Effect of macrophage differentiation and exposure to mildly oxidized LDL on the proteolytic repertoire of THP-1 monocytes

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Abstract Lipid-laden monocyte/macrophages in atherosclerotic plaques can produce a range of proteinases capable of degrading components of the plaque extracellular matrix, an event that may weaken plaques, rendering them vulnerable to rupture. The effects of differentiation from monocytes to macrophages and exposure to mildly oxidized LDL (Ox-LDL) on the expression of a range of proteinases and their inhibitors were assessed in the human THP-1 cell line. Of 56 proteinases/inhibitors investigated, 17 were upregulated during macrophage differentiation, including several matrix metalloproteinases (MMPs) and cathepsins along with their native inhibitors. Similarly, expression of matrix-degrading proteinases was also increased during differentiation of human primary macrophages. In conjunction, the proteolytic capacity of the cells increased, as assessed by substrate zymography. Subsequent exposure of differentiated THP-1 cells to mildly Ox-LDL increased the expression of a control gene (adipocyte lipid binding protein) and increased the activity of nuclear factor-κB and activator protein-1 in serum-free conditions but did not significantly affect the expression of any of the proteinases or inhibitors investigated. These results indicate that in this model macrophage differentiation, rather than exposure to Ox-LDL, has a more important effect on the expression of genes involved in extracellular matrix remodeling.—Whatling, C., H. Björk, S. Gredmark, A. Hamsten, and P. Eriksson. **Effect of macrophage differentiation and exposure to mildly oxidized LDL on the proteolytic repertoire of THP-1 monocytes. *J. Lipid Res.* 2004.** 45: **1768–1776.**

Supplementary key words atherosclerosis • proteinases • matrix metalloproteinase • cathepsin • microarray • low density lipoprotein

The clinical event most commonly associated with acute coronary syndromes is plaque rupture and subsequent thrombosis (1). Rupture is often associated with the breakdown of extracellular matrix (ECM) in the shoulder region of a plaque (2–6). Localized destruction may weaken the plaque, perhaps placing it at risk of disruption. ECM breakdown is mediated by proteinases, in particular matrix metalloproteinases (MMPs). Currently, 22 different MMPs have been characterized that together have the capacity to degrade all components of the ECM (7). In addition, cathepsins may be secreted from cells in which they retain some activity and can degrade elastin (8, 9). Serine proteinases such as plasmin, tissue plasminogen activator, and urokinase plasminogen activator may also influence ECM breakdown by activating other proteinases (10). The activity of these proteinases is tightly controlled at several levels, including transcription, zymogen processing, and interaction with specific inhibitors. In the case of MMPs, inhibition is mediated by tissue inhibitors of MMPs (TIMPs). Cathepsins can be inhibited by several proteins, including cystatins, and serine proteinases can be inhibited by serpins such as plasminogen activator inhibitor-1 and α1-anti-trypsin.

Macrophage foam cells are an important source of matrix-degrading proteinases in atherosclerotic plaques (11). The principal agent promoting foam cell formation is believed to be LDL that has become oxidized after entrapment in the arterial wall (12). This oxidized LDL (Ox-LDL) is recognized by macrophage scavenger receptors that can facilitate its uptake, leading to cholesterol accumulation in cytoplasmic inclusions and lysosomes (13). Ox-LDL does not exist as a single entity but as a continuous spectrum of molecules ranging from minimally modified to highly oxidized forms (14). Moreover, a wide range of different modifications may occur during oxidation (14). It is therefore not straightforward to identify the ef-

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Abbreviations: ALBP, adipocyte lipid binding protein; AP-1, activator protein-1; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; MDA, malondialdehyde; MMP, matrix metalloproteinase; NFκB, nuclear factor-κB; Ox-LDL, oxidized low density lipoprotein; PBMC, peripheral blood mononuclear cell; TBARS, thiobarbituric acid-reactive species; TIMP, tissue inhibitor of matrix metalloproteinases.

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fects of Ox-LDL on macrophage phenotype. Many studies have assessed the effect of highly Ox-LDL on foam cell formation. However, this may contain low amounts of cholesterol and as a consequence may not be the ideal substrate for foam cell formation (15). In contrast, several reports have demonstrated that mildly Ox-LDL retains significant amounts of unmodified cholesterol and can effectively induce foam cell formation in human and mouse macrophages (16, 17).

Several studies have investigated the effect of Ox-LDL on proteinase gene expression. In vascular endothelial cells, an increase in the expression of MMP-1 and -14 has been reported (18–20). MMP-14 expression may also increase in vascular smooth muscle cells in response to Ox-LDL (21). In macrophages derived from primary monocytes, MMP-9 was found to be increased 2-fold after exposure to Ox-LDL (22, 23). In the same experiment, TIMP-1 expression was reduced by Ox-LDL, suggesting an imbalance toward matrix degradation, although the absolute levels of MMP-9 and TIMP-1 were not compared (23). In contrast, a recent study found that Ox-LDL had no effect on the expression of MMP-1, MMP-9, or TIMP-1 in primary monocytes (24). Ox-LDL did significantly increase MMP-1 expression when an inflammatory stimulus was provided. However, MMP-9 expression was only slightly increased and expression of TIMP-1 was not affected (24). In all of these studies, candidate proteinases have been investigated. To address the effect of Ox-LDL on proteinase production more comprehensively, we have monitored the expression of a range of proteinases and their inhibitors in the monocyte/macrophage cell line THP-1. The high variability in the nature of Ox-LDL necessitated that a large number of replica experiments be performed. On this basis, we reasoned that the most reliable findings would be obtained by using a cell line, minimizing additional variability attributable to cell phenotype. Using this model, we investigated whether differentiation from monocytes to macrophages is associated with an increase in the proteinase repertoire and whether subsequent exposure to mildly Ox-LDL could modulate this.

METHODS

Cell culture and differentiation of THP-1 cells

THP-1 cells were grown in RPMI-1640, 10% FCS supplemented with 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml). To differentiate monocytes into macrophages, cells were plated at 4×10^5 cells/ml and stimulated with 50 ng/ml PMA for 24 h. Cells were washed twice with PBS before adding fresh medium and growing for up to 5 days. As an alternative method to differentiate cells, 24 h conditioned medium from THP-1 cells prepared in this way was added to THP-1 monocytes followed by incubation for 24 h. This differentiation protocol precluded direct effects of PMA on protein kinases.

Establishment of macrophage cultures

Peripheral blood mononuclear cells (PBMCs) from healthy donors (25) were plated (Primaria, Falcon; Becton Dickinson, San Jose, CA) at 18×10^6 cells/ml in Iscove's modified medium with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY), and 10% AB serum. After incubation at 37°C for 1 h, nonadherent cells were removed and the cultures were extensively washed and treated with supernatant containing "allo-cytokines" produced by the allogeneic reaction between T-cells and monocytes to induce macrophage differentiation (26). Briefly, PBMCs from different donors were mixed and incubated for 24 h in Iscove's complete medium. The supernatant was collected, cleared by centrifugation, and used to stimulate separate monocyte cultures. Twenty-four hours after stimulation, the cells were washed with Iscove's medium and cultured in complete 60/30 medium (60% AIM-V medium, 30% Iscove's modified medium, 10% AB serum, L-glutamine, penicillin, and streptomycin) for another 5 days. RNA samples were prepared from the monocyte cultures (cells incubated for 1 h) and macrophage cultures using the RNeasy minikit (Qiagen) according to the manufacturer's protocol.

LDL preparation and addition to cells

Human LDLs were isolated by sequential density gradient ultracentrifugation using KBr solution (1.030–1.053 g/ml) and dialyzed against PBS (27). Protein content was measured using the Bradford assay. Preparations were stored in the dark at 4°C and used within 4 days. LDL, Ox-LDL (50 μg/ml), or PBS carrier was added to THP-1 cells in fresh culture medium 24 h after the removal of PMA, followed by incubation for up to 4 days. When serum-free conditions were used, cells were incubated in serum-free medium for 2 h before adding LDL preparations or PBS carrier.

LDL oxidation

LDLs (500 μg/ml) were exposed to ultraviolet irradiation at 254 nm for a specified time (1–5 h), then sterilized by filtration (0.45 μM). The extent of oxidation was determined by measuring the amount of thiobarbituric acid-reactive species (TBARS), using malondialdehyde (MDA) standard (28, 29). TBARS were recorded as nanomoles of MDA per milligram of LDL protein.

RNA isolation

mRNAs were isolated using a MicroFastTrack 2.0 kit (Invitrogen). Total RNAs were isolated using an RNeasy Mini kit (Qiagen).

cDNA microarrays

A total of 136 cDNAs were PCR amplified and printed, of which 57 corresponded to genes encoding proteinases or proteinase inhibitors. In addition, the arrays contained cDNAs for a number of matrix proteins, genes involved in lipid metabolism, transcription regulators, and housekeeping genes. Microarrays were printed in triplicate on glass slides using a GMS 417 arrayer. Probes were prepared from mRNA by reverse transcription using a CyScribe First-Strand cDNA labeling kit (Amersham Pharmacia) incorporating either Cy3-UTP or Cy5-UTP. Microarrays were scanned using a GMS 418 scanner, and fluorescence was quantified using ScanAlyse 2.0 (M. Eisen, Stanford University) and ArrayGauge version 1.2 (Fujifilm) software. Data were normalized by using housekeeping genes and/or by assuming that the total spot intensity should be approximately the same for both probes. A gene was quantified only if the signal in one channel was at least 2-fold above the local background. Genes were also excluded if the signal was above the maximum detection threshold.

Northern hybridizations

Northern transfers and hybridizations were performed as described (30). Probes were prepared from the same cDNA clones used to prepare microarrays. Quantification was performed after exposure to a phosphorimage plate (Fujifilm) using ImageGauge software (Fujifilm). Transcript levels were normalized to GAPDH.

Real-time polymerase chain reaction

RNA from each sample (0.5 μg) was reverse transcribed using Superscript II according to the manufacturer's manual (Invitrogen). After dilution of the cDNA to 100 μl, 3 μl of cDNA was amplified by real-time PCR with 1× TaqMan universal PCR mastermix (Applied Biosystems). For CD11b, MMP-9, MMP-14, and cathepsin D, the Assay on Demand Kit from Applied Biosystems was used. RPLP0 was used as a housekeeping gene to normalize for RNA loading. For β-actin, 1 μM of each primer and 0.25 μM of probe were used. Primers were designed using Primer Express software (Applied Biosystems). The primers for RPLP0 were as follows: FW, 5′-CCATTCTATCATCAACGGGTACAA-3′; RW, 5′-AGCAAGTGGGAAGGTGTAATCC-3′; and the probe was 6FAM5′-TCTCCACAGACAAGGCCAGGACTCGT-3′TAMRA. Each sample was analyzed in triplicate using ABI Prism 7000 (Applied Biosystems). The PCR amplification was related to a standard curve.

Zymography

Ten percent SDS-PAGE (acrylamide-bisacrylamide, 29:1) gels were prepared containing gelatin (type A; Sigma) or casein (technical grade; Sigma) at 1 mg/ml or type I collagen (acid soluble; Sigma) at 0.5 mg/ml. Electrophoresis and zymography were performed as described elsewhere using 15 μl aliquots of culture medium (31, 32). Concentration of medium supernatants for analysis of MMP-1 was performed using Microcon-YM 30 columns (Millipore).

Western blotting

Culture medium (15 μl) was electrophoresed using 10% SDS-PAGE (acrylamide-bisacrylamide, 29:1). Western transfer to polyvinylidene difluoride membranes (Millipore), blocking, and incubation with antibodies were performed according to standard protocols (33). To detect MMP-9, monoclonal anti-human MMP-9 (MAB911; R&D Systems) was used at 2 μg/ml. Horseradish peroxidase-labeled secondary antibody was detected using enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech).

Quantification of MMP-9 and TIMP-1 protein levels in medium supernatants

MMP-9 and TIMP-1 were measured using a BIOTRAK MMP-9 activity assay and BIOTRAK TIMP-1 ELISA system (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay

Nuclear extract preparations and electrophoretic mobility shift assays (EMSAs) were performed as described elsewhere (34). Probe sequences (forward strand) were as follows: nuclear factor-κB (NFκB) consensus, 5′-AGTTGAGGGGACTTTCCCAGGC-3′; activator protein-1 (AP-1) consensus, 5′-CGCTTGATGACTCAGCCGGAA-3′. Supershift reactions were performed using 2 μg of NFκB p50 (NLS) or NFκB p65 (A) (Santa Cruz Biotechnology, Inc.).

Statistical analyses

Data are shown as means ± SD. Differences in gene expression between groups were compared using a paired Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Differentiation to macrophages increases the proteinase repertoire of THP-1 cells

cDNA microarrays were used to compare gene expression in THP-1 macrophages and monocytes (**Table 1**).

TABLE 1. Changes in gene expression when THP-1 monocytes differentiate to macrophages

Activity	Gene	Fold Change
Upregulated	Proteinases	
	MMP-1	6.6
	MMP-2	3.4
	MMP-9[a]	4.2, 5.0
	MMP-14	3.2, 2.2
	Cathepsin A	8.8, 7.8
	Cathepsin B[a]	1.6, 1.3
	Cathepsin D[a]	10.4, 11.2
	Cathepsin H[a]	2.5, 1.7
	Cathepsin L[a]	1.5, 1.6
	Cathepsin O[a]	2.3, 1.7
	Cathepsin S[a]	6.9, 7.8
	ADAM-15	5.0, 4.2
	Inhibitors	
	TIMP-1	6.2, 7.7
	TIMP-2	2.1, 2.0
	Cystatin B	2.3, 1.9
	Cystatin C	2.9, 1.9
	Neutrophil elastase inhibitor	2.0, 2.2
Unchanged	Proteinases	
	MMP-17	1.0, 1.3
Downregulated	Proteinases	
	Cathepsin G	0.37, 0.15
	Neutrophil elastase	0.36, 0.20
	Inhibitors	
	TIMP-3	0.33, 0.42
Not expressed	Proteinases	
	Other MMPs	NA
	Cathepsin K	NA
	ADAMTS-1, -4, -5, -7, -8	NA
	ADAM-17	NA
	Plasminogen	NA
	t-PA and u-PA	NA
	Inhibitors	
	TIMP-4	NA
	PAI 1 and 2	NA
	α1-proteinase inhibitor	NA
	Elafin	NA
	Kininogen	NA

ADAM, a disintegrin and metalloproteinase domain; ADAMTS, a disintegrin-like and metalloproteinase with thrombospondin type I motif; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; TIMP, tissue inhibitor of matrix metalloproteinases; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator. Probes were prepared using mRNA isolated from THP-1 monocytes and THP-1 macrophages 24 h after the removal of PMA and cohybridized to microarrays. Fold change refers to the expression level in macrophages relative to the expression level in THP-1 monocytes. Data from two separate experiments are indicated. For MMP-1 and MMP-2, fold change was calculated based upon the result of Northern hybridization using the same RNA. NA, not applicable.

[a] Background intensity was used to calculate the fold change.

Monocytes expressed high levels of cathepsin G and neutrophil elastase. However, the other proteinases examined were either not expressed or expressed at low levels. Differentiation to macrophages resulted in the expression of a larger repertoire of proteinases and proteinase inhibitors. In contrast, the expression of TIMP-3, cathepsin G, and neutrophil elastase decreased after differentiation. Only MMP-17 was expressed at a similar level in monocytes and macrophages. Because PMA is a potent regulator of protein kinase C activation, it is possible that some of the effects observed are attributable to protein kinase C phosphorylation status rather than to differentiation per

se. To address this, an alternative method was used to differentiate THP-1 cells, in which conditioned medium from PMA-differentiated THP-1 cells was added to THP-1 monocytes. As shown in **Fig. 1A** changes in gene expression akin to those observed after PMA stimulation were found. Because the conditioned medium did not contain any PMA, these results support the idea that at least some of the effects seen in the microarray experiment reflect differentiation. Furthermore, to validate THP-1 cells as a model for macrophage differentiation, the expression of some proteases was analyzed in human primary monocytes and macrophages. As shown in Fig. 1B, changes in gene expression almost identical to those observed in THP-1 cells were found.

Gene expression was followed for up to 5 days after the removal of PMA (**Fig. 2A**). In conjunction with an increase in gene expression, an increase in the proteolytic activity against gelatin, type I collagen, and casein was detected in the culture supernatants (Fig. 2B, C, D; note that medium was changed each day for the analysis of superna-

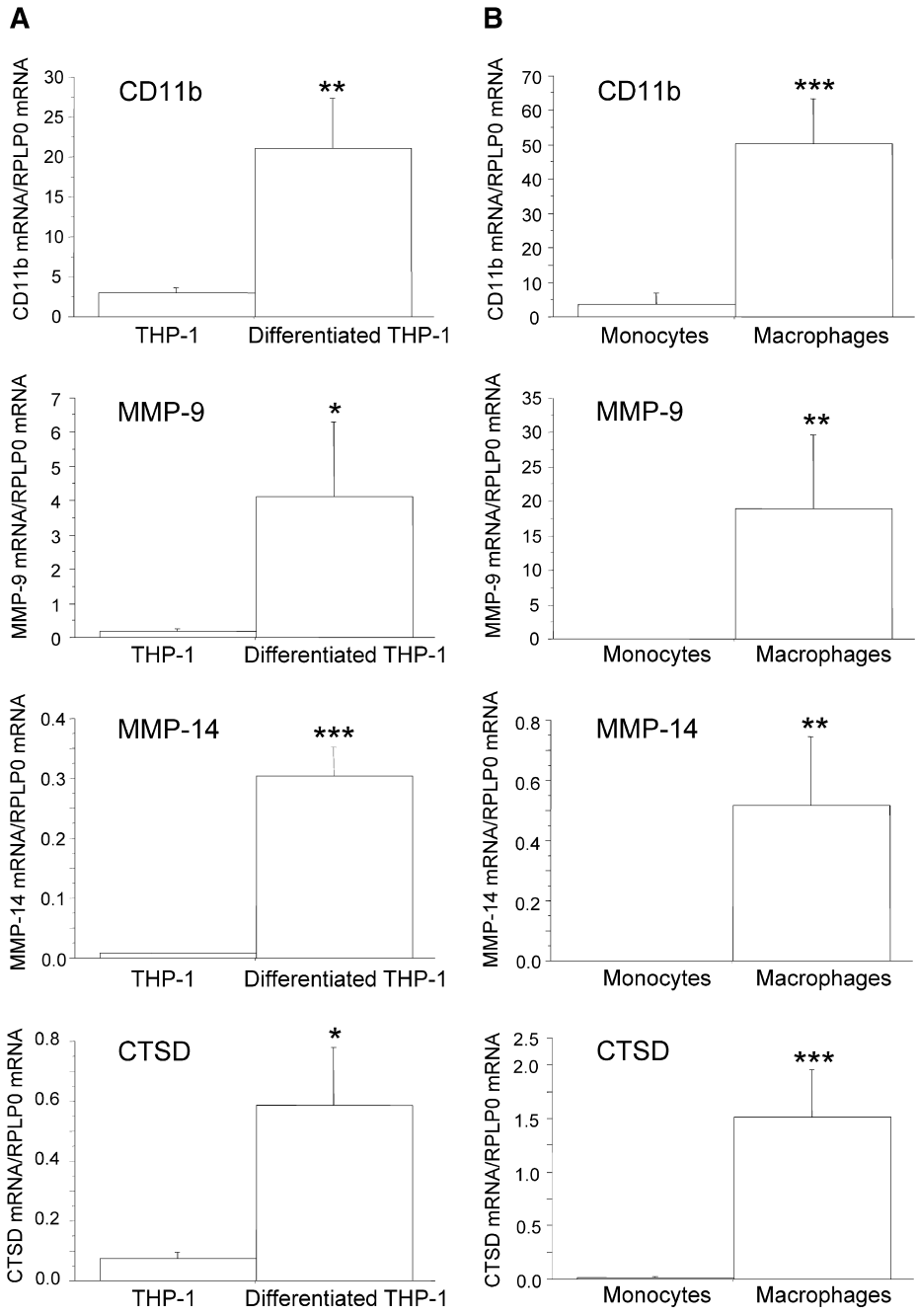

Fig. 1. TaqMan analysis of protease gene expression in monocytes/macrophages. A: Gene expression in undifferentiated THP-1 cells and THP-1 cells incubated with conditioned medium from PMA-differentiated THP-1 cells. B: Gene expression in human primary monocytes and macrophages. CTSD, cathepsin D; MMP, matrix metalloproteinase. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$ versus undifferentiated cells.

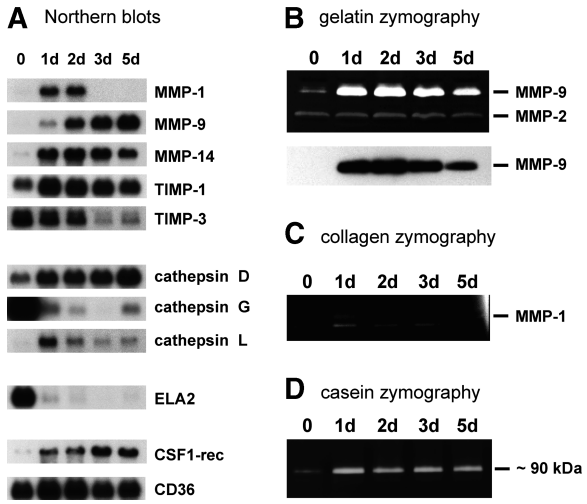

Fig. 2. Analysis of the proteinase repertoire of THP-1 cells during differentiation to macrophages. A: Northern blots to detect mRNA levels in THP-1 monocytes (0) and in THP-1 cells at intervals over a 5 day period after the removal of PMA stimulus (1d–5d). Colony-stimulating factor-1 receptor (CSF1-rec) and CD36 were measured as controls for macrophage-like differentiation. TIMP, tissue inhibitor of matrix metalloproteinases. B, C, D: Gelatin, collagen, and casein zymography to detect the release of proteinases into the culture medium by THP-1 monocytes (0) and by macrophages at intervals 5 days after the removal of PMA (1d–5d). Medium was changed each day, such that only proteinases released in each 24 h period were measured. Proteolytic activities in the size range of MMP-9 (92 kDa), MMP-2 (72 kDa), and MMP-1 (52 kDa) were detected by gelatin and collagen type I zymography. The presence of MMP-9 was confirmed by Western blotting. In the case of MMP-1, culture supernatants were concentrated 10-fold before zymography. A proteolytic band migrating at ~90 kDa was detected by casein zymography. This proteinase was also detected when 10 mM EDTA was included in the incubation buffer, indicating that it is probably not an MMP. A possible candidate is plasminogen, but this has not been validated.

tants by zymography and that in the case of MMP-9 this reduced levels of the transcript). By the end of the 5 days, some loss of macrophage phenotype was evident, exemplified by the reexpression of cathepsin G. However, cells remained attached to the plastic surface.

Lipid accumulation by and viability of THP-1 cells incubated with Ox-LDL

LDL with TBARS between 6.0 and 34.0 was classified as mildly oxidized (35, 36). Ox-LDLs were added to THP-1 cells 24 h after the removal of PMA. Oil Red O staining was used to monitor cholesterol accumulation, and trypan blue staining was used to monitor viability. Accumulation of lipid was observed by 24 h (data not shown). No significant effect of mildly Ox-LDL on THP-1 viability was observed throughout the experiments.

Mildly Ox-LDL does not have a significant effect on the expression of ECM-remodeling genes in THP-1 cells

Mildly Ox-LDLs were added to THP-1 cells in complete medium, and RNA was isolated 6, 24, 48, and 96 h later. Time-matched RNAs from control (PBS carrier) and Ox-LDL-treated cells were compared by cDNA microarrays in three separate experiments (Ox-LDL TBARS 13.7 ± 4.4). It was not possible to identify any genes affected by the Ox-LDLs (data not shown). Northern hybridizations were also used to monitor the expression of 11 selected genes in response to 9 different mildly Ox-LDLs (TBARS 19.5 ± 9.0) after 6 and 24 h (**Fig. 3**). In agreement with the cDNA microarray analyses, mildly Ox-LDL did not substantially affect gene expression. As a positive control, the expression of adipocyte lipid binding protein (ALBP; FABP4) was measured in the same RNA samples (35, 37, 38). ALBP was modestly affected after 6 h (on average 1.3-fold, $P < 0.05$), and levels after 24 h were on average 1.6-fold above the level in the presence of native LDL ($P = 0.10$) (Fig. 3). A significant correlation between ALBP levels at 24 h and TBARS between 6.2 and 19.7 was found ($r = 0.91$, $P < 0.01$, N = 7). MMP-9 and MMP-14 expression showed a small but statistically significant increase after exposure of Ox-LDL (on average, 1.3-fold, $P < 0.05$).

Gene expression was also monitored in two experiments in the absence of serum. Of 10 genes analyzed, only expression of ALBP was increased and only after 24 h of exposure to Ox-LDL (**Fig. 4**). Transcript levels of MMP-9 and MMP-14 in the presence of Ox-LDL were also above control levels after 24 h. However, in the absence of serum the expression of both genes was significantly lower at 24 h than at 6 h, implying a reduction in gene expression after serum removal. No significant effect on MMP-9 protein levels was found [MMP-9 levels in culture supernatants after 24 h in the presence of LDL, Ox-LDL (TBARS 14.8), and Ox-LDL (TBARS 34.0): 13.9 ± 1.2, 13.7 ± 0.7, and 15.8 ± 0.3 ng/ml, respectively (n = 3)]. Measurement of TIMP-1 in the same samples indicated a substantial excess of inhibitor to MMP-9 [mean TIMP-1 levels: 854.6, 872.1, and 792.5 ng/ml, respectively (n = 2)].

Mildly Ox-LDL can increase the activity of NFκB and AP-1 in THP-1 cells

As an alternative method to evaluate whether exposure to Ox-LDL had an effect on THP-1 cells, the levels of NFκB and AP-1 were measured by EMSA. Both Ox-LDL (TBARS 14.8) and Ox-LDL (TBARS 34.0) increased the level of NFκB and AP-1 in serum-free conditions (data not shown). The activity of NFκB and AP-1 was consistently higher when cells were incubated in medium containing serum. However, there was no evidence for any additional effect of mildly Ox-LDL on the activity of NFκB or AP-1 in the presence of serum (data not shown).

DISCUSSION

Using the monocytic cell line THP-1, we have investigated whether differentiation of monocytes to macrophages increases the potential for these cells to remodel ECM and whether subsequent exposure to Ox-LDL can modulate this. Whereas macrophage differentiation was associated with a significant increase in the proteolytic repertoire of THP-1 cells, exposure of cells to mildly Ox-LDL did not substantially modulate this capacity.

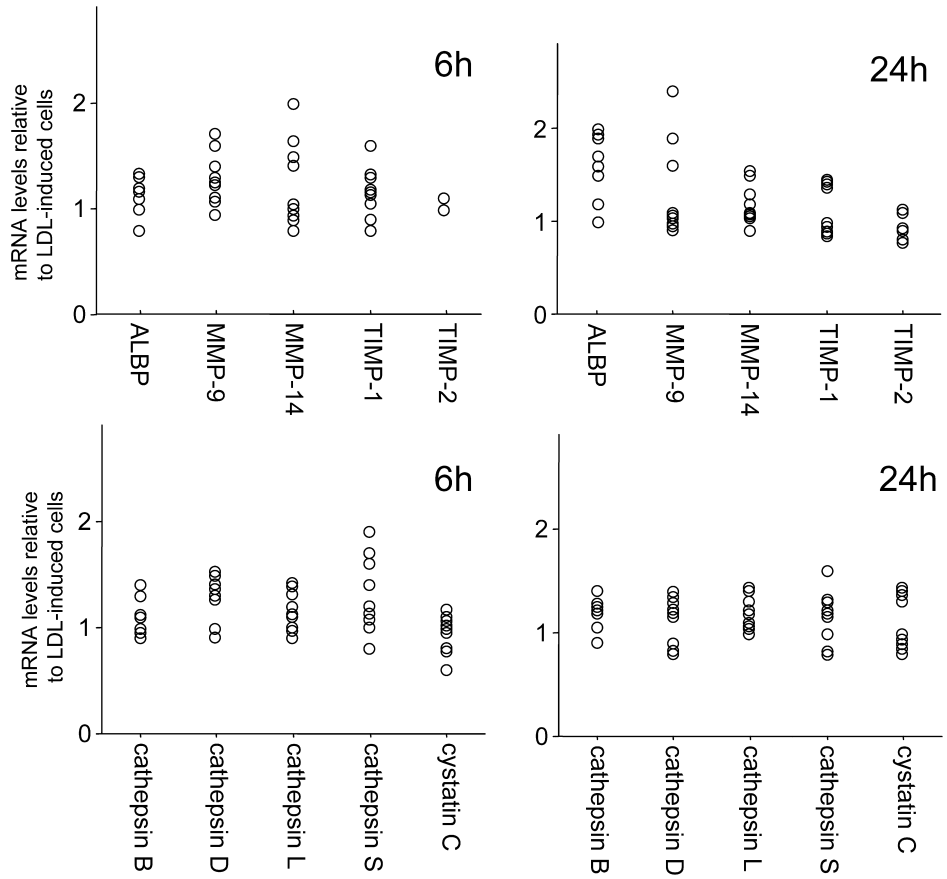

Fig. 3. The effect of mildly oxidized LDL (Ox-LDL) on the expression of selected genes in THP-1 macrophages. Cells were incubated with nine different Ox-LDLs [thiobarbituric acid reactive species (TBARS) 19.5 ± 9.0] for 24 h. RNA was isolated at 6 and 24 h, and transcript levels were measured by Northern hybridization. Values correspond to the GAPDH-normalized level in the presence of mildly Ox-LDL relative to the GAPDH-normalized level in the presence of the corresponding native LDL. Expression of TIMP-2 was often low, and values are shown only for experiments in which TIMP-2 mRNA levels could be reliably quantified. A significant correlation between adipocyte lipid binding protein (ALBP) levels at 24 h and TBARS was found ($r = 0.70$, $P < 0.05$).

In monocytic THP-1 cells, cathepsin G, neutrophil elastase, and MMP-17 were the only proteinases expressed at significant levels. Although myeloid progenitor cells synthesize cathepsin G and neutrophil elastase, they are produced primarily by neutrophils in the circulation (39). Their production by THP-1 cells is an indication that this cell line is arrested at an early stage of monocyte development (40) rather than a measure of the proteolytic capacity of monocytes. MMP-17 (MT4-MMP) has previously been identified in monocytes (41), in which its role may be associated with cell surface processing of cytokines (41, 42). In contrast to monocytes, THP-1 macrophages produced a repertoire of proteinases capable of degrading the major components of ECM. In particular, MMP-1, -2, -9, and -14 were all expressed as a result of differentiation. In parallel, expression of the specific inhibitors TIMP-1 and -2 was increased. Seven cathepsins were also upregulated during macrophage differentiation, perhaps reflecting an increase in the number of lysosomes during macrophage differentiation (43, 44). As for the MMPs, an increase in cathepsin gene expression was associated with an increase in the expression of their native inhibitors cystatin B and C. Although these studies demonstrate a dramatic increase in the proteolytic repertoire of macrophages, it is probable that this is an underestimate of the full potential. In addition to proteinases not addressed in our experiments, MMP-3, -7, -8, -12, and -13 and cathepsin K were all undetectable in THP-1 macrophages, in contrast to earlier findings that macrophages can express them (2, 5, 45–48). Expression of these genes may require additional factors not provided in our in vitro system, such as stimulation by cytokines or interaction of cells with an ECM, or may require differentiation to proceed further than was achieved here.

Macrophage differentiation progresses over several days, and in this model it may regress as soon as day 5. This is consistent with a similar study that indicated that THP-1 macrophages retain ~64% differentiated status up to 6 days after removal of PMA, on the basis of flow cytometric analysis of CD14 (37). Proteinase gene expression appeared as an early event in THP-1 differentiation; RT-PCR indicated that most of the proteinases were expressed as early as 6 h after the addition of PMA, although this could include direct effects of PMA on transcription in addition

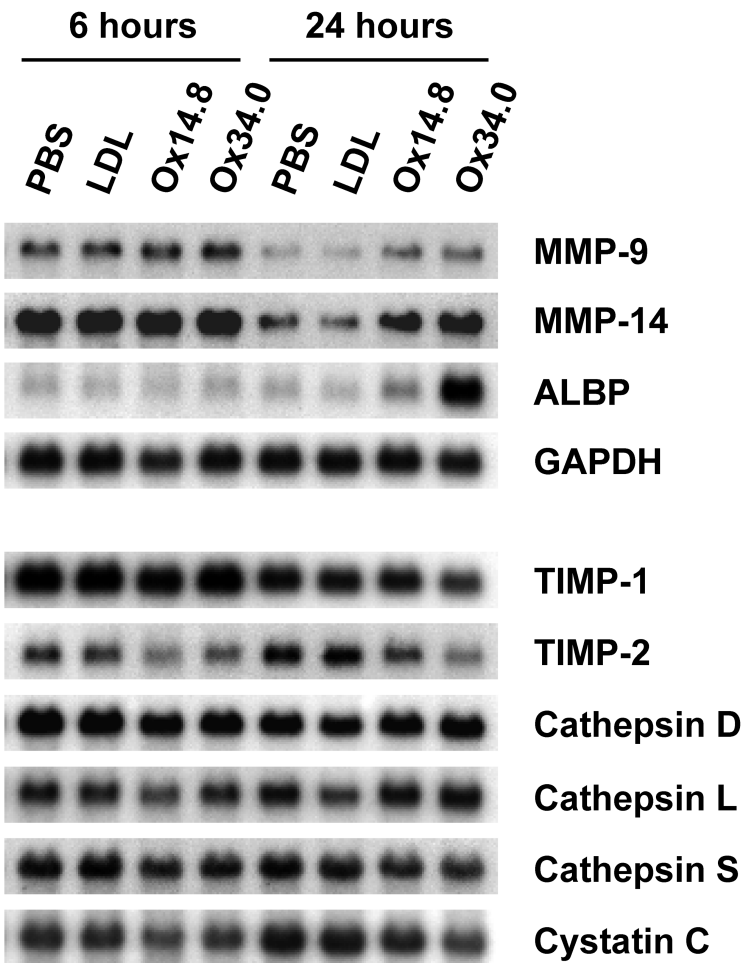

Fig. 4. The effect of mildly Ox-LDL on the expression of selected genes in THP-1 macrophages in serum-free conditions. Cells were incubated with PBS carrier, native LDL, or two mildly Ox-LDLs (TBARS 14.8 and 34.0) for 24 h. RNA was isolated at 6 and 24 h, and transcript levels were measured by Northern hybridization.

to its effect on differentiation. This would appear consistent with the life cycle of monocytes in vivo, where they may require an increase in proteolytic capacity to invade target tissues within which they can subsequently mature fully to macrophages.

Ox-LDL has been identified as a modulator of macrophage phenotype when taken up by cells within atherosclerotic plaques. In the present investigation, mildly Ox-LDL was not found to have a major effect on the proteinase repertoire of THP-1 macrophages. Based upon previous studies (18–20, 23), a more dramatic effect of Ox-LDL was expected. Several factors could explain the different conclusions. First, we used a monocytic cell line as opposed to primary monocytes in an effort to reduce variability between experiments. However, it is known that THP-1 cells differ in a number of respects from peripheral blood monocytes (40) and it is probable that differentiated macrophages also display differences. This could explain the absence of expression of certain proteinases, and it is possible that the response of signaling cascades differs. However, other studies using THP-1 cells have uncovered a variety of responses to Ox-LDL and have demonstrated activation of signaling pathways reported to be important in the response of primary monocytes to Ox-LDL (22, 37). Second, we have used ultraviolet exposure to produce mildly Ox-LDLs, reasoning that these would retain significant quantities of cholesterol and therefore promote foam cell formation (16, 17). Most studies have used copper to oxidize LDL, treatment that may produce a different range of modifications to LDL (49). As an index of oxidative modification, we measured MDA formation, and the TBARS we used were within the range previously shown to have an effect on MMP-9 and -14 in monocyte-derived macrophages and endothelial cells (20, 23). However, other oxidative events, such as the formation of oxysterols and lysophosphatidylcholine, and physical changes, such as particle diameter and LDL aggregation, were not measured and could account for differences in the magnitude of the effect. Alternatively, some of the effects seen with fully Ox-LDL could be attributed directly to the copper sulfate used in the oxidation step. Third, the PMA used to induce differentiation could have a direct effect on the expression of a number of the genes investigated, in particular MMP-1 and MMP-9. Cells were washed in PBS and left for at least 24 h in an effort to minimize direct PMA effects on transcription, but it is possible that expression levels of some genes were already maximal by the time Ox-LDL was added. On the other hand, differentiation of cells with conditioned medium suggested that at least some of the changes in gene expression observed during differentiation can occur in the absence of PMA. Moreover, gene expression changes akin

to those observed during THP-1 differentiation were also found for PBMCs differentiated to macrophages. Finally, most of our studies were done in the presence of serum to address the complexity of different signals that can confront macrophages in vivo and to maintain a macrophage-like phenotype of the THP-1 cells. It was noted that expression of ALBP was only dramatically induced when mildly Ox-LDL was added in serum-free conditions. However, even in the absence of serum, mildly Ox-LDL had little effect on the majority of the proteinase genes analyzed in this study. An increase in the activity of NFκB and AP-1 was observed only when mildly Ox-LDLs were added to cells in serum-free conditions, which may partly explain the lack of an effect of mildly Ox-LDL in the presence of serum. Despite all of these caveats, our results are consistent with a recent report that Ox-LDL alone is insufficient to alter MMP-1 and MMP-9 production in primary monocytes in the absence of an additional inflammatory stimulus (24).

Several studies have reported that an increase in MMP expression in response to Ox-LDL may lead to an imbalance toward matrix destruction attributable to alteration in the MMP/TIMP balance (19, 23). Measurement of TIMP-1 levels in serum-free conditions in our experiments indicated an ~250-fold molar excess of TIMP-1 over MMP-9. Clearly, the higher level of MMP-9 mRNA we saw in response to mildly Ox-LDL would have little impact on the proteinase/inhibitor balance in this situation. Although it would be dangerous to extrapolate too much from this in vitro observation, it is probable that Ox-LDL would have to have effects other than simply increasing MMP-9 gene expression to affect the balance of matrix destruction. In this context, the observation that lipid-loaded foam cells produce high levels of reactive oxygen species that may activate MMPs could be particularly relevant (50).

In summary, we have addressed the effects of macrophage differentiation and exposure to Ox-LDLs on a broad range of proteinases and inhibitors that may participate in ECM remodeling. In our experimental system, differentiation to macrophages was identified as the major influence on proteinase gene expression, although this was balanced by a concurrent increase in expression of the corresponding inhibitors. However, exposing the cells to mildly Ox-LDL did not substantially modify the repertoire.

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