

# Clinical Investigation and Reports

## Oxidized Low-Density Lipoprotein Regulates Matrix Metalloproteinase-9 and Its Tissue Inhibitor in Human Monocyte-Derived Macrophages

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**Background**—Macrophages in human atherosclerotic plaques produce a family of matrix metalloproteinases (MMPs), which may influence vascular remodeling and plaque disruption. Because oxidized LDL (ox-LDL) is implicated in many proatherogenic events, we hypothesized that ox-LDL would regulate expression of MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) in monocyte-derived macrophages.

**Methods and Results**—Mononuclear cells were isolated from normal human subjects with Ficoll-Paque density gradient centrifugation, and adherent cells were allowed to differentiate into macrophages during 7 days of culture in plastic dishes. On day 7, by use of serum-free medium, the macrophages were incubated with various concentrations of native LDL (n-LDL) and copper-oxidized LDL. Exposure to ox-LDL (10 to 50  $\mu\text{g}/\text{mL}$ ) increased MMP-9 mRNA expression as analyzed by Northern blot, protein expression as measured by ELISA and Western blot, and gelatinolytic activity as determined by zymography. The increase in MMP-9 expression was associated with increased nuclear binding of transcription factor NF- $\kappa$ B and AP-1 complex on electromobility shift assay. In contrast, ox-LDL (10 to 50  $\mu\text{g}/\text{mL}$ ) decreased TIMP-1 expression. Ox-LDL-induced increase in MMP-9 expression was abrogated by HDL (100  $\mu\text{g}/\text{mL}$ ). n-LDL had no significant effect on MMP-9 or TIMP-1 expression.

**Conclusions**—These data demonstrate that unlike n-LDL, ox-LDL upregulates MMP-9 expression while reducing TIMP-1 expression in monocyte-derived macrophages. Furthermore, HDL abrogates ox-LDL-induced MMP-9 expression. Thus, ox-LDL may contribute to macrophage-mediated matrix breakdown in the atherosclerotic plaques, thereby predisposing them to plaque disruption and/or vascular remodeling. (*Circulation*. 1999;99:993-998.)

**Key Words:** lipoproteins ■ metalloproteinases ■ atherosclerosis

Acute coronary syndromes of unstable angina, acute myocardial infarction, and ischemic sudden death result from disruption of atherosclerotic plaques, leading to coronary thrombosis.<sup>1</sup> The potential cellular mechanisms involved in plaque disruption are complex. It has been shown that vulnerable plaques or those that have disrupted tend to have a large extracellular lipid-rich core, a reduced number of smooth muscle cells (SMCs), and a thin fibrous cap with reduced extracellular matrix.<sup>2</sup> Furthermore, the rupture-prone regions are frequently infiltrated with an increased number of activated inflammatory cells composed largely of monocyte-derived macrophages and foam cells but also containing an increased number of activated mast cells and T-lymphocytes.<sup>3</sup> Further observations have shown that atherosclerotic lesion-associated foam cells, derived primarily from monocyte-derived macrophages and to a lesser extent from SMCs, produce the matrix metalloproteinase (MMP) enzymes and tissue inhibitors of MMP (TIMPs).<sup>4</sup> MMPs have the capacity to degrade collagen and elastin, the major components of the extracellular matrix in the fibrous cap.

Monocyte-derived macrophages have been shown to secrete MMP in cell culture and induce collagen breakdown in isolated atherosclerotic fibrous caps.<sup>5</sup> In situ zymography has demonstrated a net increased matrix-degrading activity in human atherosclerotic plaques at sites of in vivo MMP expression.<sup>6</sup> Taken together, these findings suggest that macrophage-derived MMPs may increase matrix breakdown in plaques, thereby predisposing them to disruption.

Although oxidized LDL (ox-LDL) is believed to play a critical role in various cellular processes involved in atherogenesis, its effect on MMP and TIMP expression is unknown. In this study, we chose to specifically evaluate the effect of ox-LDL on MMP-9, the 92-kDa gelatinase or gelatinase B, which has been shown to be upregulated in unstable human coronary plaques,<sup>7</sup> and its inhibitor TIMP-1.

### Methods

#### Materials

Culture reagents were purchased from GIBCO. Ficoll-Paque was obtained from Pharmacia. Lipoproteins were obtained from the

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Atherosclerosis Research Unit of the University of California at Los Angeles (UCLA). MMP-9 antigen was purchased from Biogenesis. MMP-9 and TIMP-1 mice monoclonal antibodies and the peroxidase goat anti-mouse IgG were purchased from Oncogene Science. Nitrocellulose membranes and the antibody detection kit (ECL) were obtained from Amersham. ELISA starter kit was obtained from PIERCE. MMP-9 cDNA probe was provided by Dr Gregory Goldberg's laboratory at Washington University. Dr Dan Brown at Cedars-Sinai Medical Center provided TIMP-1 cDNA probe. [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol) and [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol) were supplied by Du Pont NEN. The oligonucleotide corresponding to the palindromic NF- $\kappa$ B and AP-1 consensus sequence was purchased from Promega. Prepared gelatin gels (10%) were obtained from NOVEX. E-Toxate kit was purchased from Sigma Chemical Co.

### Isolation and Culture of Monocyte-Derived Macrophages

Mononuclear cells were isolated from venous blood of normal human subjects by a Ficoll-Paque density gradient centrifugation.<sup>8</sup> After non-adherent cells were discarded, mononuclear cells were suspended in RPMI 1640 media supplemented with 10% FCS, 24 mmol/L NaHCO<sub>3</sub>, 25 mmol/L HEPES, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 1 mmol/L sodium pyruvate, 4 mmol/L glutamine, and nonessential amino acids and incubated in a humidified incubator at 37°C for 7 days. Cell viability was determined by trypan blue stain. Cells were placed into 100-mm culture dishes (1 $\times$ 10<sup>8</sup> cells per dish) for determination of MMP-9 and TIMP-1 mRNA expression and NF- $\kappa$ B and AP-1 bindings and into 6-well plates (1 $\times$ 10<sup>7</sup> cells per well) for other experiments. Fresh culture medium was replaced every 3 days.

### Preparation and Oxidation of Lipoproteins

LDL and HDL were prepared from freshly drawn normal human plasma in the Atherosclerosis Research Unit at UCLA. Briefly, venous blood was drawn into cooled vacutainer tubes containing Na<sub>2</sub>EDTA (1.4 mg/mL). Plasma was then recovered by centrifugation at 14 000g for 20 minutes at 1°C. The isolated plasma was adjusted to a density of 1.10 kg/L by addition of NaCl. A density gradient consisting of 3 mL of 1.10-kg/L-density plasma and 3 mL of 1.065-, 1.020-, and 1.006-kg/L NaCl solution, respectively, was then formed in cellulose nitrate tubes (Ultraclear tubes, Beckmen) and centrifuged (Beckman L8 to 55 ultracentrifuge, 40 000 rpm) in a Beckman SW 40 swinging-bucket rotor at 1°C overnight. The LDL fractions were harvested. Native unmodified LDL was stored in EDTA and used within 3 days of isolation with no detectable levels of endotoxin (<0.01 ng/mL). Before oxidation, EDTA was removed by desalting against EDTA-free PBS (pH 7.4) by use of PT-10 filters. The protein content was determined by the method of Bradford.<sup>9</sup> LDL was diluted to 500  $\mu$ g/mL, oxidized by exposure to 5  $\mu$ mol/L CuSO<sub>4</sub> for 24 hours at 37°C, and used immediately for the experiments.

### Measurement of LDL Oxidation and Toxicity

The degree of oxidation of LDL was evaluated by measurement of thiobarbituric acid-reacting substances (TBARS) according to Yagi.<sup>10</sup> This assay measures oxidation by determining the content of malondialdehyde (MDA), a side product of lipid oxidation. To 100- $\mu$ L aliquots of ox-LDL, 0.5 mL of 25% trichloroacetic acid was added, followed by 0.5 mL of 1% thiobarbituric acid. The mixture was incubated at 95°C for 45 minutes, cooled, and subsequently centrifuged at 1000g for 30 minutes. Absorbance was measured at 240 nm by a spectrophotometer. MDA was freshly diluted with saline and used as a standard. The standard curve was linear from 0 to 10 nmol MDA. Results are given as TBARS and are expressed in terms of MDA equivalents per milligram of LDL. TBARS determinations were performed on duplicate aliquots from each ox-LDL batch, and the average of the values obtained was used. In addition, the endotoxin content of ox-LDL and HDL preparations was determined with the E-Toxate kit.

### Cell Viability

After the conditioned medium was removed, the cells were gently scraped and counted with a ZBI Coulter counter. Harvested cells were washed and stained by trypan blue to evaluate cell viability.

### Northern Blot Analysis

Expression of MMP-9 mRNA and TIMP-1 mRNA was determined by Northern blot analysis as previous described.<sup>11</sup> RNA was extracted from macrophages with the TRIzol reagent (Life Technologies). Total RNA was quantified spectrophotometrically, and 20  $\mu$ g RNA was separated in 1.5% formamide/agarose gels and transferred to nylon membranes. cDNA and oligonucleotide probes were labeled with [ $\alpha$ - $^{32}$ P]dCTP by use of a random-priming kit (Promega). Membranes were hybridized overnight at 65°C and washed for 20 minutes in 2 $\times$  standard saline citrate once and 0.1% SDS for 20 minutes for 3 to 4 times as required. The membranes were exposed to x-ray film at -70°C.

### Western Blot Analysis

Western blot analysis was used for detecting MMP-9 and TIMP-1 protein expression. Samples were electrophoresed on 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and incubated overnight at 4°C with blocking solution (1% nonfat dried milk in PBS containing 0.1% Tween 20). Membranes were incubated with MMP-9 or TIMP-1 antibodies (1  $\mu$ g IgG per 1 mL) while they were shaken in PBS buffer containing 0.1% Tween 20 for 1 hour at room temperature. The membranes were washed with PBS buffer, treated with 1:1000 dilution of peroxidase goat anti-mouse IgG for 1 hour, and washed again. Immunodetection was accomplished with the Enhanced Chemiluminescence kit.

### ELISA Procedure

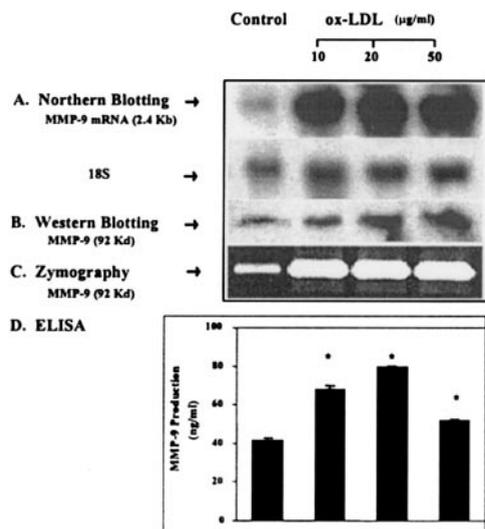
MMP-9 and TIMP-1 production was also measured by ELISA<sup>12</sup> with the ELISA Starter Kit. MMP-9 antigen (100 ng/mL) and the unknown sample volumes (adjusted according to protein content) were added into microtitration plate wells in 100  $\mu$ L of coating buffer and incubated overnight at 4°C. MMP-9 or TIMP-1 antibodies at a concentration of 50 ng/mL were added to each well and incubated overnight at 4°C to achieve maximal binding. The peroxidase goat anti-mouse IgG (diluted to 1:1000) was added to each well and incubated overnight at 4°C. The plate was washed, and 100  $\mu$ L of peroxidase substrate solution was added to initiate the colorimetric reaction for 30 minutes. Absorbance was measured at 405 nm by spectrophotometer. Data on production of MMP-9 were expressed as nanograms per milliliter. Data on production of TIMP-1 was expressed as percent change compared with control.

### Zymography

The matrix-degrading activity of MMP-9 was assayed by zymography.<sup>13</sup> Aliquots of conditioned medium (1  $\mu$ g per lane with volumes adjusted according to protein content) were denatured at room temperature for 10 minutes in an equal volume of electrophoresis sample buffer and run on 10% gelatin gels provided by NOVEX. A sample (20  $\mu$ L) was loaded into the gel and subjected to electrophoresis at a constant voltage of 125 V. The gel was then washed in 2.5% Triton X-100 solution with gentle agitation for 6 hours at room temperature, followed by replacement with developing buffer (g/L dH<sub>2</sub>O: Tris base 12.1, Tris HCl 63, NaCl 117, CaCl<sub>2</sub> 7.4, and 0.2% Brij 35). The gel was agitated at room temperature for 30 minutes, placed into fresh developing buffer, and incubated at 37°C overnight. The gel was stained with 0.5% coomassie blue, destained in destaining solution containing 5% methanol and 7% acetic acid, photographed, and dried for permanent records.

### Electrophoretic Mobility Shift Assay

Macrophage nuclear extracts were prepared, and the protein content was determined by the method of Bradford.<sup>9</sup> Cells were harvested, homogenized in 1 mL hypotonic lysis buffer, incubated on ice for 10 minutes, and centrifuged at 9000g for 30 seconds. The pellet was collected, washed, and resuspended in KCl buffer. Nuclear proteins were extracted by gentle agitation in 4°C for 30 minutes, followed by



**Figure 1.** Effects of ox-LDL on MMP-9 expression. Macrophages were treated with various doses of ox-LDL (10, 20, and 50  $\mu\text{g}/\text{mL}$ ; TBARS, 16.48 nmol/mg). In separate experiments, cells were harvested at 4 hours for assay of MMP-9 mRNA expression by Northern blot (A,  $n=3$ ), and culture medium was collected at 24 hours for measurement of MMP-9 protein expression by Western blot (B,  $n=6$ ) and ELISA (D,  $n=6$ ). Its matrix degrading activity was determined by zymography (C,  $n=6$ ). \* $P<0.05$  vs untreated control.

centrifugation at 9000g for 15 minutes. EMSA was applied for determining NF- $\kappa$ B and AP-1 bindings.<sup>14</sup> The NF- $\kappa$ B oligonucleotide (consensus, 5'-AGTTGAGGGGACTTCCAGG-3') and AP-1 oligonucleotide (consensus, 5'-CGC TTG ATG ATG CAG CCG GAA-3') were end labeled with [ $\gamma$ -<sup>32</sup>P]ATP and T<sub>4</sub> kinase, respectively. Nuclear extracts (6  $\mu\text{g}$ ) were added to [ $\gamma$ -<sup>32</sup>P]-labeled NF- $\kappa$ B and AP-1 oligonucleotide in buffer containing 0.25 mg/mL of poly(dI-dC), 50 mmol/L Tris-HCl (pH 7.5), 250 mmol/L NaCl, 5 mmol/L MgCl<sub>2</sub>, 2.5 mmol/L dithiothreitol, 2.5 mmol/L EDTA, and 20% glycerol (total volume of 30  $\mu\text{L}$ ), and the binding reaction was incubated for overnight at 4°C. Specificity was verified by the addition of 100-fold excess unlabelled oligonucleotide as competitor. Anti-NF- $\kappa$ B subunits p50 and p65 antibodies and anti-AP-1 subunits *c-fos* and *c-jun* antibodies were added to samples treated by 20  $\mu\text{g}/\text{mL}$  ox-LDL. The DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gel and electrophoresed at 12 V/cm for 2.5 hours in low-ionic-strength buffer (0.5 $\times$  tris-borate-ethylenediaminetetraetic acid) at 4°C. The gel was then taken down, dried under vacuum, and exposed to x-ray film (X-OMAT, Kodak) overnight at -70°C.

### Experimental Protocol

Culture medium was replaced on day 7 by serum-free medium supplemented with 0.5% BSA for an additional 24 hours before incubation with lipoproteins. Macrophages were exposed to various concentrations of ox-LDL or unmodified LDL (10, 20, and 50  $\mu\text{g}/\text{mL}$ ) in the presence or absence of HDL (100  $\mu\text{g}/\text{mL}$ ). Phorbol myristate acetate (125 ng/mL), a potent activator of AP-1, served as a positive control. For determination of transcription factor NF- $\kappa$ B and AP-1 binding, cells were cultured for 2 hours, and nuclear fractions were extracted for electrophoretic mobility gel shift assay. For determination of MMP-9 mRNA and TIMP-1 mRNA expression, cells were cultured for 4 hours, and RNA was extracted for Northern blot analysis. For measurement of MMP-9 and TIMP-1 protein expression and performance of bioassay, cells were cultured for 24 hours, and the media were collected for Western blot analysis, ELISA, and zymography. Finally, lipid uptake by macrophages was determined by Oil Red O stain.

### Statistical Analysis

Optical densities of MMP-9 and TIMP-1 signals on x-ray films or dried gels were measured by computer-assisted densitometry for

quantification of changes in protein and mRNA expression. Data are expressed as mean $\pm$ SEM. For repeated measures, differences between groups were determined by use of ANOVA for multiple comparisons, followed by Bonferroni's correction. A corrected value of  $P<0.05$  were considered statistically significant.

## Results

### LDL Oxidation and Toxicity and Cell Cytotoxicity

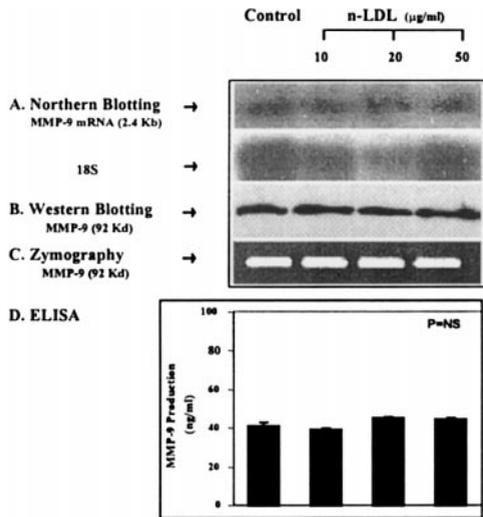
Oxidation of copper-oxidized LDL (500  $\mu\text{g}/\text{mL}$ ) was measured by TBARS, and the degree of LDL oxidation corresponded to 16.48 $\pm$ 0.8 nmol MDA/mg LDL protein ( $n=6$ ). The endotoxin concentration of ox-LDL and HDL was 100 pg/100 mg lipoprotein per 1 mL. Similarly, endotoxin in the medium was <100 pg/mL. Cell number and viability were examined after incubation of lipoprotein. Cell viability was >90% in all the groups. There was no significant difference in the number of human macrophages among the different groups. Oil Red O staining showed visible vacuoles in the macrophages after incubation of with oxidized lipoproteins, indicating uptake of lipoproteins by macrophage (data not shown).

### Effects of ox-LDL and Native LDL on MMP-9 Expression

The effect of ox-LDL on MMP-9 expression was investigated in human monocyte-derived macrophages. Within 7 days, cultured monocytes were differentiated into macrophages and then treated with ox-LDL at concentrations of 10, 20, and 50  $\mu\text{g}/\text{mL}$ . MMP-9 mRNA expression was determined by Northern blot analysis after 4 hours of incubation with ox-LDL. The results indicate that macrophages express the 2.4-kb mRNA species for MMP-9. When exposed to ox-LDL, MMP-9 mRNA expression increased at concentrations of 10, 20, and 50  $\mu\text{g}/\text{mL}$  (Figure 1A). MMP-9 protein expression was then determined in conditioned media by ELISA and Western blot analysis. Figure 1D shows that MMP-9 protein expression increased in response to ox-LDL at a concentration of 10 to 50  $\mu\text{g}/\text{mL}$  ( $n=6$ ,  $P<0.05$ ). These data were confirmed by Western blot analysis, which showed an increase in protein expression (Figure 1B). Moreover, MMP-9 matrix-degrading activity in the conditioned medium from macrophages incubated with ox-LDL was assayed by gelatin zymography. The zymogram demonstrated increased gelatinolytic activity with exposure to ox-LDL at 10 to 50  $\mu\text{g}/\text{mL}$ , whereas macrophages under control conditions demonstrated low levels of gelatinolytic activity (Figure 1C, lane 1). In contrast, when cultured human macrophages were treated with native (n-LDL), MMP-9 mRNA and protein expression and gelatinolytic activity were unaffected (Figure 2).

### Effects of ox-LDL and n-LDL on TIMP-1 Expression

We used Northern blotting, Western blotting, and ELISA to assay the expression of TIMP-1 in macrophages. TIMP-1 mRNA expression was determined by Northern blot after 4 hours of incubation with ox-LDL. The results indicate that macrophages express the 900-bp mRNA species for TIMP-1, and ox-LDL (10 to 50  $\mu\text{g}/\text{mL}$ ) decreased TIMP-1 mRNA expression (Figure 3A). After exposure to various doses of ox-LDL for 24 hours, conditioned medium was collected for measurement of TIMP-1 protein by ELISA. The data showed that ox-LDL decreased the level of TIMP-1 expression at

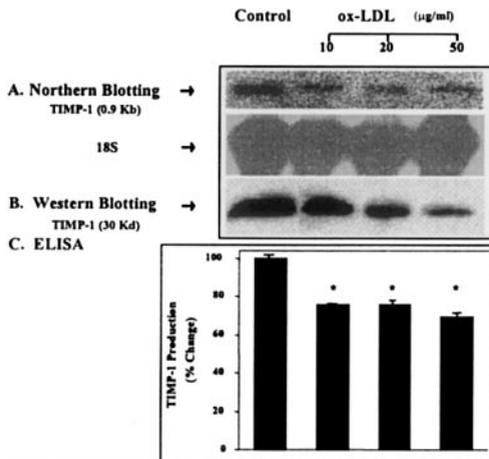


**Figure 2.** Effects of n-LDL on MMP-9 expression. Macrophages were treated with various doses of n-LDL (10, 20, and 50 µg/mL). In separate experiments, cells were harvested at 4 hours for assay of MMP-9 mRNA expression (A, n=3). Culture medium was collected at 24 hours for measurement of MMP-9 protein expression (B and D, n=6) and its matrix-degrading activity (C, n=6).

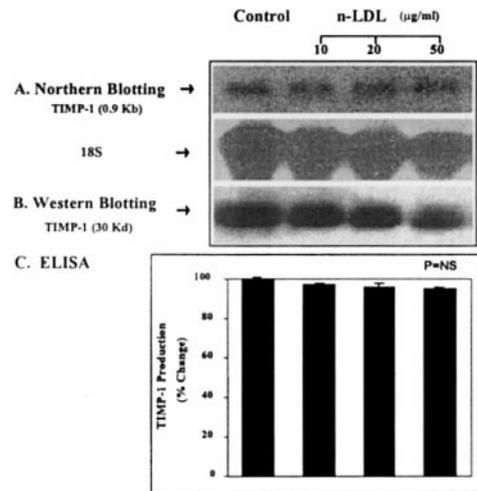
ox-LDL doses of 10 to 50 µg/mL compared with control (n=6,  $P<0.05$ ; Figure 3C). The Western blot analysis data supported this observation (Figure 3B). On the other hand, n-LDL did not alter TIMP-1 expression significantly (Figure 4).

**Effects of HDL on ox-LDL–Induced MMP-9 Expression**

When HDL (100 µg/mL) was added to culture medium, the ox-LDL–induced increase in MMP-9 protein expression was significantly inhibited. As determined by ELISA, ox-LDL (20 µg/mL) increased MMP-9 protein expression, but this increase was reduced by HDL (n=3,  $P<0.05$ ; Figure 5, lane 4). Western blot analysis confirmed the above observations



**Figure 3.** Effects of ox-LDL on TIMP-1 expression. Macrophages were treated with various doses of ox-LDL (10, 20, and 50 µg/mL). In separate experiments, cells were harvested at 4 hours for assay of TIMP-1 mRNA expression (A, n=3). Culture medium was collected at 24 hours for measurement of TIMP-1 protein expression (B and D, n=6) and its matrix-degrading activity (C, n=6). \* $P<0.05$  vs untreated control.

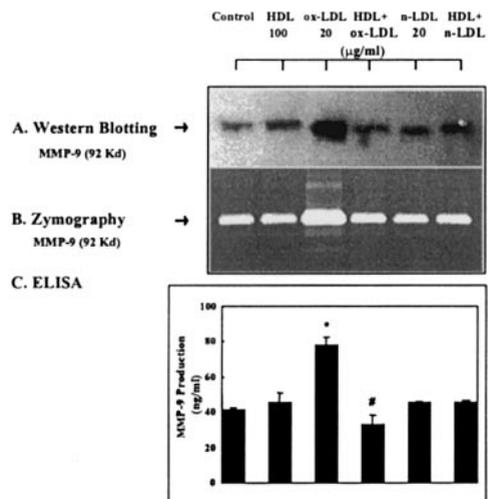


**Figure 4.** Effects of n-LDL on TIMP-1 expression. Macrophages were treated with various doses of n-LDL (10, 20, and 50 µg/mL). In separate experiments, cells were harvested at 4 hours for assay of TIMP-1 mRNA expression (A, n=3). Culture medium was collected at 24 hours for measurement of TIMP-1 protein expression (B and D, n=6) and its matrix-degrading activity (C, n=6).

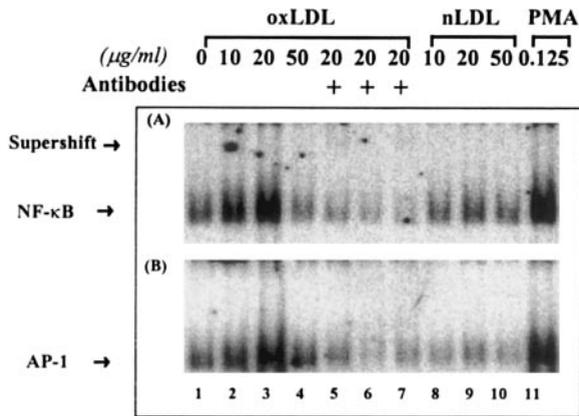
(Figure 5A). Furthermore, HDL (Figure 5B, lane 4) suppressed matrix-degrading activity of MMP-9 induced by ox-LDL. HDL had no effect on MMP-9 activity of macrophages incubated with n-LDL (Figure 5, lane 6).

**Effects of ox-LDL and n-LDL on Nuclear Binding of Transcription Factors AP-1 and NF-κB**

After 2 hours of incubation with ox-LDL, cells were harvested for evaluation of the transcription factor NF-κB and AP-1 bindings. Exposure to PMA (125 ng/mL) served as a positive control. Autoradiographic data obtained from EMSA demonstrated a low level of NF-κB and AP-1 activation in untreated



**Figure 5.** Effects of HDL on ox-LDL–induced MMP-9 expression. Macrophages were incubated with 20 µg/mL of ox-LDL or n-LDL in presence of 100 µg/mL HDL. Culture medium was collected at 24 hours for measurement of MMP-9 protein expression (A and C, n=6) and its matrix-degrading activity (B, n=6). \* $P<0.05$  vs untreated control; # $P<0.05$  vs ox-LDL–treated culture.



**Figure 6.** Effects of ox-LDL and n-LDL on NF- $\kappa$ B and AP-1 binding. Macrophages were treated with various doses of ox-LDL or n-LDL (10, 20, and 50  $\mu$ g/mL). Cells were harvested at 2 hours for assay of transcription factors by EMSA ( $n=4$ ). A and B, NF- $\kappa$ B and AP-1 binding, respectively. Lane 1, probes alone; lane 2, clod oligonucleotides; lane 3, control; lanes 4 through 6, ox-LDL 10, 20, and 50  $\mu$ g/mL; lane 7, antibody to p50 in A and antibody to *c-fos* in B; lane 8, antibody to p65 in A and antibody to *c-jun* in B; lane 9, antibodies to p50 and p65 in A and antibodies to *c-fos* and *c-jun* in B; lanes 10 through 12, n-LDL 10, 20, and 50  $\mu$ g/mL; and lane 13, PMA.

macrophages ( $n=4$ ; Figure 6A and 6B, lane 1). Ox-LDL treatment increased NF- $\kappa$ B and AP-1 bindings at concentrations of 10, 20, and 50  $\mu$ g/mL (lanes 2 through 4). The gel supershift assay data showed that incubation with the p50 antibody resulted in supershift (Figure 6A, lanes 5 and 7). Addition of p65 antibody resulted in a reduction in the complex (Figure 6A, lane 6). Similarly, *c-fos* and *c-jun* antibodies resulted in a reduction in AP-1 complex as (Figure 6B, lanes 5 through 7). n-LDL had no significant effect on the binding of the transcription factors (Figure 6A and 6B, lanes 8 through 10).

## Discussion

### Main Findings

This study demonstrates that unlike n-LDL, copper-oxidized LDL upregulates MMP-9 mRNA, protein, and activity levels while downregulating TIMP-1 expression in human monocyte-derived macrophages. Our data also demonstrate increased nuclear bindings of NF- $\kappa$ B and AP-1 transcription factors by ox-LDL. Furthermore, MMP-9 protein upregulation induced by ox-LDL was abrogated by coincubation with HDL.

### MMP Regulation

MMPs are a family of proteases produced by macrophages and a wide variety of other cells that are secreted in zymogen form requiring extracellular activation.<sup>15-17</sup> MMPs are active at neutral pH, require zinc and calcium as cofactors, and are capable of degrading virtually all the components of extracellular matrix. The MMPs are tightly regulated not only at the transcriptional level but also by their requirement for extracellular activation and by specific inhibitors such as TIMP-1 and TIMP-2 that are cosecreted with the MMPs.<sup>15-17</sup> The MMP and TIMP genes are regulated by the transcription factors NF- $\kappa$ B and AP-1.<sup>18-20</sup> Among the MMPs, MMP-1 (interstitial collagenase) specifically cleaves collagen types I, II, and III; MMP-3 (57-kDa stromelysin-1) is active on collagen type IV, IX, and X,<sup>21</sup>

whereas MMP-2 (72-kDa gelatinase) and MMP-9 (92-kDa gelatinase) degrade denatured collagen and elastin.<sup>22</sup> Several lines of evidence support the potential role of MMPs in human atherosclerosis and plaque disruption. MMP-1, MMP-2, MMP-3, MMP-9, and TIMP-1 proteins have been demonstrated in macrophages and SMCs in rupture-prone shoulder regions of plaques.<sup>7,23,24</sup> MMP-3 mRNA expression has been demonstrated in the plaque and localized to macrophages and SMCs,<sup>25</sup> whereas MMP-9 was expressed in human monocytes/macrophages.<sup>26</sup> Foam cells derived from aortas of cholesterol-fed rabbits have been shown to express MMP in cell cultures.<sup>4</sup> Human monocyte-derived macrophages have been shown to express MMP-1 and MMP-2 with the ability to induce collagen breakdown in fibrous caps of atherosclerotic plaques.<sup>5</sup> However, the precise factors that stimulate MMP elaboration in human lesion-associated macrophages have not been well characterized. Exposure to cytokines,<sup>26</sup> mechanical injury,<sup>27</sup> inflammatory mediators,<sup>28</sup> free radicals,<sup>29</sup> mast cell-derived proteases, or collagen itself<sup>30</sup> could be a potential culprit. Increased MMP activation by oxidant stress has been shown recently.<sup>31</sup> Whether cholesterol loading and/or increased oxidant stress generated by exposure to ox-LDL plays a critical role in mediating the effects observed in this study warrants further inquiry. The effect of lipoproteins on MMP or TIMP expression in macrophages has not been previously reported. Our results suggest that ox-LDL induces MMP-9 gene expression in human macrophages. The results of this study are in keeping with the observation from previous studies showing that ox-LDL alone serves as a primary regulator of cellular function and gene expression.<sup>32,33</sup>

Previous studies have shown that TIMP-1 is cosecreted with MMP-9 and that the catalytic activity of MMP-9 is controlled by the simultaneously secreted counterregulatory molecule TIMP-1.<sup>15-17</sup> However, little is known about the effect of lipoproteins on TIMP-1 expression. In the present study, we found that ox-LDL reduced TIMP-1 mRNA and protein expression in cultured human monocyte-derived macrophages, thereby favoring a net increase in matrix-degrading activity. Our data showing reduced TIMP-1 expression by ox-LDL suggest that the regulation of MMP-9 and TIMP-1 expression may be under the control of separate mechanisms. The ability of HDL to inhibit ox-LDL-induced MMP-9 expression is consistent with a body of work that demonstrates the ability of HDL to abrogate ox-LDL-mediated cellular events.<sup>34</sup> Although the mechanism of this effect of HDL remains to be determined, it is possible that HDL scavenges or alters the biologically active components of ox-LDL such as lysophosphatidylcholine or exerts an antioxidant effect through HDL-associated enzymes PAF acetylhydrolase and paraoxonase.<sup>35,36</sup> Alternatively, it could be that HDL influences ox-LDL-induced MMP-9 expression by enhancing cholesterol efflux from intracellular pools to macrophage surface.<sup>37</sup>

### Clinical Implications

The findings of the present study suggest that ox-LDL may influence matrix turnover in atherosclerotic plaques by upregulating MMP-9 and downregulating TIMP-1 expression, thereby favoring net matrix degradation and thus predisposing plaque to rupture and remodeling the arterial wall. These studies also provide evidence for another mechanism by which HDL may have favorable effects in atherosclerosis.

## Conclusions

Unlike unmodified LDL, ox-LDL upregulates MMP-9 and downregulates TIMP-1 expression in monocyte-derived macrophages, and this effect is abrogated by HDL.

## Study Limitations

Our data do not provide insight into the signaling pathways through which ox-LDL regulates MMP-9 expression. Although the focus of this study was on evaluating the effect of ox-LDL on MMP-9 expression, ox-LDL may influence other members of the MMP family such as MMP-1, MMP-2, MMP-3, and membrane type of MMP. We are currently engaged in studies designed to evaluate the potential effects of ox-LDL on other members of the MMP family.

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