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Minimally modified low density lipoprotein is biologically active in vivo in mice.

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Research Article

Minimally modified low density lipoprotein (MM-LDL), derived by mild iron oxidation or prolonged storage at 4 degrees C, has been shown to induce certain inflammatory responses in vascular cells in tissue culture. These include induction of monocyte (but not neutrophil) adherence to endothelial cells (EC), induction of EC production of colony stimulating factors (CSF), and induction of EC and smooth muscle cell production of monocyte chemotactic protein (MCP-1). To test for biologic activity in vivo, microgram quantities of MM-LDL were injected into mice, sera were assayed for CSF activity, and tissues were subjected to Northern analysis. After injection of MM-LDL, CSF activity increased approximately 7-26-fold but remained near control levels after injection of native LDL. Essentially all of the induced CSF activity was due to macrophage CSF as judged by antibody inhibition. Injection of MM-LDL into a mouse strain (C3H/HeJ) that is resistant to bacterial LPS gave similar results, indicating that the induction of CSF was not due to contaminating LPS and suggesting that there are differences in the pathways by which LPS and MM-LDL trigger cytokine production. In addition, after injection of MM-LDL, mRNA for JE, the mouse homologue [...]

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Minimally Modified Low Density Lipoprotein Is Biologically Active In Vivo in Mice

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Abstract

Minimally modified low density lipoprotein (MM-LDL), derived by mild iron oxidation or prolonged storage at 4°C, has been shown to induce certain inflammatory responses in vascular cells in tissue culture. These include induction of monocyte (but not neutrophil) adherence to endothelial cells (EC), induction of EC production of colony stimulating factors (CSF), and induction of EC and smooth muscle cell production of monocyte chemotactic protein (MCP-1). To test for biologic activity in vivo, microgram quantities of MM-LDL were injected into mice, sera were assayed for CSF activity, and tissues were subjected to Northern analysis. After injection of MM-LDL, CSF activity increased ~ 7–26-fold but remained near control levels after injection of native LDL. Essentially all of the induced CSF activity was due to macrophage CSF as judged by antibody inhibition. Injection of MM-LDL into a mouse strain (C3H/HeJ) that is resistant to bacterial LPS gave similar results, indicating that the induction of CSF was not due to contaminating LPS and suggesting that there are differences in the pathways by which LPS and MM-LDL trigger cytokine production. In addition, after injection of MM-LDL, mRNA for JE, the mouse homologue of MCP-1, was markedly induced in various tissues, but was not induced after injection of native LDL. We conclude, therefore, that MM-LDL is biologically active in vivo and may contribute to the early stages of atherosclerosis by acting as an inflammatory agent. (*J. Clin. Invest.* 1991. 87:2253–2257.) Key words: atherosclerosis • monocytes • monocyte colony stimulating factors • JE/monocyte chemotactic protein-1 • oxidized low density lipoprotein

Introduction

The earliest event observed in experimental atherogenesis is the retention of apolipoprotein B-containing lipoproteins in the extracellular matrix of the subendothelial space (1–3). There is an increasing body of evidence that oxidative modification of LDL may play a significant role in atherogenesis (4–12).

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Most studies have focused on highly oxidized LDL which is no longer recognized by the LDL receptor but is recognized by the scavenger (acetyl-LDL) receptor and has been postulated to play a role in foam cell formation (13). We (J. A. Berliner) hypothesized that if LDL retention was the first step in lesion development initially there would be relatively few cells present in the subendothelial space to oxidize LDL to the extent that it would be recognized by the scavenger receptor. Therefore we studied the biologic consequences of minimally oxidizing LDL. We found that this LDL was not sufficiently modified to be recognized by the scavenger receptor and that it appeared to interact normally with the LDL receptor (14). However, in vitro this minimally modified LDL (MM-LDL)¹ was biologically quite different from native LDL. As a result of the oxidation of lipid(s) in MM-LDL it induced the binding of monocytes but not neutrophils to cultured endothelial cells (EC) (14). It also induced EC to produce colony stimulating factors (CSF) (15), and a monocyte chemotactic protein (MCP-1) (16). Since the results of in vitro studies can often be different from those in vivo, we sought to further test the biologic activity of MM-LDL in vivo. We chose the mouse model for this in vivo work because of the convenience of working with these animals and the availability of mouse strains that are known to be genetically susceptible or resistant to the development of atherosclerosis and to cytokine induction by bacterial LPS (17–19).

Methods

Reagents. Anti-mouse macrophage CSF (M-CSF) antibody, raised against M-CSF purified from the conditioned medium of L cells, was a generous gift from Dr. E. R. Stanley, Albert Einstein College of Medicine, Bronx, NY. Lipopolysaccharide prepared from *Escherichia coli* 0111:B4 was purchased from List Biological Laboratories, Inc., Campbell, CA. All other reagents were from previously described sources (14–16).

Mice. BALB/cHS, C3H/HS, and C57BL/6HS female mice, 3–6 mo of age, were purchased from Harlan Sprague Dawley, Inc., Indianapolis, IN, and C3H/HeJ and C57BL/6J female mice, 3–6 mo of age, were purchased from Jackson Laboratories, Bar Harbor, ME. The test substances were injected into the tail veins 4 or 5 h before killing unless otherwise indicated.

Lipoproteins. Human LDL was isolated as previously described (14) from the serum of normal donors after an overnight fast and after

1. Abbreviations used in this paper: CSF, colony stimulating factors; EC, endothelial cells; JE, the mouse homologue of the gene for MCP-1; MCP-1, monocyte chemotactic protein; M-CSF, macrophage CSF; MM-LDL, minimally modified LDL; TBARS, thiobarbituric acid-reactive substances.

obtaining written permission. MM-LDL was prepared as previously described by either cold storage or iron oxidation (14) and contained 2.0–3.7 nmol of thiobarbituric acid reactive substances (TBARS) as malondialdehyde equivalents per mg cholesterol, and < 4 pg of contaminating LPS per μg of LDL protein. MM-LDL prepared by cold storage was kept sterile in plastic tubes at 4°C for 6–11 mo in phosphate buffered 0.15 M NaCl containing 0.01% EDTA. MM-LDL was also prepared by iron oxidation by dialyzing native LDL against 9 μM FeSO_4 in phosphate buffer, pH 7.2, for 72 h at 4°C as described by Kosugi and colleagues (20). Each preparation of MM-LDL was tested in vitro for its ability to induce monocyte binding to EC as described previously (14) or for the induction of mRNA for JE (the mouse homologue of the gene for MCP-1) in mouse L cells by Northern analysis as described below. Not all preparations of LDL that were stored under these conditions became biologically active. Only a minority (on the order of one in five) of the preparations demonstrated biologic activity even though almost all of the preparations demonstrated a small increase in TBARS (2–4 nmol of TBARS as malondialdehyde equivalents per mg cholesterol) characteristic of MM-LDL. Similarly, activity after iron oxidation varied from preparation to preparation as a function of the time of exposure to FeSO_4 . Therefore, the optimum time for exposing native LDL to FeSO_4 must be determined empirically for each LDL preparation in order to produce biologically active MM-LDL. As previously reported (14) the biologically active component in MM-LDL appears to be a polar lipid(s) presumably formed by oxidation of unsaturated lipid contained in native LDL. With continued oxidation (i.e., continued exposure to FeSO_4 beyond the time required to induce biologic activity) the induced biologic activity was lost, as would be expected if the biologically active agent in MM-LDL were an oxidized polar lipid. Between one in four and one in two preparations can be expected to show biologic activity after exposure to FeSO_4 for periods up to 72 h. Moreover, in testing these preparations in vitro we found that as previously described (14) one can induce resistance in cultured cells to MM-LDL and, therefore, the preparations of MM-LDL must be tested against cells that have not been induced to be resistant. Each preparation of MM-LDL used in these studies was found to be active in vitro before testing in vivo.

Serum CSF assay. Blood was obtained from the mice by heart puncture under Halothane anesthesia. CSF activity was determined by mouse bone marrow cell colony formation assay as previously described (21). Serum CSF activity was expressed as CFU per ml of serum. One CFU is defined as the amount of CSF stimulating formation of one colony per 10^5 plated mouse bone marrow cells (within the linear range of the assay).

RNA analysis. A 445-bp JE cDNA containing the complete coding sequence was obtained by polymerase chain reaction amplification of cDNA synthesized from total NIH 3T3 cell RNA. Density arrested cell cultures grown in DME supplemented with 10% fetal bovine serum were transferred to fresh DME supplemented with 5% platelet-poor plasma, free of platelet derived growth factor (PDGF), for 16 h. Cells were then treated with 0.1 ng/ml rPDGF (BB-homodimer; Genzyme Corp., Cambridge, MA) for 2 h (22) before RNA extraction (23). The cDNA synthesis was performed using oligo(dT) primer followed by polymerase chain reaction amplification for 30 cycles with Taq DNA polymerase (Perkin-Elmer/Cetus, Emeryville, CA) using a denaturation temperature of 95°C (1 min), an annealing temperature of 55°C (1 min), and an extension temperature of 72°C (3 min). The amplification buffer contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 0.01% (wt/vol) gelatin, and 200 μM each dATP, dTTP, dGTP, and dCTP. Reactions were performed in a 100- μl vol containing 100 pmol of the two primer oligonucleotides. The synthetic oligomers used for the amplification were based on the JE nucleotide sequence determined by Kawahara and Deuel (24): (5') 5'ATGCAGGTCCCTGT-CATGCTTCTGGGC3' and (3') 5'GTTCAGTGCACACTGGT-CACTCCTAC 3'. The resulting double stranded cDNA of the expected size of 445 bp was gel purified and labeled with ^{32}P to a sp act of $\sim 10^9$ cpm/ μg using random priming. Northern blot analysis was performed as previously described (16). In addition, the identity of the gel-purified

cDNA was confirmed to be JE by sequence analysis following subcloning of the fragment into a PUC 18 vector.

Results

Induction of M-CSF activity in serum after injection of MM-LDL. Within 5 h after injection of MM-LDL into BALB/cHS or into C57BL/6HS mice there was a concentration-dependent increase in serum CSF activity which was not seen after the injection of native LDL. In addition, injection of LPS in concentrations that were determined to be the maximum that could have contaminated the MM-LDL preparations failed to induce CSF activity (Fig. 1). As shown in Fig. 2, CSF activity peaked ~ 9 h after injection of MM-LDL and was declining by 20 h. Experiments with neutralizing antibody (Table I) indicated that almost all of the induced CSF activity was due to M-CSF. As shown in Fig. 3, BALB/cHS mice readily induced CSF activity when injected with LPS (at a concentration 2,000-fold greater than that determined to be the maximum LPS that could have contaminated the MM-LDL) whereas the C3H/HeJ mice that are genetically resistant to LPS showed poor induction of CSF at even higher concentrations. In contrast CSF induction was equal in the two strains after injection of MM-LDL. These results provide definitive proof that the biologic activity of MM-LDL was not due to contaminating LPS. Moreover, they indicate that there are differences in the pathways by which LPS and MM-LDL induce cytokines. The MM-LDL used for the experiments in Figs. 1–3 and in Table I were prepared by cold storage. Fig. 4 demonstrates that MM-LDL prepared by iron oxidation also gave similar results.

MM-LDL induces JE mRNA. The mouse homologue of the human MCP-1 gene is known as JE (25). As shown in Fig.

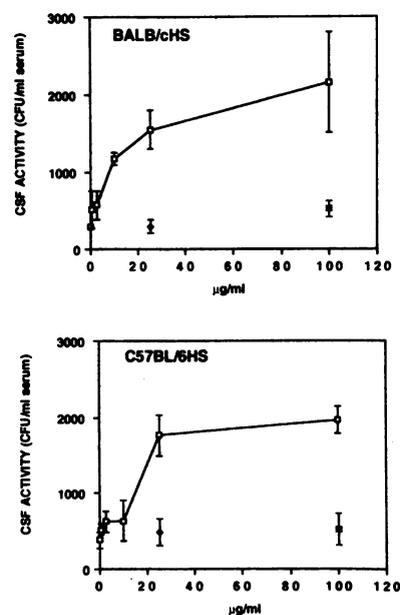


Figure 1. Serum CSF activity after the injection of LDL, MM-LDL, or LPS. BALB/cHS and C57BL/6HS mice were injected intravenously with LDL (\blacklozenge), MM-LDL (prepared by cold storage and containing 3 nmol of TBARS as malondialdehyde equivalents per mg cholesterol) (\blacksquare), or LPS (\blackstar) 5 h before killing. The concentrations shown on the abscissa are the concentration of MM-LDL or LDL in mouse blood immediately after injection assuming a blood vol of 2 ml. The concentration of LPS (50 $\mu\text{g/ml}$) was determined as the maximum level

that could have been present in the MM-LDL at a blood concentration of 100 $\mu\text{g/ml}$ MM-LDL. Neither MM-LDL nor LPS had CSF activity when added to the mouse bone marrow cell cultures (data not shown). The data shown are from the sera of two mice with each serum assayed in duplicate to give a total of four assays for each data point. The values are the mean \pm 1 SD of these assays.

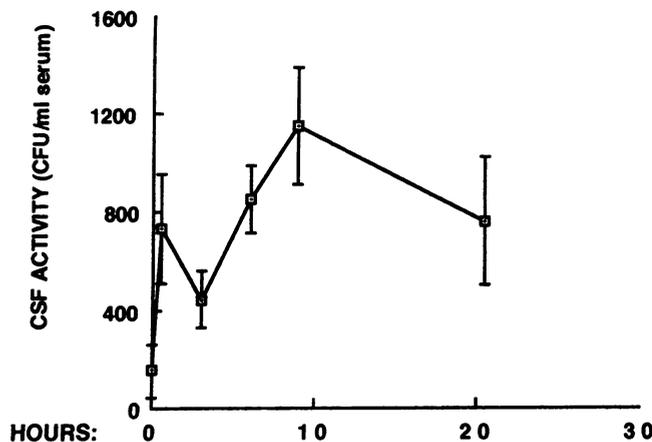


Figure 2. Time course of the induction of serum CSF activity after injection of MM-LDL. BALB/cHS were injected intravenously with MM-LDL (prepared by cold storage of LDL from a different donor from that described in Fig. 1 but also containing 3 nmol of TBARS as malondialdehyde equivalents per mg cholesterol) to give a blood concentration of 100 $\mu\text{g/ml}$ MM-LDL immediately after injection (assuming a blood vol of 2 ml). Blood was collected at the time points indicated on the abscissa and CSF activity in the sera was determined. Four mice were used for each time point and their sera was assayed in duplicate. The values shown are the mean \pm 1 SD of these assays.

5, there was a dramatic increase in JE mRNA in liver after the injection of MM-LDL (prepared by mild iron oxidation) without a significant change in tubulin mRNA. In contrast, injection of the same LDL (but without iron oxidation) did not induce mRNA for JE (Fig. 5). Similar results were seen after injection of MM-LDL prepared by cold storage and there was also induction of JE mRNA in heart, lung, spleen, and kidney in all strains tested including C57BL/6HS, BALB/cHS, and C3H/HS (data not shown). 9 h after the injection, mRNA levels for JE had returned to baseline (data not shown).

Discussion

In various animal models of atherosclerosis cholesterol-feeding results in the rapid retention of apolipoprotein B-containing lipoproteins in the subendothelial space (1-3) followed by the

Table I. MM-LDL Induces M-CSF

Preincubation	Percentage of original activity
None	100.0 \pm 1.1
Anti-M-CSF	17.9 \pm 10.5
Irrelevant IgG	83.6 \pm 3.7

Duplicate BALB/cHS mice were injected with MM-LDL (prepared by cold storage and containing 3 nmol of TBARS as malondialdehyde equivalents per mg cholesterol) to give a blood concentration immediately after injection of 50 $\mu\text{g/ml}$ (assuming a blood vol of 2 ml). Blood was collected 5 h later and the sera were preincubated for 20 min without additions, or with anti-M-CSF antibody, or with an irrelevant IgG before addition of the sera to the mouse bone marrow cell cultures. The sera were assayed in triplicate or quadruplicate and the data shown are the mean \pm 1 SD of the assays from both mice.

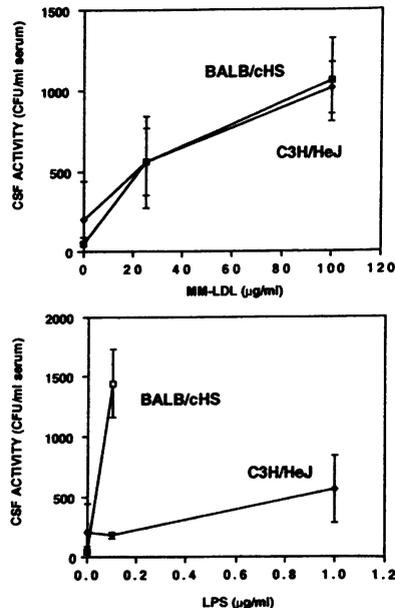


Figure 3. Induction of serum CSF activity after injection of LPS or MM-LDL into BALB/cHS and C3H/HeJ mice. Blood was collected 5 h after injection of LPS or MM-LDL (prepared by cold storage of LDL from a donor different from those used in the experiments of Figs. 1 and 2 and containing 2 nmol of TBARS as malondialdehyde equivalents per mg cholesterol) into BALB/cHS (\square) or C3H/HeJ (\blacklozenge) mice. The concentrations shown on the abscissa are those immediately after injection assuming a blood vol of 2 ml. Each data

point represents serum taken from two mice and assayed in duplicate. The values are the mean \pm 1 SD of these assays.

adherence of monocytes to the endothelium at sites of predilection such as the orifices of the intercostal arteries (26-29). Enhanced monocyte progenitor cell proliferation occurs in the bone marrow of cholesterol-fed swine and this is accompanied by increased ability of the sera from these swine to induce monocytic colonies in bone marrow cell cultures (30). Our recent

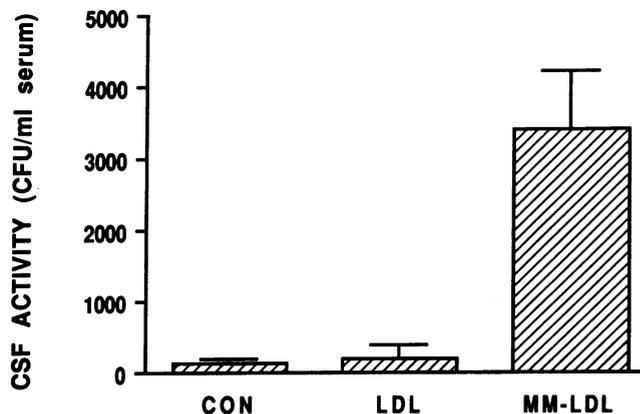


Figure 4. Serum CSF activity in control mice or after injection of LDL or MM-LDL. C57BL/6J mice were either not injected (CON) or were injected with LDL from a donor different from those used in the experiments described above and containing $<$ 2 nmol of TBARS as malondialdehyde equivalents per mg cholesterol (LDL) or the same LDL exposed to FeSO_4 as described in Methods and subsequently containing 3.7 nmol of TBARS as malondialdehyde equivalents per mg cholesterol (MM-LDL) 3-6 h before killing to give a blood concentration of 100 $\mu\text{g/ml}$ of LDL or MM-LDL immediately after injection (assuming a blood vol of 2 ml). The sera from three control mice, three mice injected with LDL, and two mice injected with MM-LDL were assayed in duplicate for CSF activity. The values are the mean \pm 1 SD of these assays.

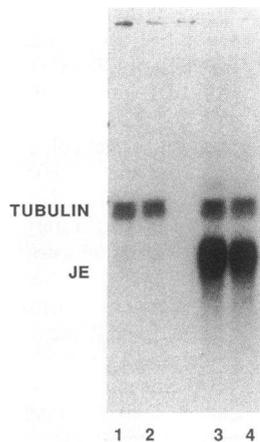


Figure 5. Induction of mRNA for JE after injection of LDL or MM-LDL into C57BL/6J mice. RNA was extracted from the livers of two of the mice described in Fig. 4 after injection with LDL (lanes 1 and 2) or from both of the mice injected with MM-LDL (lanes 3 and 4) and subjected to Northern blot analysis using probes for JE and α -tubulin.

findings that in vitro MM-LDL induced the binding of monocytes but not neutrophils to cultured EC (14), induced EC to produce CSF (15) and MCP-1 (16), together with the findings reported here that MM-LDL induced similar events in vivo, suggest that the early cellular events in atherogenesis could be due, in part, to the formation of MM-LDL. As the monocytes migrate into the subendothelial space and convert into macrophages further modification of LDL could occur as a result of the release of metabolites from the macrophages (11, 31) into the microenvironment. The resulting highly modified LDL would then be taken up by the scavenger receptor, producing foam cells.

There are genetic differences in susceptibility of inbred strains of mice to the diet-induced development of the early events of atherogenesis (monocyte adhesion to the endothelium, monocyte chemotaxis into the subendothelial space, monocyte conversion into macrophages, and monocyte foam cell formation) (17, 18). C57BL/6 mice are highly susceptible while BALB/c and C3H are relatively resistant (17, 18). Since we found that all of these strains readily induced M-CSF in their sera and mRNA for JE in their tissues after injection of MM-LDL, it is possible that the varied susceptibility to diet-induced development of these early events of atherogenesis is due to differences in the formation of oxidatively modified LDL. The inbred strains of mice that are resistant to the development of these early events of atherogenesis have significantly higher levels of HDL compared to the strains that are susceptible (18). Evidence from several laboratories suggests that HDL is a potent antioxidant that is capable of preventing the formation of oxidatively modified LDL in vitro (32–35). If this is also true in vivo, some of the genetic differences in susceptibility of inbred strains of mice to the development of these early events of atherogenesis may be due to differences in their HDL levels on the high fat diet and the resulting differences in their ability to resist oxidative modification of LDL.

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