL signals; and (3) increased coexpression of SAF-1 and MMP-14 in the lesions of atherosclerotic plaques.

Although the role of MMP-14 in the pathogenesis of cardiovascular disease is becoming increasingly clear, the activation mechanism under atherosclerotic conditions and the identity of participating factors are obscured. We demonstrate here that an atherogenic agent, ox-LDL, induces MMP-14 in monocyte/macrophage cells, a major cell type of the atherosclerotic plaque (Figure 3). The elements regulating ox-LDL–mediated induction are present within the first 213 nucleotides of *MMP-14* promoter (Figure 4) and two major elements in which transcription factors interact were located (Figure 5). We show that ox-LDL–stimulated SAF-1 transcription factor interacts with one of these elements (Figure 6A) and SAF-1 overexpression stimulates expression from *MMP-14* promoter in macrophage cells (Figure 7A), resulting in increasing endogenous MMP-14 protein level (Figure 7B) and 72-kDa MMP-2 to processed form of MMP-2 conversion activity (Figure 7C). Mutation of the SAF-1 element in *MMP-14* promoter or coexpression of antisense SAF-1 mRNA markedly reduced ox-LDL–, IL-1β–, and TNF-α–mediated induction of the MMP-14 gene (Figure 7D). Further, we show that SAF-1 and MMP-14 are coexpressed in the lesions of atherosclerotic plaque (Figure 8).

SAF-1 is a member of a recently identified family of transcription factors containing six Cys2-His2 type zinc fingers.[17] Many inflammatory agents, including cytokines,[32] lipopolysaccharide,[33] and minimally modified-LDL,[31] markedly increase the DNA-binding and transactivational functions of SAF-1. Phosphorylation of SAF-1 by protein kinases, including mitogen-activated protein kinase[34] and protein kinase C,[35] is critical for its activation. Activation of SAF-1 by different signaling pathways demonstrates its ability to respond to different inflammatory conditions. The typical SAF-1 DNA-binding element is rich in purine or pyrimidine sequences. Interestingly, several other transcription factors, including Sp1[36] and Egr-1,[37] are able to interact with purine- or pyrimidine-rich sequences, and occasionally a Sp1 or Egr-1 element is found embedded in the SAF-1 DNA-binding element of many promoters. In the mouse MMP-14 gene, the region identified as SAF-1 DNA-binding element (Figure 4B) is also shown to be a DNA-binding site of Sp1[38] and Egr-1.[21] The Sp1-binding site was found to be essential in regulating the basal expression of human MMP-14 gene[38] and negatively regulating shear stress–mediated inhibition of mouse *MMP-14* expression in endothelial cells.[39] In contrast, Egr-1 is identified as a positive regulator of extracellular matrix driven and cyclic strain–mediated induction of mouse *MMP-14* expression in endothelial cells.[21,40] The roles of Egr-1 and Sp1, although not directly studied in this report, could not be ignored, especially in light of the observation that mutation of the SAF-1 element does not completely abolish ox-LDL–, IL-1β–, and TNF-α–mediated induction of *MMP-14* promoter (Figure 7D). Further, the M1 element of *MMP-14* promoter could interact with Oct-1 (consensus binding element, AAGACAAAT) and CIZ/Nmp4 transcription factors (consensus binding element, G/CAAAAAA). Of these two factors, CIZ/Nmp4 is of particular interest because this protein is reported to interact with the promoters of type I collagen α1 (I) polypeptide chain[41] and MMP-1, MMP-3, and MMP-7 genes.[42] What role this family of proteins plays in regulating MMP-14 expression is currently being investigated.

In summary, we have identified a novel enhancer region that binds SAF-1 and is essential for ox-LDL–, IL-1β–, and TNF-α–mediated induction of *MMP-14* in monocyte/macrophage cells. Future studies in vivo using wild-type and SAF-1–deficient mice should enable us to determine the precise role of SAF-1 in augmenting MMP-14 synthesis and its role in atherosclerosis.

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