Role of Nuclear Factor-κB Activation in Metalloproteinase-1, -3, and -9 Secretion by Human Macrophages In Vitro and Rabbit Foam Cells Produced In Vivo

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Abstract—Metalloproteinase secretion by macrophages is believed to play a key role in the matrix degradation that underlies atherosclerotic plaque instability and aneurysm formation. We studied the hypothesis that nuclear factor-κB (NF-κB), a transcription factor, is necessary for metalloproteinase secretion and, hence, is a target for pharmacological intervention. Adenovirus-mediated gene transfer of the inhibitory NF-κB subunit, I-κBα, was achieved into human monocyte-derived macrophages in vitro and into foam cells produced in vivo in cholesterol-fed rabbits. Human macrophages and rabbit foam cells secreted matrix-degrading metalloproteinase (MMP)-9 without further stimulation, and this was not inhibited by I-κBα (11±16% and 8±10%, respectively; $P>0.05$). MMP-1 secretion from human macrophages increased in response to recombinant human CD40 ligand and was inhibited 92±5% by I-κBα (n=3, $P<0.05$). Rabbit foam cells secreted MMP-1 and -3 without further stimulation, and this was inhibited 83±12% and 69±11%, respectively, by I-κBα (n=6 or 7, $P<0.001$). I-κBα did not significantly affect the expression or activity of tissue inhibitor of metalloproteinases-1 or -2. Overexpression of I-κBα inhibited collagenolytic and β-caseinolytic activity by 42±2% and 41±7%, respectively (n=3, $P<0.05$). Secretion of MMP-1 and MMP-3 from macrophages stimulated in vitro or in vivo depends on the activation of NF-κB. Because the inhibition of NF-κB reduces proteolytic activity, it appears to be an attractive pharmacological target in unstable atheromas. **(*Arterioscler Thromb Vasc Biol*. 2002;22:765-771.)**

Key Words: macrophages ■ foam cells ■ metalloproteinases ■ nuclear factor-κB

Rupture of the fibrous cap of the atherosclerotic plaque is a key event in triggering coronary thrombosis and acute coronary syndromes. Two characteristic pathological features of the rupture-prone plaque are increased macrophage density and reduced collagen content, associated with reduced tensile strength.[1,2] Overexpression of several matrix-degrading metalloproteinases (MMPs), including MMP-1, MMP-3, and MMP-9, has been demonstrated in human atherosclerotic plaques and in animal models[3,4] and is particularly associated with macrophages. Therefore, excessive production of macrophage-derived MMPs probably links increased macrophage density and loss of collagen. Consequently, inhibition of MMP production from macrophages could prove a valuable strategy for promoting plaque stability. Our recent work[5,6] has shown that inhibiting nuclear factor-κB (NF-κB), a transcription factor, prevents upregulation of MMP-1, MMP-3, and MMP-9 secretion from fibroblasts and vascular smooth muscle cells (SMCs). However, it is unknown whether this strategy would also reduce MMP secretion from macrophages. To investigate this possibility, we subjected human monocyte-derived macrophages to adenovirus-mediated overexpression of the inhibitory subunit, I-κBα, which has been shown previously to inhibit the secretion of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 from macrophages.[7] Although several available agents, including aspirin, statins, fibrates, and thiazolidinediones, probably exert part of their effects though reducing NF-κB activity, there are presently no highly selective inhibitors that can be used in vivo. As a surrogate to evaluate the likely impact of NF-κB inhibition in vivo, we extended our study of I-κBα overexpression to the established model[8] of foam cells elicited in cholesterol-fed rabbits.

Methods

Reagents

Recombinant human CD40 ligand (rhCD40L) was obtained from Alexis. Sheep polyclonal anti-rabbit MMP-1 and MMP-3 antibodies were a generous gift from Dr G. Murphy, University of East Anglia, Norwich, UK. Recombinant adenovirus capable of overexpressing the porcine I-κBα gene (rAd:IκBα) was a kind gift from Dr Rainer De Martin, Department of Vascular Biology and Thrombosis Research, University of Vienna, Vienna, Austria.[9] Recombinant adenoviruses capable of overexpressing the bacterial *Lac*Z gene (rAd:βgal)

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and a control virus with a silent expression cassette (rAd:null) were kind gifts from Dr G. Wilkinson, University of Wales College of Medicine, Cardiff, UK.[10] All other reagents were purchased from Sigma Chemical Co unless otherwise stated.

Tissue Culture

Monocytes were isolated from peripheral blood donated from healthy volunteers by density centrifugation with the use of Nycoprep (Nycomed) according to the manufacturer's instructions. Cells were resuspended in macrophage-full medium (2 g/L bicarbonate-buffered RPMI 1640 media supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 10% [vol/vol] FCS, and 100 ng/mL macrophage colony stimulating factor [MCSF]) and counted via a Neubauer hemocytometer. Cell viability was determined by trypan blue exclusion, and monocyte lineage was confirmed by immunocytochemistry for CD68 expression after cytocentrifugation. A 40-mL whole-blood donation yields 5 to 10×10^6 monocytes by this method. Monocyte purity was routinely $\geq$95%, as assessed by cytocentrifugation and analysis for CD68 expression (clones KP1 and PG-M1, Dako) by immunocytochemistry. Monocytes were cultured in macrophage-full medium for 10 to 14 days to allow maturation/differentiation to macrophages and were then referred to as monocyte-derived macrophages. Conditioned media were generated by incubating cells at 5×10^5 per well in 24-well plates in serum-free media supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 0.25% (vol/vol) lactalbumin, and 100 ng/mL MCSF for 48 hours in the presence or absence of stimuli as stated.

Rabbit experimental foam cells were isolated from subcutaneous granulomas of cholesterol-fed New Zealand White rabbits as previously described.[8] Briefly, rabbits began a 1% cholesterol diet 2 weeks before implantation of 2 to 6 polyurethane sponges (Baxter Scientific) under the dorsal skin. Sponges remained in place for 4 to 5 weeks to allow macrophage accumulation while the animal remained on a 1% cholesterol diet throughout. The recovered sponges were gently squeezed over sterile test tubes, and the exudates were layered onto a discontinuous metrizamide gradient (bottom cushion 10 mL of 10% metrizamide [wt/vol], top 3-mL cell suspension) and centrifuged at 1200*g* for 15 minutes at 10°C. Foam cells were recovered from the floating layer and washed 3 times, and aliquots were prepared for oil red O staining to confirm lipid content and immunocytochemistry by using the rabbit macrophage-specific marker RAM 11. One rabbit yielded $\approx2\times10^7$ foam cells. Nonfoamy macrophages were isolated by adherence. Briefly, exudate cells were allowed to adhere to 100-mm Petri dishes (tissue culture grade) for 35 minutes at 37°C. Nonadherent cells were discarded, and the adherent macrophages were harvested by incubation on ice for 30 minutes and gentle scraping with a rubber policeman.

Infection of Monocyte-Derived Macrophages and Granuloma Foam Cells

Cultured human monocyte-derived macrophages and rabbit granuloma foam cells were incubated with purified adenovirus diluted in serum-free medium for 4 hours at different ratios of plaque-forming units (pfu) per cell. Inoculum was aspirated, and cells were washed with warmed serum-free medium and cultured in fresh full medium for a further 24 hours before the experiments.

Western Blotting for Rabbit MMP-1 and MMP-3

Concentrated supernatants (10×) were separated by SDS-PAGE and blotted onto a Hybond-nitrocellulose membrane (Amersham) with the use of a semidry blotting apparatus. Blocking of nonspecific binding and dilutions of the primary (40 μg/mL) anti–MMP-1 or anti–MMP-3 and secondary (1:2000, Dako) antibodies used 5% defatted dry milk/Tris-buffered saline/0.2% Tween 20. Proteins were visualized by using an enhanced chemiluminescence system (ECL, Amersham). Bands were quantified by densitometry.

ELISA for Human MMP-1, MMP-3, and IL-12

Culture supernatants were analyzed for human MMP-1, MMP-3, and IL-12 production by ELISA kits purchased from Amersham Pharmacia according to the manufacturer's instructions.

Zymography for MMP-9

Gelatinase activity was detected in conditioned media as previously described.[11] Briefly, 15 μL aliquots of nonreduced conditioned media were electrophoresed at 4°C in 7.5% SDS-polyacrylamide gels containing 2 mg/mL gelatin. After the removal of SDS, gelatinase activity was revealed by overnight incubation at 37°C and staining with 0.1% Coomassie brilliant blue. Zymograms were quantified in the linear range by densitometry with a GS 690 Image Analysis software system (Bio-Rad).

Reverse Zymography for TIMP Activity

Culture supernatants from rabbit foam cells were concentrated 20-fold. Aliquots (40 μL) of nonreduced media were electrophoresed at 4°C in 10% SDS-polyacrylamide gels containing 0.5 mg/mL gelatin and 10% baby hamster kidney cell, serum-free, conditioned media as a source of gelatinase. After removal of SDS, gelatinase activity was revealed by 24-hour incubation at 37°C and staining with 0.1% Coomassie brilliant blue. Zymograms were quantified in the linear range by densitometry with a GS 690 Image Analysis software system (Bio-Rad).

Semiquantitative Analysis by RT-PCR

Rabbit experimental foam cells were cultured in 6-well plates (5×10^6 cells per well) and uninfected or infected with either rAd:null or rAd:IκBα at 200 pfu per cell. Total mRNA was extracted by using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Procedures for reverse transcription (RT)–polymerase chain reaction (PCR) have been described.[12] PCR amplification was performed with *Taq* polymerase (Qiagen) for 35 cycles (MMP-1, MMP-3, MMP-9, and tissue inhibitor of metalloproteinases [TIMP]-1) or, to avoid saturation, for 22 cycles (GAPDH) and 25 cycles (TIMP-2) at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The primers used to measure rabbit MMP-1, MMP-3, MMP-9, TIMP-1, TIMP-2, and GAPDH mRNA levels are listed in online Table I (which can be accessed at http://atvb.ahajournals.org). Results are expressed in arbitrary units and are adjusted for GAPDH mRNA levels.

Collagenolytic and β-Caseinolytic Assays

Freshly isolated culture supernatants were assayed for collagenolytic and β-caseinolytic activity on the basis of the cleavage of fluorescently labeled substrates by using the Type I Collagenase Assay Kit and Stromelysin Activity Assay Kit (Yagai Corp), respectively, according to the manufacturer's instructions.

Cell Viability and Proliferation Studies

After the harvest of conditioned media, viable cell numbers were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,-5-diphenyltetrazolium bromide (MTT) assay (Sigma) according to the manufacturer's instructions. Apoptosis was assayed by using Cell Death Detection Elisa^{PLUS} (Roche), a photometric enzyme immunoassay for the quantitative determination of cytoplasmic histone-associated DNA fragments.

Nuclear Extracts and EMSA

To maximize cell yield, rabbit foam cells used for electrophoretic mobility shift assay (EMSA) were cultured and infected in polytetrafluoroethylene (Teflon) bags. This avoided the loss of cells by damage seen in the recovery of adherent cells by scraping (with the high lipid burden increasing cell fragility). Nuclear proteins were extracted from $\approx5\times10^6$ rabbit foam cell nuclei as previously described.[13] Binding reactions (20 μL) containing 2 μg poly(dI-dC), 10 mmol/L HEPES, pH 7.9, 50 mmol/L NaCl, 0.5 mM dithiothreitol, 2.5 mmol/L EDTA, 7 mmol/L $MgCl_2$, 4% glycerol, and 4 to 6 μg

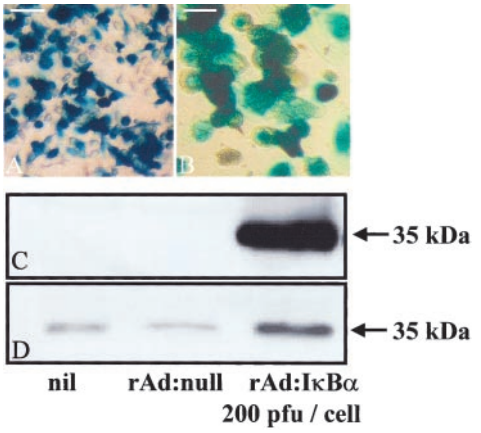

Figure 1. Adenoviral gene delivery of I-κBα to human monocyte-derived macrophages and granuloma foamy macrophages from cholesterol-fed rabbits. β-Galactosidase activity of infected human monocyte-derived macrophages (A) or rabbit foam cells (B) was assayed 48 hours after infection with rAd:βgal by X-Gal staining (blue). Bars=100 μm (A) and 25 μm (B). Human monocyte-derived macrophages (C) or rabbit foam cells (D) were uninfected or infected with 200 pfu per cell of either rAd:null or rAd:IκBα. Whole-cell lysates were prepared 48 hours after infection, and I-κBα protein expression was determined by Western blotting.

nuclear extract were incubated on ice for 30 minutes with 20 000 cpm of ^{32}P-labeled oligonucleotide corresponding to the consensus NF-κB element (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) or the consensus activator protein (AP)-1 element (5′-CGC TTG ATG AGT CAG CCG GAA-3′), Promega. Complexes were separated on 6% nondenaturing polyacrylamide gels and visualized by autoradiography.

NF-κB p65 Activity ELISA

Human monocyte-derived macrophages were either uninfected or infected with rAd:null or rAd:IκBα at 200 pfu per cell. Twenty-fours after infection, cells were placed into fresh serum-free media with or without 1 μg/mL rhCD40L for 48 hours. Whole-cell lysates were prepared and stored at −70°C. Equal quantities of total cellular protein (25 μg) were assayed for p65 NF-κB binding activity by using the Trans-AM NF-kB p65 Transcription Factor Assay kit (Active Motif) according to the manufacturer's instructions. ELISA was preferred to EMSA because it is more sensitive.

Results

Efficient Gene Transfer Into Human Monocyte-Derived Macrophages and Rabbit Foam Cells

Infection of human macrophages with 200 pfu adenovirus per cell transduced 95% to 100% of cells, as measured by β-galactosidase expression 48 hours later (Figure 1A). Uninfected macrophages and macrophages infected with adenovirus containing an empty expression cassette (rAd:null) did not express I-κBα protein at levels measurable by Western blotting. However, macrophages infected with adenovirus containing an I-κBα expression cassette (rAd:IκBα) overexpressed the I-κBα protein (Figure 1C).

Among cells isolated from subcutaneous granulomas of cholesterol-fed rabbits, 100% were stained with the rabbit macrophage marker RAM 11 (please see online Figure IA, which can be accessed at http://atvb.ahajournals.org) and 100% with oil red O (see online Figure IC), which confirmed

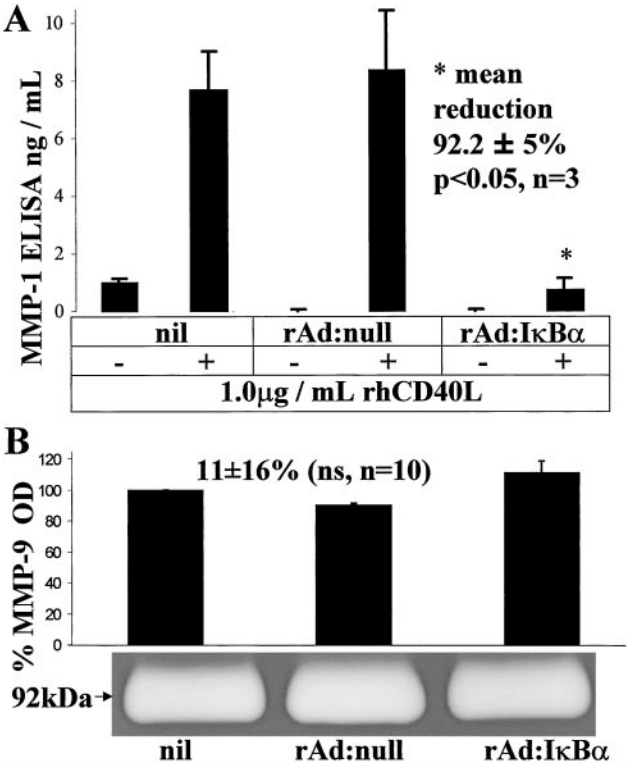

Figure 2. Overexpression of I-κBα inhibits CD40 ligand–mediated induction of MMP-1 in human monocyte-derived macrophages but not the constitutive secretion of MMP-9. A, Conditioned media from human monocyte-derived macrophages uninfected or infected with rAd:null or rAd:IκBα at 200 pfu per cell treated with or without rhCD40L (1 μg/mL) and CD40 ligand enhancer (1 mg/mL) were analyzed for MMP-1 secretion by ELISA. B, Conditioned media from human monocyte-derived macrophages uninfected or infected with rAd:null or rAd:IκBα were analyzed for MMP-9 secretion by 0.2% gelatin zymography and quantified by densitometry.

their macrophage lineage and "foamy" characteristics. Infection of rabbit foam cells with 200 pfu per cell of adenovirus yielded 95% to 100% transduction (Figure 1B). Western blotting revealed detectable levels of I-κBα protein in uninfected and rAd:null-infected foam cells. However, I-κBα protein was overexpressed 10.1±4.1-fold (n=6) 48 hours after rAd:IκBα infection (Figure 1D).

I-κBα Overexpression Inhibits CD40 Ligand–Induced MMP-1 Secretion by Human Macrophages but Not Constitutive MMP-9 Secretion

The human monocytes used in the present study were differentiated in the presence of 100 ng/mL MCSF partially to upregulate the receptors necessary for adenovirus infection.[14] In agreement with previous work involving macrophages differentiated by serum,[15] our unstimulated human macrophages secreted very low levels of MMP-1 (Figure 2A). Among the potential agonists investigated, namely, IL-1α, TNF-α, lipopolysaccharide, and oxidized LDL, only rhCD40L produced a robust upregulation of MMP-1 (Figure 2A). rhCD40L also potently induced IL-12 secretion, which was used as a positive control,[16] from undetectable levels in

untreated cells to 70.7±0.5 ng/mL ($P<0.05$, n=4). Overexpression of I-κBα significantly reduced total MMP-1 secretion by 92.2±5% ($P<0.05$, n=3; Figure 2A). In contrast, unstimulated macrophages showed constitutive secretion of MMP-9, which arose by de novo synthesis because it was abolished by 10 μg/mL of cycloheximide (data not shown). MMP-9 secretion was not significantly inhibited by I-κBα overexpression (11±16%, n=10; Figure 2B). No basal secretion of MMP-3 was seen in unstimulated human macrophages, and in contrast to a previous study,[15] we observed no significant upregulation of MMP-3 in response to rhCD40L (data not shown). There was no significant difference in viable cell numbers between rAd:Iκβα-infected and rAd:null-infected macrophage cultures used for these experiments, as determined by microscopy or MTT assay (2.050±0.021 and 2.102±0.034 optical density [OD] units, respectively; $P=0.23$, n=5).

I-κBα Overexpression Inhibits Cholesterol-Feeding–Elicited MMP-1 and MMP-3 Secretion but Not MMP-9 or TIMP-1 and TIMP-2 Secretion From Foam Cells

A previous study[8] demonstrated that cholesterol feeding elicited similar MMP-1 and MMP-3 secretion from aortic atherosclerotic plaque and granuloma macrophages without the need for further exogenous stimuli. Because granuloma macrophages are more abundant and easier to isolate, we used these for our studies. Granuloma macrophages isolated from New Zealand White rabbits maintained on a normal chow diet did not elaborate MMP-1 or MMP-3 but secreted MMP-9 without further stimulation, similar to human macrophages (please see online Figure II, which can be accessed at http://atvb.ahajournals.org). Granuloma foam cells also expressed MMP-9 without further stimulation (see online Figure II). In contrast, after cholesterol feeding, MMP-1 and MMP-3 were secreted even without stimulation (see online Figure II). By Western blotting zymography, the overwhelming majority of MMP-1, MMP-3, and MMP-9 was secreted as the pro form. Overexpression of I-κBα after infection by rAd:IκBα resulted in a highly significant reduction in MMP-1 and MMP-3 secretion by 83±12% ($P<0.001$, n=6) and 69±11% ($P<0.001$, n=7), respectively, whereas rAd:null infection had no effect (Figure 3). In keeping with the observation in human macrophages, constitutive MMP-9 secretion was unaffected by the overexpression of I-κBα (Figure 3). Similarly, TIMP-1 and TIMP-2 activities were unaffected by the overexpression of I-κBα (Figure 3). No significant differences in cell number or viability were observed between rAd:Iκβα-infected and rAd:null-infected foam cells as determined by MTT assay (2.672±0.006 and 2.666±0.021 OD units, respectively; $P=0.78$, n=3), and there was no significant difference in levels of apoptosis by cell death detection by ELISA^{PLUS} (0.3800±0.019 and 0.4077±0.007 OD units, respectively; $P=0.2218$, n=5).

I-κBα Overexpression by Rabbit Foam Cells Decreases mRNA Levels of MMP-1 and MMP-3 but Not MMP-9 or TIMP-1 and TIMP-2

Steady-state mRNA levels were measured by RT-PCR under nonsaturating conditions. Consistent with the pattern of

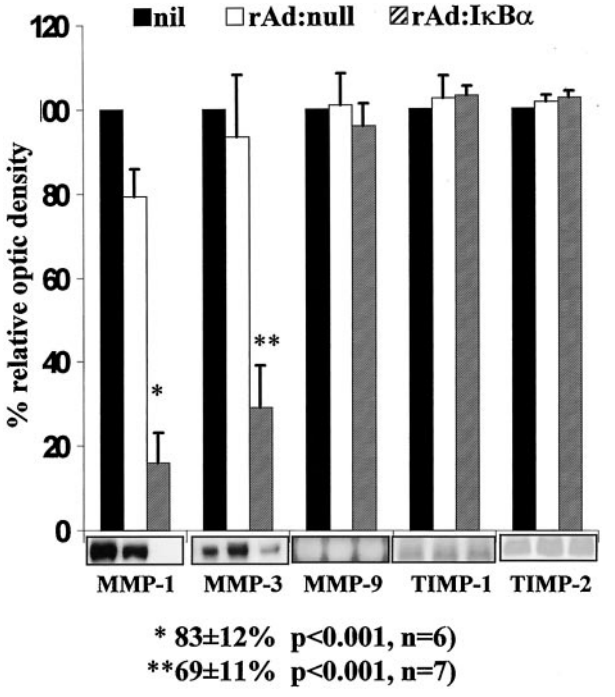

Figure 3. Cholesterol-elicited upregulation of MMP-1 and MMP-3 in rabbit foam cells is inhibited by overexpression of I-κBα. Rabbit foam cells were uninfected or infected with rAd:null or rAd:IκBα at 200 pfu per cell. Conditioned media were harvested at 48 hours and analyzed for MMP-1 and MMP-3 secretion by Western blotting, MMP-9 secretion by gelatin zymography, and TIMP-1 and TIMP-2 activity by reverse gelatin zymography. Blots and gels were quantified by densitometry.

secreted MMP proteins, overexpression of I-κBα resulted in a profound reduction in MMP-1 and MMP-3 steady-state mRNA levels by 99±1% ($P<0.05$, n=3) and 82±9% ($P<0.05$, n=3), respectively (Figure 4). There was no sig-

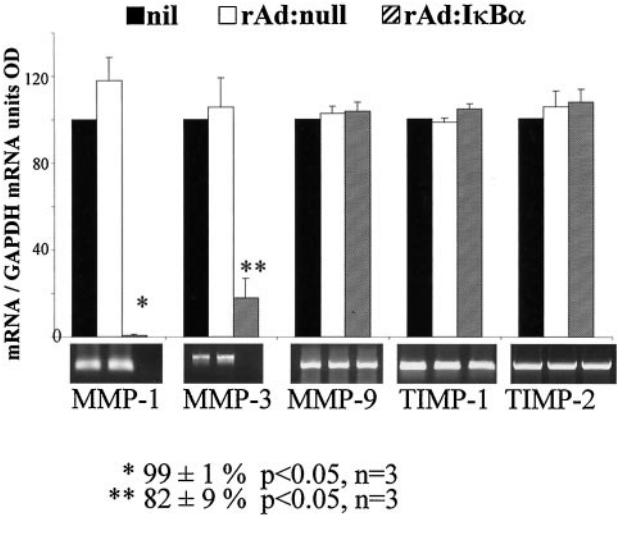

Figure 4. Overexpression of I-κBα in rabbit foam cells inhibits MMP-1 and MMP-3 mRNA production but not MMP-9 or TIMP-1 or TIMP-2. Rabbit foam cells were uninfected or infected with rAd:null or rAd:IκBα at 200 pfu per cell. Forty-eight hours after infection, total RNA was extracted. Semiquantitative mRNA analysis was performed by RT-PCR. mRNA levels are expressed in arbitrary units and adjusted for GAPDH mRNA.

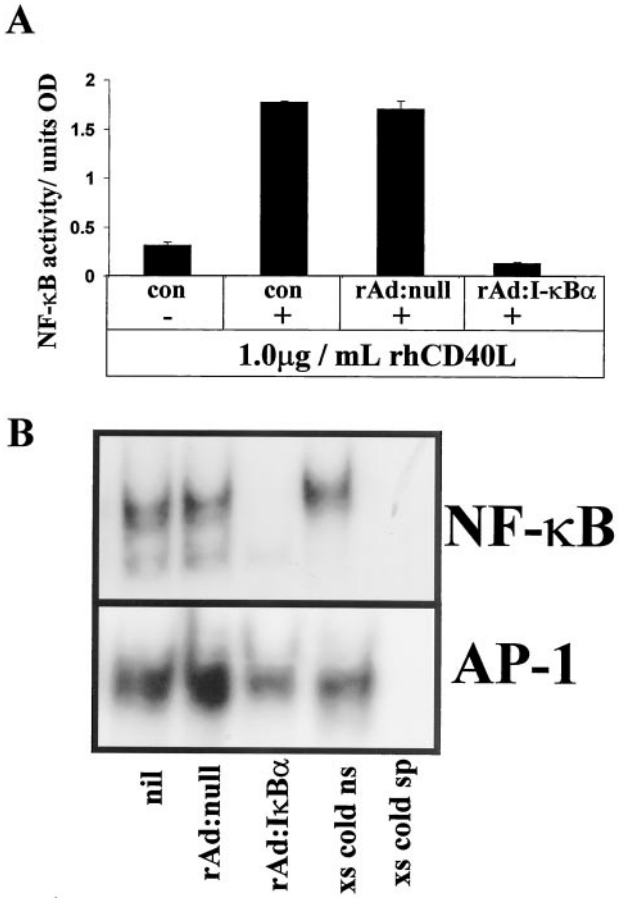

Figure 5. Overexpression of I-κBα inhibits CD40 ligand–induced NF-κB activity in human macrophages and constitutive NF-κB activity in rabbit foam cells. A, Human monocyte-derived macrophages were uninfected or infected with 200 pfu per cell of either rAd:null or rAd:IκBα. Twenty-four hours after infection, cells were transferred to serum-free media with or without rhCD40L (1 μg/mL) and CD40 ligand enhancer (1 mg/mL). Cells were lysed, and 25 μg total cellular protein was assayed for p65 NF-κB binding activity by ELISA with the use of the Trans-AM NF-κB p65 Transcription Factor Assay kit. B, Rabbit foam cells (5×10^6) were uninfected or infected with rAd:null or rAd:IκBα at 200 pfu per cell. After 48 hours, cells were recovered, and nuclear extracts were prepared. Protein (5 μg) was analyzed for NF-κB or AP-1 activity by EMSA. Nuclear protein binding was inhibited by 100-fold excess (xs) specific (cold sp), but not nonspecific (cold ns) unlabeled digonucleotides.

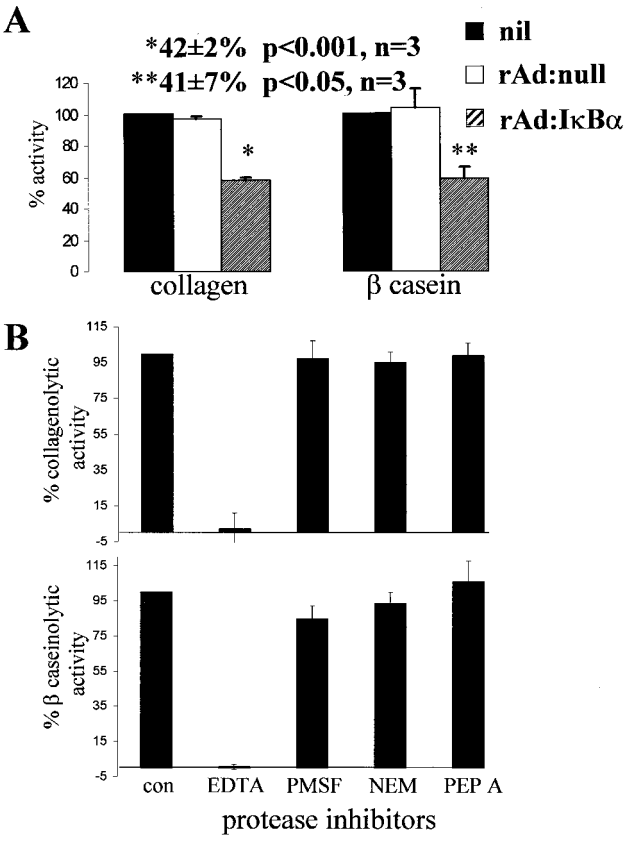

Figure 6. Overexpression of I-κBα reduces collagenolytic and β-caseinolytic activity in foam cell–conditioned medium. A, Freshly isolated conditioned media from granuloma foam cells either uninfected or infected with rAd:null or rAd:IκBα at 200 pfu per cell were analyzed for collagenolytic (MMP-1) and β-caseinolytic (MMP-3) activity by using fluorescently labeled substrate digestion as described in Methods. B, To demonstrate that substrate digestion was attributable to MMP activity as opposed to other proteases, the collagenolytic and β-caseinolytic activity assays were conducted by using conditioned media from uninfected cells (con) in the presence or absence of the specific protease inhibitors EDTA (20 mmol/L), phenylmethylsulfonyl fluoride (PMSF, 1 mmol/L), *N*-ethylmaleimide (NEM, 5 mmol/L), and pepstatin A (PEP A, 5 mmol/L).

nificant reduction in MMP-9, TIMP-1, and TIMP-2 mRNA (Figure 4).

Overexpression of I-κBα Decreases NF-κB Binding Activity in Human Macrophages and Rabbit Foam Cells

In the absence of exogenous stimuli, low levels of NF-κB binding activity were detectable in human monocyte-derived macrophages. Stimulation with rhCD40L resulted in a potent induction of NF-κB binding activity, which was inhibited in cells overexpressing I-κBα (Figure 5A). Previous studies have indicated a dual role for NF-κB and AP-1 transcription factors in the regulation of MMP-1, MMP-3, and MMP-9 secretion.[5,6] Therefore, nuclear extracts from rabbit foam cells were assayed for NF-κB and AP-1 DNA binding by EMSA with the use of consensus NF-κB and AP-1 binding site oligonucleotides. Consistent with the pattern of MMP secretion, NF-κB DNA binding in human macrophages is seen only after challenge with exogenous stimuli such as rhCD40L (Figure 5A) or lipopolysaccharide,[14] whereas rabbit granuloma foam cells exhibited NF-κB and AP-1 DNA binding ability even without added stimulation (Figure 5B). Moreover, adenovirus-mediated overexpression of I-κBα abolished NF-κB but not AP-1 DNA binding (Figure 5B). Nuclear extracts from granuloma cells isolated from New Zealand White rabbits maintained on a normal chow diet did not exhibit NF-κB binding activity (results not shown).

Overexpression of I-κBα Reduces Foam Cell Collagenolytic and β-Caseinolytic Activity

Conditioned media from rabbit granuloma foam cells contained measurable proteolytic activity against type I collagen and β-casein substrates (Figure 6A). Activity was inhibited

by EDTA but not by phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, or pepstatin A, showing it to be the result of metalloproteinase activity (Figure 6B). Overexpression of I-κBα by rAd:IκBα resulted in a significant reduction in collagenolytic and β-caseinolytic activity by 42.1±2% ($P<0.001$, n=3) and 41.4±7% ($P<0.05$, n=3), respectively, whereas rAd:null had no effect (Figure 6A).

Discussion

Although they are secreted from a wide variety of cells, it is generally believed that MMPs derived from macrophages are the most significant contributors to plaque instability, acute coronary syndromes, and aneurysm formation. For example, MMP-1, MMP-3, and MMP-9 are prominently located in macrophage foam cells in the shoulder region of human and experimental plaques,[4,17,18] and areas rich in macrophages show direct evidence of collagen cleavage.[19] Furthermore, knockout mice models directly demonstrate that deficiency in MMP-3 or MMP-9 is associated with reduced aneurysm formation.[20,21]

Our present results demonstrate for the first time that NF-κB is a key factor in macrophage-derived MMP-1 and MMP-3 secretion. Inhibition of NF-κB dramatically reduced MMP-1 secretion from healthy human macrophages in response to CD40 ligation. Moreover, NF-κB was necessary for the pathology-related upregulation of MMP-1 and MMP-3 in foam cells elicited during atherosclerosis formation in vivo, thereby giving an indirect indication of the likely impact of NF-κB inhibition in vivo. Agents as diverse as salicylic acid, antioxidants such as *N*-acetylcysteine, and proteosome inhibitors have all been suggested to be capable of inhibiting NF-κB in vivo, although not without other unrelated effects.[22] Similarly, several drug classes, including statins, fibrates, and thiazolidinediones, may also exert their anti-inflammatory effects, in part, through reducing NF-κB activity. However, none of these agents is a selective inhibitor of NF-κB, in contrast to molecular approaches, such as the one used here. The potential for direct in vivo studies using molecular interventions is limited because knockouts of NF-κB cause severe phenotypes or lethality.[23] In addition to the absence of clean and selective pharmacological inhibitors of NF-κB, there is, as yet, no established in vivo model of plaque rupture in which to test the impact of selective NF-κB inhibition. Therefore, we adopted a hybrid strategy in which the effect of I-κBα overexpression was investigated in foam cells elicited in vivo. Our results suggest that NF-κB inhibition would inhibit MMP-1 and MMP-3 upregulation and, hence, decrease matrix turnover in vivo. However, we discovered that constitutive MMP-9 secretion by human macrophages and rabbit foam cells is independent of NF-κB, which might limit the effectiveness of NF-κB inhibition. In part to overcome this limitation, we measured the effect of I-κBα overexpression on the degradation of MMP substrates by rabbit foam cells and demonstrated a significant, albeit somewhat less profound, inhibition.

One concern with NF-κB inhibition would be an increase in apoptosis[24] that might add to plaque instability. However, we found no evidence of decreased viability or increased apoptosis of macrophages in our experiments, which is encouraging and consistent with a previous study.[14]

Although the mechanisms underlying MMP secretion have been widely studied in several cell types, including SMCs, they are less well understood in macrophages. Binding of transcription factors to an AP-1 site in the core promoter appears to be a common regulatory mechanism in many MMP genes.[25] We recently proposed that synergy between AP-1 and NF-κB transcription factors underlies upregulation of MMP-1, MMP-3, and MMP-9 in SMCs.[6] The present results extend this concept to macrophages. Indeed, CD40 ligation is known to upregulate NF-κB activity in leukocytes, probably via a downstream effect of TNF–receptor associated factor (TRAF) family members.[26] Moreover, overexpression of I-κBα has been previously shown to abolish NF-κB activity in human macrophages and to downregulate the secretion of IL-1, TNF-α, IL-6, and IL-8, all of which are known to be regulated by NF-κB. The exact mechanisms of NF-κB–dependent MMP-1 and MMP-3 gene regulation remain elusive and form the focus of separate ongoing studies in SMCs. An atypical NF-κB binding *Drosophila* dorsal-like element in the distal (−3029-bp) MMP-1 promoter has been implicated in the IL-1β induction of MMP-1 in rabbit fibroblasts,[27] although not in the human breast cancer cell line BC-8701.[28] The importance of NF-κB for increased secretion of MMP-3 is also surprising because no consensus NF-κB element has been identified in its proximal promoter. However, NF-κB is also required for the upregulation in fibroblasts of the α_2-integrin subunit, which also lacks a known NF-κB promoter element. Bondeson et al[7] demonstrated that overexpression of I-κBα resulted in the inhibition of MMP-1 and MMP-3 from a rheumatoid synovium coculture containing fibroblasts, T lymphocytes, and macrophages. Interestingly, the secretion of MMP-3 (but not MMP-1) was mediated by NF-κB–dependent secretion of IL-1 from macrophages, which then acted on fibroblasts.[7] This is paralleled by studies demonstrating that macrophages stimulate vascular SMCs to produce MMP-1, MMP-3, and MMP-9 via the secretion of IL-1.[29,30] In our experiments, highly homogeneous populations of either human monocytes or macrophage foam cells were used to avoid such indirect effects.

Secretion of MMP-9 in macrophages depends largely on cell-to-cell and cell-to-matrix interactions mediated by the engagement of integrins rather than inflammatory mediators.[31,32] Hence, its independence from NF-κB, shown in the present study, is perhaps not surprising. This differential regulation of MMP-9 versus MMP-1 and MMP-3 probably has functional significance. Early migration of monocytes/macrophages from the circulation to the site of injury involves traversing basement membranes, and this probably requires MMP-9 activity. Consistent with this, there is evidence of impaired monocyte migration in MMP-$9^{-/-}$ mice.[33] Subsequent upregulation of MMP-1 and MMP-3 production by macrophages reflects a further stage of activation and can, as we have shown in the present study, be reversed by the inhibition of NF-κB. In conclusion, therefore, the present study not only advances the mechanistic understanding of MMP regulation in macrophages but also suggests that

further studies are merited to assess the therapeutic impact of NF-κB inhibition in unstable atheroma.

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