Inhibition of lipopolysaccharide-induced interleukin-1β mRNA expression in mouse macrophages by oxidized low density lipoprotein

Loren G. Fong,[1,*] T. Annie T. Fong,[2,†] and Allen D. Cooper[*,]**

Research Institute,* Palo Alto Medical Foundation, Palo Alto, CA 94301; DNAX Research Institute of Molecular and Cellular Biology,† Palo Alto, CA 94304; and Department of Medicine,** Stanford University, Stanford, CA 94305

Abstract Recent studies have demonstrated the expression of messenger RNA (mRNA) for several cytokines within atherosclerotic arteries. Since cytokines have been shown to modulate functions of cultured arterial wall cells in a manner that could influence atherogenesis, this suggests that factors that modulate cytokine production would influence the atherosclerotic process. To examine whether lipoproteins can modulate cytokine production, the effect of lipoproteins on mouse macrophage interleukin-1β (IL-1β) mRNA expression was examined by dot blot and Northern blot analyses. Low density lipoprotein (LDL), acetylated-LDL, or malondialdehyde-LDL did not induce IL-1β mRNA expression or affect the expression in response to lipopolysaccharide (LPS). Similarly, copper ion-oxidized LDL did not stimulate the production of IL-1β mRNA. However, oxidized LDL inhibited the LPS-induced expression in a concentration- and time-dependent manner with a maximum inhibition (>90%) observed after a 2.5 h preincubation with 25 μg protein/ml. These conditions did not affect protein synthesis or phagocytosis and the inhibition was partially reversible after 24 h, which together suggest that the inhibition was not due to cell death. An inhibition of IL-1α and IL-6 mRNA expression was also observed while there was no change in γ-actin mRNA levels. The level of inhibition of IL-1β mRNA was dependent upon the extent of LDL oxidation, but did not correlate with recognition by the scavenger receptor. A non-receptor pathway was supported by two lines of evidence: *1*) the inhibition could be reproduced with a lipid extract, and *2*) oxidized LDL also inhibited scavenger receptor negative THP-1 cell IL-1β mRNA expression. Finally, oxidized LDL had no effect on the turnover of IL-1β mRNA, suggesting that the decreased accumulation of IL-1β mRNA is due to a decrease in gene transcription. Together these studies suggest that as macrophages become foam cells their immune responsiveness is attenuated. —**Fong, L. G., T. A. T. Fong, and A. D. Cooper.** Inhibition of lipopolysaccharide-induced interleukin-1β mRNA expression in mouse macrophages by oxidized low density lipoprotein. *J. Lipid Res.* 1991. **32:** 1899–1910.

Supplementary key words atherosclerosis • cytokines • lipid peroxidation • THP-1 cells • IL-1α • IL-6

Ultrastructural and immunologic studies of the developing atherosclerotic lesion have documented the temporal sequence of cellular events leading to foam cell formation (1–3). It is evident from these studies that the initiation and progression of the fatty streak lesion is dependent upon the close interaction of the cells involved and predicts that modulatory factors secreted by these cells would play an underlying role to regulate cellular function. Recent studies have suggested that cytokines might participate in this capacity. First, mRNA encoding cytokines have been identified in atherosclerotic lesions including IL-1β (4), IL-1α (5), macrophage-colony stimulating factor (M-CSF) (6), and tumor necrosis factor alpha (TNFα) (7). Second, tissue culture studies have demonstrated that artery wall cells can secrete cytokines (8–10) and that individual cytokines can modulate artery wall cell function of likely importance to the atherosclerotic process such as the induction of cell adhesion molecule expression (11), stimulation of PDGF secretion (12), and modulation of the LDL and scavenger receptor pathways (13–15). Together, these studies provide support for a potential role of cytokines in atherogenesis and suggest that modulation of cytokine production would affect the atherosclerotic process.

Lipoproteins can modulate a number of cellular processes including the secretion of factors by endothelial cells, smooth muscle cells, and macrophages (16–19). Included in this is the production of cytokines. Minimally

Abbreviations: mRNA, messenger RNA; LDL, low density lipoprotein; β-VLDL, beta-very low density lipoprotein; IL, interleukin; M-CSF, macrophage-colony stimulating factor; TNFα, tumor necrosis factor alpha; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; LPS, lipopolysaccharide; PLA_2, phospholipase A_2; PDGF, platelet-derived growth factor; BHT, butylated hydroxytoluene; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid; RM, reductively methylated.

[1]To whom correspondence should be addressed at: Research Institute, Palo Alto Medical Foundation, 860 Bryant Street, Palo Alto, CA 94301.

[2]Current address: Inflammation Biology and Immunology, Syntex Research, 3401 Hillview Avenue, Palo Alto, CA 94303.

Downloaded from www.jlr.org by guest, on June 18, 2012

oxidized LDL induces the secretion and mRNA expression of M-CSF by endothelial cells (6). LDL stimulates the production of TNF by smooth muscle cells (20). And after this work was begun, Hamilton, Ma, and Chisolm (21) reported that oxidized LDL could inhibit the expression of IL-1α and TNFα mRNA by macrophages. Thus the localized production of cytokines in the artery wall and the presence of native or modified forms of lipoproteins could together provide a mechanism for the regulation of cytokine production and, indirectly, artery wall function. To study further the modulation of cytokine production by lipoproteins and to examine the basis for the modulation of macrophage cytokine production by oxidized LDL, we have examined the effect of lipoproteins on macrophage IL-1β mRNA expression.

EXPERIMENTAL PROCEDURES

Materials

F-10 medium, RPMI 1640, endotoxin-tested Dulbecco's Modified Eagle's medium (DMEM), methionine-free DMEM, and fetal calf serum (FCS) were purchased from GIBCO Laboratories (Grand Island, NY). FCS was heat-inactivated before use (56°C for 30 min). *E. coli* lipopolysaccharide (LPS; serotype 026:B6), actinomycin D, and agarose-coupled phospholipase A_2 (4000 U/g agarose) were purchased from Sigma Chemical Co. (St. Louis, MO). The LPS was resuspended with sterile saline (2 mg/ml) and stored in aliquots at −20°C until use. Acetic anhydride and malondialdehyde bis(dimethyl acetal) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Carrier-free $Na^{125}I$, [S^{35}]methionine, [α-^{32}P]dATP, and [α-^{32}P]dCTP were purchased from Amersham (Arlington, IL).

Animals

Female Swiss Webster mice (27–30 g) were purchased from Simonsen Laboratories (Gilroy, CA) and given standard mouse chow and water ad libitum.

Methods

Oligonucleotides. Complementary DNA strands encoding for murine IL-1β (nucleotides 830–927), murine IL-1α (nucleotides 811–918), murine IL-6 (nucleotides 592–681), murine γ-actin (nucleotides 1262–1363), and human IL-1β (nucleotides 829–922) were synthesized and radiolabeled by fill-in polymerization. The specific activities of the labeled probes were approximately 100 μCi/μg DNA.

Lipoproteins. Human LDL (d 1.019–1.063 g/ml) and HDL (d 1.070–1.21 g/ml) were isolated from EDTA-treated plasma by density gradient ultracentrifugation (22) in the presence and absence of butylated hydroxytoluene (BHT, 20 μM). BHT was added to a portion of the plasma (usually one-third of the total amount) to prevent lipid peroxidation during the isolation procedure. All lipoproteins were dialyzed against PBS containing 0.01% EDTA, and for LDL isolated in the presence of the antioxidant, BHT was also added during dialysis. All lipoproteins were radiolabeled with $Na^{125}I$ using Iodogen (Pierce Chemical Co.) to specific activities of 100–200 cpm/ng.

Copper ion-mediated oxidation of LDL. LDL (100 μg protein/ml) was incubated in F-10 medium (2 ml) with added $CuSO_4$ (10 μM) in 6-well dishes at 37°C for 18–24 h. Usually, 12 ml of oxidized LDL was prepared at one time. The extent of lipid peroxidation was estimated by measuring the amount of thiobarbituric acid-reactive substances (TBARS) generated. The average amount of TBARS generated for the 26 different oxidized LDL preparations used in this study was 74.8 nmol MDA equivalents/mg LDL protein ± 2.6 (mean ± SE).

Chemical modifications of LDL. Acetylated LDL and malondialdehyde (MDA)-modified LDL were prepared essentially as described by Basu et al. (23) and Fogelman et al. (24), respectively, except that BHT was added during the incubations and dialysis. The modification of LDL was monitored by measuring the electrophoretic mobility of the lipoproteins on agarose gels. LDL was reductively methylated with two additions of formaldehyde and borohydride as described by Weisgraber, Innerarity, and Mahley (25).

Aggregation of LDL. LDL was aggregated as described by Khoo et al. (26). Briefly, LDL was resuspended in PBS containing 0.01% EDTA and 20 μM BHT at a concentration of 500 μg protein/ml. The solution was then vortexed at room temperature for 30 sec and then placed on ice immediately. The aggregated LDL was used within 15 min. Aggregation stimulated LDL degradation by macrophages greater than 10-fold relative to LDL without increasing the amount of TBARS generated.

Filtration of oxidized LDL. Oxidized LDL (2 ml) was added to an Amicon microconcentrator (Centricon 10) and centrifuged in a fixed-angle rotor at 4000 *g* for 1.5 h. The filtrate was saved and 2 ml of fresh F-10 medium was added to the retained oxidized LDL. The filtration unit was centrifuged again and the filtrate was discarded. The washed, retained oxidized LDL was then resuspended with fresh medium to 2 ml. After filtration, less than 10% of the total TBARS was located in the retained oxidized LDL fraction.

Phospholipase A_2 treatment of LDL. LDL (0.5 mg/ml) was incubated with agarose-bound PLA_2 (1 U/ml) in 0.1 M TRIS (pH 7.4) and 2 mM $CaCl_2$ (27) with the addition of BHT at 37°C for 0.5, 1, and 2 h. The suspension was centrifuged to sediment the enzyme and the solution containing the LDL was incubated with macrophages. Analysis by thin-layer chromatography showed that the amount of lysolecithin generated between 1 and 2 h incubation was similar to the amount of lysolecithin present in oxidized LDL (data not shown).

Downloaded from www.jlr.org by guest, on June 18, 2012

Cell lines. Human THP-1 cells were obtained from the ATCC and cultured in RPMI 1640 containing 10% FCS, 2 mM glutamine, and antibiotics.

Measurement of macrophage lipoprotein degradation. The degradation of lipoproteins by resident mouse peritoneal macrophages was performed as described previously (14). The rate of lipoprotein degradation was expressed as the amount of degradation products generated in micrograms per milligram cell protein after 4 h. Unless indicated, the mean and standard deviation of triplicate samples of a representative experiment are shown.

Measurement of macrophage cytokine mRNA expression by dot blot hybridization. Resident peritoneal macrophages were harvested and resuspended in DMEM supplemented with 10% FCS, 2 mM glutamine, and antibiotics (medium A) at 2.0×10^6 cells/ml and 3 ml was added to 6-well tissue culture plates (Costar). After incubation at 37°C for 30 min, the adherent macrophages were washed three times with medium A and incubated in the presence or absence of lipoproteins in medium A at 37°C for 2.5 h. The cells were then rinsed three times with medium B, consisting of DMEM supplemented with glutamine and antibiotics, and then incubated with freshly diluted LPS (0.3 μg/ml) in medium B at 37°C for 2 h. The cells were next washed three times with sterile PBS and total cytoplasmic RNA was isolated by NP40 lysis (28). Cytokine mRNA was detected by dot blot hybridization, essentially as described by Cherwinski et al. (29). RNA equivalent to 6.0, 2.0, and 0.67×10^5 cells was applied to Nytran filters (Schleicher and Schuell) by vacuum and the filters were baked for 2 h at 80°C. The filters were prehybridized overnight at 42°C, and then hybridized with ^{32}P-labeled synthetic oligonucleotides at 42°C for at least 16 h. The washed filters were exposed to X-ray film at −70°C. The autoradiograms were scanned using a laser densitometer and the area under the curve was tabulated.

Measurement of macrophage cytokine mRNA expression by Northern blot hybridization. Resident mouse peritoneal macrophages were harvested and cultured, as described for dot blot hybridization, except that the cells were added to T-75 flasks (18 ml cell suspension/flask). After the incubation with lipoproteins, total cytoplasmic RNA was isolated, as described by Chomczynski and Sacchi (30). Briefly, the washed cells were scraped into 2.8 ml of solution D and transferred to a 15-ml polypropylene tube containing 0.28 ml 2 M sodium acetate. The samples were extracted with 2.8 ml water-saturated phenol and 0.56 ml chloroform–isoamyl alcohol 49:1, centrifuged (2,000 *g* for 30 min), and the upper aqueous phase was transferred to 15-ml glass Corex tubes. Isopropanol (2.8 ml) was added and the mixture was incubated overnight at −20°C to precipitate RNA. The RNA was sedimented by centrifugation (10,000 *g* for 30 min) and the pellet was resuspended in 0.25 ml of solution D. The RNA was reprecipitated with an equal volume of isopropanol and the pellet was washed with 0.5 ml ice-cold 75% ethanol. The air-dried RNA pellet was resuspended with 10 μl of water and the RNA concentration was determined by measuring the absorbance at 260 nm. Between 15 μg and 19 μg RNA was denatured by heating at 65°C for 10 min and separated on a 1% agarose/formaldehyde gel. The RNA was blotted overnight, baked, and then probed, exactly as described for the dot blot filters.

Effect of oxidized LDL on the rate of degradation of IL-1β mRNA. Mouse macrophages were preincubated in the presence or absence of oxidized LDL (2.5 μg/ml) for 2.5 h at 37°C and stimulated with LPS. The cells were then washed and actinomycin D (5 μg/ml) was added to all cells, followed by incubation at 37°C for 0 h, 0.5 h, 1 h, and 1.5 h. RNA was harvested and probed for IL-1β by dot blot hybridization.

Measurement of macrophage phagocytosis. Adherent macrophages in 6-well tissue culture plates were incubated with rhodamine-labeled latex beads (0.9 μm diameter; Polysciences Inc.) in medium B (20×10^7 particles/ml) at 37°C for 1 h. The cells were washed 3 times and then incubated for an additional 1 h to allow endocytosis of cell surface-associated beads. The cells were washed a second time with PBS and then visualized by fluorescence microscopy. Cells that were fluorescent were defined as phagocytic. The percentage of cells that were phagocytic from three different wells was then calculated. Incubations at 4°C were also included to provide a measure of background fluorescence.

Other procedures. Protein was measured by the method of Lowry et al. (31) using albumin as a standard. Lipid peroxidation was estimated by measuring the amount of TBARS. Briefly, 25 μg LDL protein (0.25 ml) was incubated with 0.75 ml 0.67% thiobarbituric acid in 50 mM NaOH and 0.75 ml 20% TCA for 15 min in a boiling water bath. The samples were centrifuged and the absorbance of the supernatant was measured at 532 nm. Dilutions of tetramethoxypropane were used as a standard and the results were expressed as the amount of MDA equivalents in nmol per mg LDL protein. Protein synthesis was measured by incubating adherent macrophages in methionine-deficient medium A with [S^{35}]methionine (20 μCi) at 37°C for 30 min. The cells were washed three times with PBS, lysed, precipitated with 10% TCA, and the precipitate was collected onto GF/A disks (Whatman). The disks were washed three times with TCA followed by 95% ethanol, air dried, and then counted in a scintillation counter. Lipid extracts of lipoproteins were prepared by acidification of the lipoprotein-containing solution and extraction by the method of Bligh and Dyer (32). The chloroform phase was collected and evaporated under N_2 and the lipid residue was dissolved in ethanol.

Statistics. Statistical analysis was done using non-paired Student's *t*-test.

Downloaded from www.jlr.org by guest, on June 18, 2012

RESULTS

Inhibition of macrophage IL-1β mRNA expression by oxidized LDL

Intimal macrophages are the predominant cell type that express IL-1α and IL-1β mRNA in the atherosclerotic artery (5). To examine whether macrophage IL-1 mRNA expression can be modulated by lipoproteins, macrophages were preincubated with lipoproteins that bind to either the LDL receptor or the scavenger receptor and then stimulated with LPS to induce IL-1β mRNA expression. Prior incubation with LDL, acetyl-LDL, or MDA-LDL did not affect IL-1β mRNA levels; however, there was a substantial decrease in subsequent expression of IL-1β mRNA when cells were preincubated with oxidized LDL (**Fig. 1**). The decrease in expression was not due to a lower amount of RNA loaded since comparable amounts of γ-actin mRNA were present (not shown). The absence of an inhibition was not due to the induction of mRNA by the lipoproteins since their preincubation with macrophages did not stimulate IL-1β mRNA expression (Fig. 1). In addition to the lipoproteins above, rat β-VLDL, human HDL, and aggregated human LDL (26) were also tested and had no effect (not shown). Thus, oxidized LDL by itself does not stimulate IL-1β mRNA, but can inhibit the stimulated expression induced by LPS.

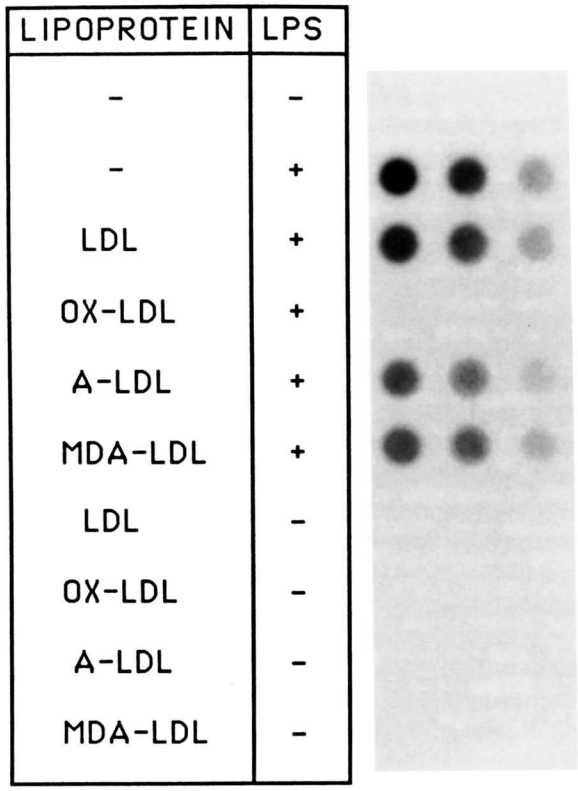


Fig. 1. Effect of LDL, acetyl-LDL, MDA-LDL, and oxidized LDL on macrophage IL-1β mRNA expression. Macrophages were preincubated in medium or with 25 μg/ml of either LDL, oxidized LDL, acetyl-LDL, or MDA-LDL and then stimulated with LPS (0.3 μg/ml). Total cytoplasmic RNA was then isolated and IL-1β mRNA was measured by dot blot hybridization (see Methods). Macrophages that were preincubated with the above lipoproteins, but not stimulated with LPS, were also included.

The inhibition of IL-1β mRNA expression by oxidized LDL was both concentration- and time-dependent. A decrease in IL-1β mRNA could be observed with as little as 2.5 μg/ml oxidized LDL (preincubation for 2.5 h) or after a 15–30 min preincubation (25 μg/ml oxidized-LDL). The extent of inhibition steadily increased with increasing concentration or time reaching a maximal inhibition after approximately 2 h of incubation with 20–25 μg/ml oxidized-LDL. For all subsequent incubations to maximally inhibit IL-1β mRNA expression, macrophages were preincubated with 25 μg/ml oxidized LDL for 2.5 h.

Inhibition of IL-1β mRNA expression is not due to cell death

Oxidized LDL is cytotoxic to some cells in culture (33). Thus it was possible that the inhibition of IL-1β mRNA expression was related to its cytotoxicity. This was examined in three ways: first, the effect of oxidized LDL on the rate of protein synthesis was measured; second, the effect of oxidized LDL on the percentage of phagocytic cells was studied, and third, whether the inhibition of IL-1β mRNA expression was reversible was investigated. The incorporation of [S^{35}]methionine into TCA-precipitable cellular proteins during the final 30 min of the 2.5-h incubation with oxidized LDL (25 μg/ml) was used to assess overall protein synthesis. Oxidized LDL did not suppress the rate of protein synthesis (control, 11,277 $\pm$ 1979 cpm/1.5 $\times$ 10^5 cells; oxidized LDL, 10,789 $\pm$ 804 cpm/1.5 $\times$ 10^5 cells). Similarly, oxidized LDL (25 μg/ml for 2.5 h) did not affect the percentage of cells that were phagocytic (control, 92.4 $\pm$ 2.9%; oxidized LDL, 95.5 $\pm$ 2.0%). To determine whether the inhibition was reversible, cells were incubated with oxidized LDL under conditions that result in maximal inhibition of IL-1β mRNA expression and then incubated in the absence of oxidized LDL for 0, 12, or 24 h. The cells were then stimulated with LPS and IL-1β mRNA levels were measured. Macrophages incubated for an additional 12 h expressed detectable IL-1β mRNA, but not nearly as much as control incubated cells (**Fig. 2**). However, cells incubated for 24 h expressed almost the same amount of IL-1β mRNA as did the paired control (Fig. 2). Preincubation with a larger amount of oxidized LDL (50 μg/ml) did not delay the recovery time for IL-1β mRNA expression (not shown). This demonstrates that the inhibition induced by oxidized LDL is largely reversible with time. All of the above suggests that the decreased expression is not due to cell death or selected survival of an altered phenotype.

LPS can interact with lipoproteins to form a complex that is quite stable (34). Thus it is possible that the inhibi-

Downloaded from www.jlr.org by guest, on June 18, 2012

INCUBATION TIME (hours)	OX-LDL
0	-
0	+
12	-
12	+
24	-
24	+

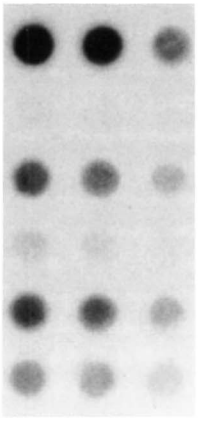


Fig. 2. Reversibility of oxidized LDL-induced inhibition of macrophage IL-1β mRNA expression. Macrophages were preincubated in the absence (-) or presence (+) of oxidized LDL (25 μg/ml) for 2.5 h. The cells were washed and then incubated in fresh medium for 0, 12, or 24 h. The macrophages were then stimulated with LPS and IL-1β mRNA was measured by dot blot hybridization.

tion induced by oxidized could be due to the complexing of LPS by surface-bound oxidized LDL. To test this possibility, macrophages were first incubated with oxidized LDL (25 μg/ml) at 4°C to allow surface binding. The cells

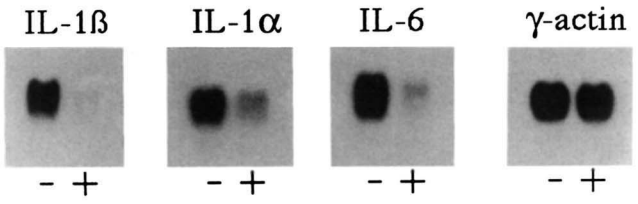
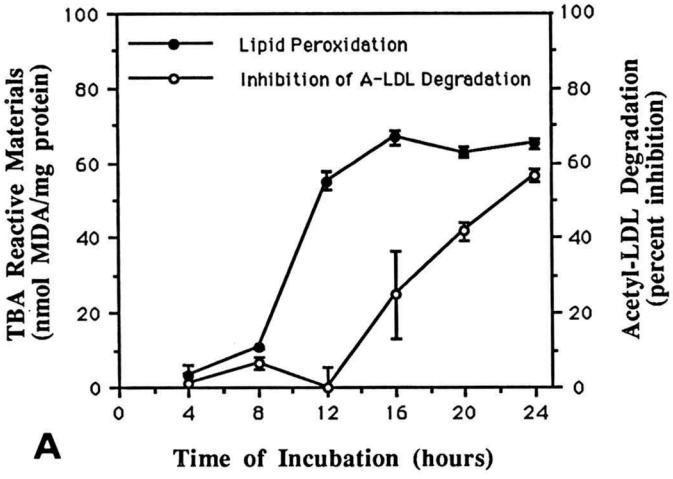


Fig. 3. Inhibition of macrophage IL-1α, IL-1β, and IL-6 mRNA expression by oxidized LDL. Macrophages were preincubated in the absence (-) or presence (+) of oxidized LDL (25 μg/ml) and then stimulated with LPS (0.3 μg/ml). Total cytoplasmic RNA was isolated and RNA (15 μg) was analyzed for IL-1α, IL-1β, IL-6, and γ-actin mRNA by Northern blot hybridization (see Methods).

were then washed and stimulated with LPS. If surface-bound oxidized LDL in some way inactivated LPS, then a decrease in IL-1β mRNA expression should be observed. However, the presence of surface-associated lipoprotein had no apparent effect on mRNA levels (LPS control, 100%; +oxidized LDL, 98% ± 4; average of three experiments). This suggests that oxidized LDL most likely affects a process within the cell required for LPS-induced stimulation of IL-1β mRNA expression.

Inhibition of macrophage IL-1α and IL-6 mRNA expression by oxidized LDL

LPS stimulates macrophages to express several cytokine mRNA. However, despite sharing a common stimulus, the expression of individual cytokine mRNA by mac-



INCUBATION TIME (hours)	LPS
control	+
4	+
8	+
12	+
16	+
20	+
24	+

B

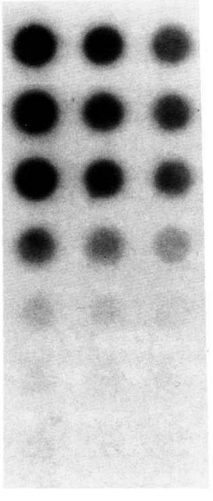


Fig. 4. Inhibition of IL-1β mRNA expression is dependent upon the extent of LDL oxidation. A. Kinetics of LDL oxidation and recognition by the scavenger receptor. LDL (100 μg/ml) was incubated with copper ion (10 μM) at 37°C for 4, 8, 12, 16, 20, or 24 h. After each incubation period, BHT was added and the lipoproteins were stored at 4°C to prevent further lipid peroxidation. The amount of TBARS generated (●) and the ability of the incubated LDL (20 μg/ml) to inhibit the degradation of radiolabeled acetyl-LDL (4 μg/ml) by macrophages (○) were measured. B. Effect of oxidized LDL on IL-1β mRNA expression. Macrophages were preincubated with the differently oxidized LDL samples (25 μg/ml) for 2.5 h. After stimulation with LPS, IL-1β mRNA was measured by dot blot hybridization.

Downloaded from www.jlr.org by guest, on June 18, 2012

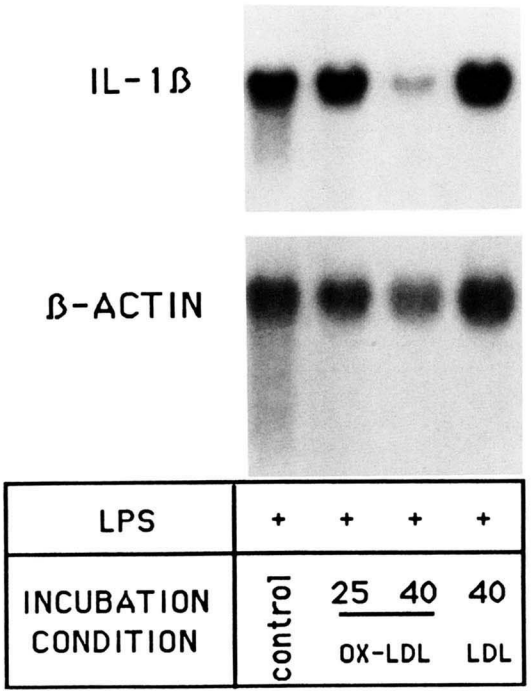


Fig. 5. Inhibition of human THP-1 IL-1β mRNA expression by oxidized LDL. Human THP-1 cells (1×10^6 cells/ml) were preincubated with medium alone, oxidized LDL (25 and 40 μg/ml), or LDL (40 μg/ml) at 37°C for 23 h in RPMI 1640 containing 10% FCS. The cells were then washed and stimulated with LPS (5 μg/ml) for 1 h and total cytoplasmic RNA was isolated. RNA (25 μg) was analyzed for IL-1β and β-actin mRNA by Northern blot hybridization.

rophages can be regulated differentially (35). To examine whether oxidized LDL inhibits IL-1β mRNA selectively or coordinately with other LPS-induced cytokines, the effect of oxidized LDL on IL-1α or IL-6 mRNA expression was analyzed by Northern blot hybridization. LPS stimulated the expression of IL-1β, IL-1α, and IL-6 mRNA by macrophages (**Fig. 3**). As observed previously for IL-1β, the expression of IL-1α and IL-6 mRNA was inhibited by oxidized LDL without affecting γ-actin mRNA levels (Fig. 3). Thus, oxidized LDL is a potent inhibitor of expression of a broad group of cytokine mRNAs by macrophages.

Inhibition of IL-1β mRNA expression is associated with the extent of LDL oxidation

The copper ion-catalyzed modification of LDL converts the LDL into a modified form that is avidly taken up by macrophages (36; also see Table 1) and this has been shown to be dependent upon LDL oxidation (36). Similarly, the generation of a modified LDL that inhibits IL-1β mRNA expression was also found to be dependent upon LDL oxidation; the antioxidant BHT (20 μM) inhibited LDL oxidation (82.0 nmol MDA/mg ± 2.2 vs. 9.4 ± 0.3) and its conversion to an inhibitory LDL (not shown). However, these studies do not provide any information on whether an inhibition is dependent only on LDL oxidation or whether both oxidation and recognition by the scavenger receptor are required. To examine this, LDL was oxidized to different degrees by incubating LDL with copper ion for 4, 8, 12, 16, 20, and 24 h. The kinetics of inhibition of IL-1β mRNA expression was then compared with that of the extent of LDL oxidation and recognition by the scavenger receptor. There was a substantial increase in TBARS generated between 8 and 12 h of incubation which reached a maximum approximately after 16 h (**Fig. 4A**). In contrast, recognition by the scavenger receptor did not occur until after 12 h but steadily increased thereafter (Fig. 4A). The onset of inhibition of IL-1β mRNA expression occurred between 8 and 12 h and the extent of inhibition closely paralleled the kinetics of LDL oxidation (Fig. 4B). Together, these results suggest that the inhibition of IL-1β mRNA expression is closely associated with the extent of LDL lipid peroxidation but that the inhibition is not entirely dependent upon recognition of the oxidized LDL by the scavenger receptor.

The requirement for processing by the scavenger receptor was, therefore, further examined. First, the effect of oxidized LDL on THP-1 cells was studied. THP-1 cells are a human monocytic cell line that does not express the scavenger receptor (37) but does express IL-1β mRNA in response to LPS. Thus these cells were used to examine whether oxidized LDL could inhibit IL-1β mRNA expression in the absence of the scavenger receptor. As with mouse macrophages, the expression of IL-1β mRNA was inhibited by oxidized LDL, but not LDL (**Fig. 5**). However, a longer preincubation period with a higher concentration of oxidized LDL was required; an overnight incubation with 40 μg/ml of oxidized LDL inhibited THP-1 IL-1β mRNA expression whereas a 2.5 h incubation with 25 μg/ml oxidized LDL was without effect (not shown). Nevertheless, oxidized LDL was inhibitory. Second, oxidized, reductively methylated LDL was tested for inhibitory activity. Reductive methylation of lysine amino groups of LDL (40–90%) prevents the modification(s) required to stimulate macrophage uptake without affecting its oxidation (38). Therefore, reductively methylated LDL (RM-LDL) was oxidized and then tested for inhibitory activity. Reductive methylation derivatized more than 75% lysine residues (control LDL, 157 ± 4 nmol NH_2/mg protein; RM-LDL, 34 ± 1 nmol NH_2/mg protein) but did not substantially affect the extent of LDL oxidation (control, 51.8 nmol MDA/mg protein; RM-LDL, 46.5 nmol MDA/mg protein; average of two experiments) nor the inhibition of IL-1β mRNA expression (OX-LDL, >90% inhibition; OX-RM-LDL, >90% inhibition; representative of two experiments). Third, the effect of MDA-LDL on the inhibition induced by oxidized LDL

Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 1. Lipoprotein characteristics: recognition by the scavenger receptor, electrophoretic mobility, and lipid peroxidation

Lipoprotein	Electrophoretic Mobility (Migration relative to LDL)	Scavenger Receptor Recognition (Percent inhibition of acetyl-LDL degradation)	Lipid Peroxidation (nmol MDA/mg protein)
LDL	1.0	5.6 ± 2.1	ND[a]
OX-LDL	3.18 ± 0.05	92.6 ± 2.5	83.2 ± 9.6
A-LDL	3.14 ± 0.02	81.7 ± 7.0	ND
MDA-LDL	3.40 ± 0.20	96.3 ± 0.6	172.3 ± 17.8

Lipoproteins were prepared as described in Methods and the electrophoretic mobility, recognition by the scavenger receptor, and the extent of lipid peroxidation were measured. The electrophoretic mobility was measured on agarose gels and the results are expressed as the distance migrated relative to LDL. Recognition by the scavenger receptor was assessed by measuring the inhibition of macrophage degradation of ^{125}I-labeled acetyl-LDL (4 μg/ml) by added unlabeled lipoprotein (20 μg/ml). The results are expressed as a percentage of the rate of degradation in the absence of any added lipoprotein (control rate, 3.3 μg/mg cell protein per 4 h ± 0.3; mean ± SE, n = 3). The extent of lipid peroxidation was determined by measuring the amount of TBARS and the results are expressed as nmol MDA equivalents/mg lipoprotein. The mean ± SE for three different preparations is shown.

[a]ND, not detectable; limit of detection = 2.5 nmol MDA/mg protein.

was examined. MDA-LDL competes for the degradation of oxidized LDL (**Table 1**) but does not inhibit nor stimulate IL-1β mRNA expression (Fig. 1). Thus, to examine whether MDA-LDL can also suppress the inhibition induced by oxidized LDL, macrophages were incubated with a submaximal concentration of oxidized LDL (10 μg/ml) in the absence or presence of a concentration of MDA-LDL (60 μg/ml) that largely prevents the uptake by the scavenger receptor and then IL-1β mRNA expression was measured. MDA-LDL (60 μg/ml) inhibited the degradation and binding of oxidized LDL (10 μg/ml) by 90% and 73%, respectively. However, MDA-LDL did not affect the inhibition induced by oxidized LDL (oxidized LDL, 31% inhibition; oxidized LDL+MDA-LDL, 26% inhibition; average of two experiments). Together these experiments provide strong support for the suggestion that the extent of inhibition is not dependent upon the processing of oxidized LDL by the scavenger receptor pathway.

Localization of the inhibitory component to the lipid fraction of oxidized LDL

Substantial amounts of lipid peroxidation products are generated during the copper ion modification of LDL and some of these disassociate from the LDL particle (36). To examine whether the inhibitory activity of oxidized LDL is actually associated with the lipoprotein or is free in solution, oxidized LDL was either dialyzed against PBS containing EDTA (0.01%) and the lipoprotein was tested or oxidized LDL was filtered through a microconcentrator (Amicon; 10 kDa cut off) and both the retained oxidized LDL and filtrate (flow through) were tested. Dialysis decreased the amount of TBARS by more than 90% while greater than 90% of the total TBARS could be recovered in the filtrate after filtration. When these samples were compared for their ability to inhibit IL-1β mRNA expression, both the dialyzed and reisolated oxidized LDL were inhibitory while the filtrate was without effect (**Fig. 6**). Thus, the inhibitory activity was still associated with the lipoprotein and was not in a soluble moiety.

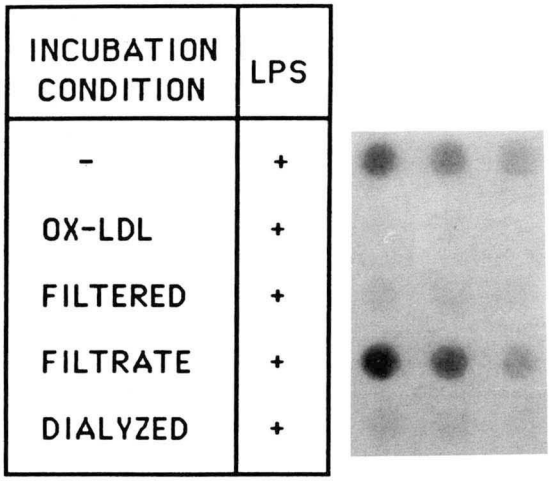


Fig. 6. Localization of the inhibitory activity of oxidized LDL. Macrophages were preincubated with 25 μg/ml native oxidized-LDL, filtered oxidized LDL, a filtrate of oxidized LDL, or dialyzed oxidized LDL at 37°C for 2.5 h. The cells were then stimulated with LPS and IL-1β mRNA expression was measured by dot blot hybridization. Neither the lipoproteins nor the filtrate stimulated IL-1β mRNA expression in the absence of LPS (not shown). The dialyzed and filtered oxidized LDL inhibited the degradation of radiolabeled oxidized LDL to the same extent as native oxidized LDL, whereas the filtrate had no effect (not shown). In three other dialyzed preparations, dialyzed oxidized LDL also inhibited the binding of acetyl-LDL to the same extent as native oxidized LDL (not shown).

Downloaded from www.jlr.org by guest, on June 18, 2012

Although the active component is not free in solution, it remains possible that the lipoprotein-associated component could inhibit macrophage cytokine mRNA expression indirectly through the modification of a serum constituent in the incubation medium. However, when oxidized LDL was preincubated with macrophages in the absence of serum, the extent of inhibition was identical to that produced in the presence of serum (inhibition with serum, 85.2% ± 8.6; inhibition without serum, 87.0% ± 6.5; mean ± SD of three experiments).

Modifications to both apoB and the lipid constituents of LDL occur during its oxidation. To examine whether changes to the lipids of oxidized LDL are responsible for the inhibitory activity of oxidized LDL, a lipid extract of oxidized LDL was prepared and tested. The extract inhibited IL-1β mRNA expression in a concentration-dependent manner whereas lipids from non-oxidized LDL were without effect (**Fig. 7**). The inhibition by the lipid extract was, however, somewhat less than that of the intact lipoprotein (Fig. 7). These findings are consistent with others that lipids of oxidized LDL can mimic specific biologic activities of the intact lipoprotein (39–43) including the inhibition of TNFα mRNA expression (21). There are several lipid products generated during LDL oxidation (44–46) including an increase in lysolecithin (36). To examine whether the inhibition could be attributed to an increase in lysolecithin content, LDL was treated with the enzyme phospholipase A_2 (PLA_2) to generate lysolecithin in amounts equivalent to oxidized LDL (see Methods section). However, no inhibition was observed with PLA_2-treated LDL (control, 100%; +PLA_2-LDL, 119%; average of two experiments). Similar results were obtained when acetyl-LDL was treated with PLA_2 (not shown). Thus these studies suggest that a major portion of the inhibition of macrophage cytokine mRNA expression by oxidized LDL is due to the production of a non-dialyzable lipid extractable compound that is not lysolecithin.

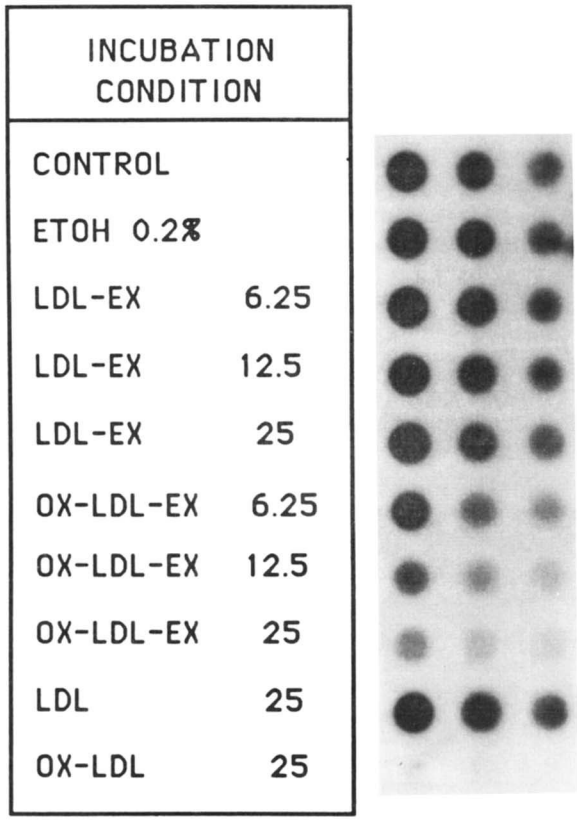


Fig. 7. Inhibition of macrophage IL-1β mRNA expression by a lipid extract of oxidized LDL. Oxidized LDL or LDL were extracted with chloroform and methanol (32) and the chloroform phases were dried under N_2. The residues were resuspended in ethanol and the lipid extracts (EX) equivalent to 6.25, 12.5, and 25 μg protein/ml were added to macrophages and incubated at 37°C for 2.5 h. In each case the concentration of ethanol was 0.2%. The cells were then stimulated with LPS and IL-1β mRNA expression was measured by dot blot hybridization.

Effect of oxidized LDL on the rate of degradation of IL-1β mRNA

The decrease in IL-1β mRNA levels could be due to a decreased rate of transcription or to an increased rate of IL-1β mRNA degradation. To examine whether oxidized LDL stimulates the turnover of IL-1β mRNA, the rate of decay of IL-1β mRNA was measured. Macrophages were first preincubated with oxidized LDL at a concentration that submaximally inhibits IL-1β mRNA expression ($\approx$40% at 2.5 μg/ml). After stimulation with LPS, actinomycin D (5 μg/ml) was added to inhibit further gene transcription. Preliminary studies showed that this concentration was sufficient to completely inhibit LPS-induced IL-1β mRNA expression. The amount of IL-1β mRNA was then measured during the next 1.5 h. The expression of IL-1β mRNA by control incubated macrophages decreased rapidly following a short lag period with an approximate disappearance $t_{1/2}$ of about 0.75 h (**Fig. 8**). Oxidized LDL had very little effect on the rate of degradation of IL-1β mRNA. These studies suggest that the inhibition of IL-1β mRNA expression by oxidized LDL is not due to a stimulated rate of IL-1β mRNA degradation, but most likely to a decreased rate of transcription.

DISCUSSION

One currently attractive hypothesis to explain the mechanism of foam cell formation in atherosclerosis lesions proposes that lipoproteins become oxidized after penetrating the arterial wall. This renders them substrates for one or more receptors on macrophages, and this leads to their avid uptake by these cells without receptor down-regulation (rev. in 47). In addition to being substrates for rapid uptake, oxidized forms of LDL have a number of other important effects on macrophage behavior, including a change in chemotaxis (39, 40), altered cholesterol

Downloaded from www.jlr.org by guest, on June 18, 2012

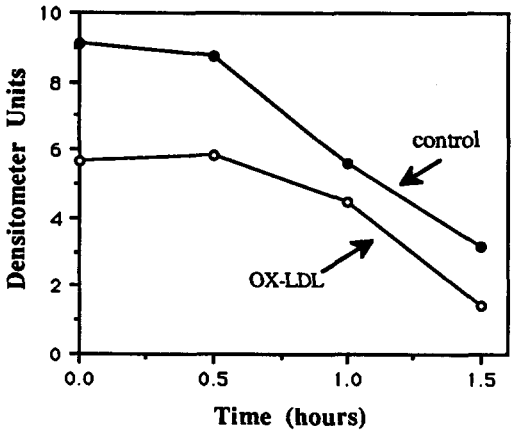


Fig. 8. Effect of oxidized LDL on the rate of degradation of macrophage IL-1β mRNA. Macrophages were preincubated in the presence (○) or absence (●) of oxidized LDL (2.5 μg/ml) for 2.5 h, washed, and then stimulated with LPS for 2 h. The cells were then washed again and actinomycin D (5 μg/ml) was added and the cells were incubated at 37°C for 0, 0.5, 1, and 1.5 h. IL-1β mRNA expression was measured by dot blot hybridization and the autoradiograms were scanned by laser densitometry. The results are expressed as relative densitometric units.

metabolism (46), and enhanced secretion of bioactive substances (18). In the present studies, LDL, β-VLDL, HDL, and modified forms of LDL were compared for their ability to modulate the expression of IL-1β mRNA by mouse macrophages. None of the lipoproteins tested were themselves able to stimulate the expression of IL-1β mRNA or to synergize with LPS-induced IL-1β mRNA expression. However, there was a concentration-dependent inhibition of IL-1β mRNA expression only with oxidized LDL. The decreased IL-1β expression was accompanied by an inhibition of IL-1α and appears to be part of a general phenomenon in that we observed that another macrophage activation gene product, IL-6, was suppressed by oxidized LDL. Also, Hamilton et al. (21) have reported that the maleylated albumin-induced stimulation of TNFα mRNA was also suppressed by oxidized LDL. We have found the same thing for LPS induction of TNFα. There was no change in γ-actin mRNA levels or in protein synthesis or phagocytosis by the incubations, suggesting that viability and general function were not being altered. Consistent with this, the effect was reversible. Finally, the inhibition of IL-1β mRNA accumulation was not due to a stimulation of IL-1β mRNA turnover and suggests that oxidized LDL suppresses IL-1β gene transcription. Together, these findings demonstrate that if macrophages become foam cells because of exposure to oxidized LDL, they are likely to lose a specific complement of immune response functions.

The processing of lipoproteins by the macrophage scavenger receptor was not itself an adequate signal to produce the inhibition; nonoxidized modified forms of LDL that also bind to the scavenger receptor were not inhibitory. Moreover, the extent of inhibition by oxidized LDL was not related to processing by the scavenger receptor. This is suggested by three lines of evidence: first, the kinetics of inhibition did not parallel the kinetics of receptor recognition; second, the inhibition of oxidized LDL binding by MDA-LDL had very little, if any, effect on IL-1β mRNA expression; and third, prior methylation of LDL did not affect the extent of inhibition. This is not to say that the scavenger receptor or other surface receptors that recognize oxidized LDL (48, 49) have no role in the inhibition since it remains possible that occupancy of a low threshold number of receptors may be required as a concomitant signal. Nevertheless, it is clear that the extent of inhibition is not proportional to receptor binding. On the other hand, evidence for receptor-independent inhibition makes this the probable pathway. First, oxidized LDL was able to inhibit the expression of IL-1β mRNA by THP-1 cells, cells that do not express the scavenger or oxidized LDL receptor and degrade modified forms of LDL slowly (relative to LDL) (37). Second, lipid extracts of oxidized LDL were able to inhibit IL-1β mRNA expression. Together these studies suggest that the generation of peroxidized lipids within the artery wall may provide another level of control on macrophage function and perhaps other cytokine-producing cells.

The active component of oxidized LDL appears to be a modified lipid or a lipid breakdown product. This is emerging as an important component of oxidized LDL in that many of the modifications of cellular functions induced by oxidized LDL have now been reproduced using a lipid extract. These include effects on chemotaxis (39, 40), cell adhesion (41), PDGF secretion (42), cell growth (43), and TNFα mRNA expression (21). However, the active lipid has been identified in only one case (27). There are several newly generated byproducts of LDL lipid peroxidation that could contribute to the inhibition of IL-1β mRNA expression including lysolecithin (36), lipid hydroperoxides (45), oxysterols (46), and aldehydes (44). It appears unlikely though that lysolecithin is the active component since the enzymatic generation of lysolecithin was unable to produce an inhibitory lipoprotein. Similarly, small molecular weight unreacted aldehydes do not appear to be responsible since dialysis to remove these products had no effect and the incubation medium containing these materials was not inhibitory. A fraction of oxidized LDL lipids that is highly active has been obtained (Fong, L. G., and A. D. Cooper, unpublished observation); however, the identification of the lipid(s) in this fraction has not yet been accomplished and will require further analysis.

In addition to identifying the active compound in oxidized LDL, it will be important to elucidate its mechanism of action. Regulation at the level of transcription, translation, and secretion have all been reported for IL-1. In the present study, gene transcription appears to be one mode of IL-1 regulation in macrophages. This is sup-

Downloaded from www.jlr.org by guest, on June 18, 2012

ported by the observation that oxidized LDL suppressed steady state levels of IL-1β mRNA without affecting its turnover. It is too early to speculate on the mode of regulation by oxidized LDL but general mechanisms can be suggested. Oxidized LDL could interfere with the LPS signal pathway after receptor binding. This is consistent with the generalized inhibitory effect of oxidized LDL on LPS-induced cytokine mRNA expression. Although the intracellular signal(s) responsible for LPS induced cytokine production are not entirely clear, changes in protein kinase activity (50), cyclic nucleotide levels (51), and prostaglandin metabolism (52) have been reported to alter IL-1 production. A mechanism of action through the prostaglandin pathway is particularly attractive since oxidized LDL has already been found to affect this pathway (18). Alternatively, oxidized LDL could initiate intracellular events that interfere directly with IL-1 gene transcription including perhaps the activation of a repressor (53). It is also possible that oxidized LDL produces a generalized decrease in mRNA synthesis. While γ-actin mRNA levels were not affected by oxidized LDL, the relatively long half-life of actin message precludes assessment of a potential effect on overall mRNA synthesis.

The monocyte and monocyte-derived macrophage are central to the development of the fatty streak lesion, and the interaction between these and the other cells of the artery wall may play a key role in determining the rate of this process. A logical mechanism for governing this interaction is the modulation of cell function by cytokines. Based on this report, as well as that of Hamilton et al. (21), the generation and interaction of oxidized LDL with macrophages within the artery wall at some stage will inhibit the production of macrophage inflammatory cytokines (i.e., IL-1, IL-6, TNFα). The atherogenicity of oxidized LDL in this context, however, is difficult to judge. Argument for both pro- and anti-atherogenic activities can be made based on studies of tissue culture cells. For example, the inhibition of IL-1-induced endothelial cell surface procoagulant activity (rev. in 54) could be considered as beneficial. On the other hand, the inhibition of IL-1-mediated T-cell activation and interferon-gamma production could favor foam cell formation (14). It is apparent that further studies will be required to elucidate the role of cytokines and their modulation by oxidized LDL in order to understand their role in atherogenesis.

We wish to thank Jeanne Gill for preparation of the manuscript. We also thank the G-3 for valuable discussion. This work was supported in part by grants DK36659, DK38707-02, and funds provided by the Cigarette and Tobacco Surtax Fund of the State of California through the Tobacco-Related Disease Research Program of the University of California.

Manuscript received 13 February 1991, in revised form 18 July 1991, and in re-revised form 29 August 1991.

REFERENCES

1. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103:** 180–190.
2. Faggiotto, A., R. Ross, and L. Harker. 1984. Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation. *Arteriosclerosis.* **4:** 323–340.
3. Rosenfeld, M. E., T. Tsukada, A. M. Gown, and R. Ross. 1987. Fatty streak initiation in Watanabe heritable hyperlipemic and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis.* **7:** 9–23.
4. Ross, R., J. Masuda, E. W. Raines, A. M. Gown, S. Katsuda, M. Sasahara, T. Malden, H. Masuko, and H. Sato. 1990. Localization of PDGF-B protein in macrophages in all phases of atherogenesis. *Science.* **248:** 1009–1012.
5. Moyer, C. F., D. Sajuthi, H. Tulli, and J. K. Williams. 1991. Synthesis of IL-1 alpha and IL-1 beta by arterial cells in atherosclerosis. *Am. J. Pathol.* **138:** 951–960.
6. Rajavashisth, T. B., A. Andalibi, M. C. Territo, J. A. Berliner, M. Navab, A. M. Fogelman, and A. J. Lusis. 1990. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature.* **344:** 254–257.
7. Barath, P., M. C. Fishbein, J. Cao, J. Berenson, R. H. Helfant, and J. S. Forrester. 1990. Tumor necrosis factor gene expression in human vascular intimal smooth muscle cells detected by in situ hybridication. *Am. J. Pathol.* **137,** 503–509.
8. Miossec, P., D. Cavender, and M. Ziff. 1986. Production of interleukin 1 by human endothelial cells. *J. Immunol.* **136:** 2486–2491.
9. Warner, S. J. C., and P. Libby. 1989. Human vascular smooth muscle cells. Target for and source of tumor necrosis factor. *J. Immunol.* **142:** 100–109.
10. Brandwein, S. R. 1986. Regulation of interleukin 1 production by mouse peritoneal macrophages. *J. Biol. Chem.* **261:** 8624–8632.
11. Doukas, J., and J. S. Pober. 1990. IFN-γ enhances endothelial activation induced by tumor necrosis factor but not IL-1. *J. Immunol.* **145:** 1727–1733.
12. Hajjar, K. A., D. P. Hajjar, R. L. Silverstein, and R. L. Nachman. 1987. Tumor necrosis factor-mediated release of platelet-derived growth factor from cultured endothelial cells. *J. Exp. Med.* **166:** 235–245.
13. Fogelman, A. M., J. Seager, J. E. Groopman, J. A. Berliner, M. E. Haberland, P. A. Edwards, and D. W. Golde. 1983. Lymphokines secreted by an established lymphocyte line modulate receptor-mediated endocytosis in macrophages derived from human monocytes. *J. Immunol.* **131:** 2368–2373.
14. Fong, L. G., T. A. T. Fong, and A. D. Cooper. 1990. Inhibition of mouse macrophage degradation of acetyl-low density lipoprotein by interferon-γ. *J. Biol. Chem.* **265:** 11751–11760.
15. Ishibashi, S., T. Inaba, H. Shimano, K. Harada, I. Inoue, H. Mokuno, N. Mori, T. Gotoda, F. Takaku, and N. Yamada. 1990. Monocyte colony-stimulating factor enhances uptake and degradation of acetylated low density lipoproteins and cholesterol esterification in human monocyte-derived macrophages. *J. Biol. Chem.* **265:** 14109–14117.
16. Fox, P. L., and P. E. DiCorleto. 1986. Modified low density lipoproteins suppress production of a platelet-derived growth factor-like protein by cultured endothelial cells. *Proc. Natl. Acad. Sci. USA.* **83:** 4774–4778.

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 18, 2012

17. Dianiak, N., H. B. Warren, S. Kreczko, M. A. Riordan, L. Feldman, J. Lawler, A. M. Cohen, and P. F. Davies. 1988. Acetylated lipoproteins impair erythroid growth factor release from endothelial cells. *J. Clin. Invest.* **81:** 834–843.
18. Yokode, M., T. Kita, Y. Kikaw. T. Ogorochi, S. Narumiya, and C. Kawai. 1988. Stimulated arachidonate metabolism during foam cell transformation of mouse peritoneal macrophages with oxidized low density lipoprotein. *J. Clin. Invest.* **81:** 720–729.
19. Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. Gerrity, C. J. Schwartz, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* **87:** 5134–5138.
20. Barath, P., M. C. Fishbein, J. Cao, J. Berenson, R. H. Helfant, and J. S. Forrester. 1990. Detection and localization of tumor necrosis factor in human atheroma. *Am. J. Cardiol.* **65:** 297–302.
21. Hamilton, T. A., G. Ma, and G. M. Chisolm. 1990. Oxidized low density lipoprotein suppresses the expression of tumor necrosis factor-α mRNA in stimulated murine peritoneal macrophages. *J. Immunol.* **144:** 2343–2350.
22. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
23. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
24. Fogelman, A. M., I. Shechter, J. Seager, M. Hokom, J. S. Child, and P. A. Edwards. 1980. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc. Natl. Acad. Sci. USA.* **77:** 2214–2218.
25. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1978. Role of the lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *J. Biol. Chem.* **253:** 9053–9062.
26. Khoo, J. C., E. Miller, P. McLoughlin, and D. Steinberg. 1988. Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis.* **8:** 348–357.
27. Quinn, M. T., S. Parthasarthy, and D. Steinberg. 1988. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc. Natl. Acad. Sci. USA.* **85:** 2805–2809.
28. White, B. A., and F. C. Bancroft. 1982. Cytoplasmic dot hybridization. *J. Biol. Chem.* **257:** 8569–8572.
29. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann. 1987. Two types of mouse helper T cell clone. *J. Exp. Med.* **166:** 1229–1244.
30. Chomczynski, P., and N. Sacchi. 1987. Single-step method for RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
32. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
33. Kosugi, K., D. W. Morel, P. E. DiCorleto, and G. M. Chisolm. 1987. Toxicity of oxidized low-density lipoprotein to cultured fibroblasts is selective for S phase of the cell cycle. *J. Cell. Physiol.* **130:** 311–320.
34. Van Lenten, B. J., A. M. Fogelman, M. E. Haberland, and P. A. Edwards. 1986. The role of lipoproteins and receptor-mediated endocytosis in the transport of bacterial lipopolysaccharide. *Proc. Natl. Acad. Sci. USA.* **83:** 2704–2708.
35. Tannenbaum, C. S., and T. A. Hamilton. 1989. Lipopolysaccharide-induced gene expression in murine peritoneal macrophages is selectively suppressed by agents that elevate intracellular cAMP. *J. Immunol.* **142:** 1274–1280.
36. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
37. Hara, H., H. Tanishita, S. Yokoyama, S. Tajima, and A. Yamamoto. 1987. Induction of acetylated low density lipoprotein receptor and suppression of low density lipoprotein receptor on the cells of human monocytic leukemia cell line (THP-1 cell). *Biochem. Biophys. Res. Commun.* **146:** 802–808.
38. Steinbrecher, U. P. 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603–3608.
39. Quinn, M. T., S. Parthasarthy, and D. Steinberg. 1985. Endothelial cell-derived chemotactic activity for mouse peritoneal macrophage and the effects of modified forms of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82:** 5949–5953.
40. Quinn, M. T., S. Parthasarthy, L. G. Fong, and D. Steinberg. 1987. Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte-macrophages during atherogenesis. *Proc. Natl. Acad. Sci. USA.* **84:** 2995–2998.
41. Berliner, J. A., M. C. Territo, A. Sevanian, S. Ramin, J. A. Kim, B. Bamchad, M. Esterson, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* **85:** 1260–1266.
42. Fox, P. L., G. M. Chisolm, and P. E. DiCorleto. 1987. Lipoprotein-mediated inhibition of endothelial cell production of platelet-derived growth factor-like protein depends on free radical lipid peroxidation. *J. Biol. Chem.* **262:** 6046–6054.
43. Hessler, J. R., D. W. Morel, L. J. Lewis, and G. M. Chisolm. 1983. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Arteriosclerosis.* **3:** 215–222.
44. Esterbauer, H., G. Jürgens, O. Quehenberger, and E. Koller. 1987. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J. Lipid Res.* **28:** 495–509.
45. Lenz, M. L., H. Hughes, J. R. Mitchell, D. P. Via, J. R. Guyton, A. A. Taylor, A. M. Gotto, Jr., and C. V. Smith. 1990. Lipid hydroperoxy and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein. *J. Lipid Res.* **31:** 1043–1050.
46. Zhang, H., H. J. K. Basra, and U. P. Steinbrecher. 1990. Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. *J. Lipid Res.* **31:** 1361–1369.
47. Steinberg, D., S. Parthasarthy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
48. Sparrow, C. P., S. Parthasarathy, and D. Steinberg. 1989. A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. *J. Biol. Chem.* **264:** 2599–2604.

Downloaded from www.jlr.org by guest, on June 18, 2012

49. Arai, H., T. Kita, M. Yokode, S. Narumiya, and C. Kawai. 1989. Multiple receptors for modified low density lipoproteins in mouse peritoneal macrophages: different uptake mechanisms for acetylated and oxidized low density lipoproteins. *Biochem. Biophys. Res. Commun.* **159:** 1375–1382.
50. Kovacs, E. J., B. Brock, L. Varesio, and H. A. Young. 1989. IL-2 induction of IL-1β mRNA expression in monocytes: regulation by agents that block second messenger pathways. *J. Immunol.* **143:** 3532–3537.
51. Ohmori, Y., G. Strassman, and T. A. Hamilton. 1990. cAMP differentially regulates expression of mRNA encoding IL-1α and IL-1β in murine peritoneal macrophages. *J. Immunol.* **145:** 3333–3339.
52. Kunkel, S. L., S. W. Chensue, and S. H. Phan. 1986. Prostaglandins as endogenous mediators of interleukin 1 production. *J. Immunol.* **136:** 186–192.
53. Fenton, M. J., B. D. Clark, K. L. Collins, A. C. Webb, A. Rich, and P. E. Auron. 1987. Transcription regulation of the human prointerleukin 1β gene. *J. Immunol.* **138:** 3972–3979.
54. Cotran, R. S. 1987. New roles for the endothlium in inflammation and immunity. *Am. J. Pathol.* **129:** 407–413.

Downloaded from www.jlr.org by guest, on June 18, 2012