Macrophage-derived factors increase low density lipoprotein uptake and receptor number in cultured human liver cells

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Abstract Recent evidence suggests the possibility that macrophages can influence lipoprotein metabolism. Therefore we investigated the ability of cultured macrophages to alter low density lipoprotein (LDL) uptake in a human liver cell line (HepG2). Conditioned media from phlogogenic-induced mouse peritoneal macrophages or from a human macrophage cell line stimulated with endotoxin increased HepG2 LDL uptake by as much as 60–70%. The increase was due, in part, to a significant macrophage-induced 40% increase in the number of LDL receptors per cell. Although macrophage conditioned media inhibited HepG2 cholesterol synthesis, the LDL receptor up-regulation did not appear to be due to the effects on cholesterol synthesis. The LDL receptor stimulatory activity was sensitive to proteolysis and heat. Its molecular mass was approximately 20 kDa based on gel filtration. Several macrophage secretory proteins were tested in HepG2 cultures for LDL uptake stimulation. Of these, oncostatin M (approximately 18 kDa by gel filtration) gave the strongest response. The rank order for LDL uptake stimulation was oncostatin M >> interleukin 6 = interleukin 1 = transforming growth factor-beta 1. A neutralizing antibody directed against oncostatin M inhibited the ability of conditioned media to up-regulate LDL receptors by 85%. Thus, our results indicate that macrophages can secrete several proteins that up-regulate LDL receptors in HepG2 cells and that most of the up-regulatory activity in macrophage conditioned media appears to be due to oncostatin M.—**Grove, R. I., C. Mazzucco, N. Allegretto, P. A. Kiener, G. Spitalny, S. F. Radka, M. Shoyab, M. Antonaccio, and G. A. Warr.** Macrophage-derived factors increase low density lipoprotein uptake and receptor number in cultured human liver cells. *J. Lipid Res.* 1991. **32:** 1889–1897.

Supplementary key words macrophage • LDL receptor regulation • HepG2 cells • oncostatin M

A number of factors influence cholesterol homeostasis, including low density lipoprotein (LDL) uptake by the liver LDL receptor pathway. Regulation of LDL uptake is thought to occur at the level of LDL receptor (LDLR) transcription and is inversely related to intrahepatocyte free cholesterol concentration (1–5). Relatively little is known about regulation of liver lipoprotein receptors by mechanisms that are unrelated to the intracellular cholesterol regulation pathway.

Recently, in vivo evidence that macrophages may affect cholesterol homeostasis has been emerging. Colony stimulating factors (GM-CSF, M-CSF) that can activate macrophages produced a dramatic decrease in total serum cholesterol in humans (6) and in primates (7). A study in diet-induced hypercholesterolemic rats revealed that injection of zymosan, another macrophage activator, caused a significant decrease in total serum cholesterol (8). It has also been reported that macrophages secrete a factor that increases both cholesterol synthesis and receptor-mediated uptake of LDL in cultured monkey arterial smooth muscle cells (9). Furthermore, it is well known that macrophages secrete factors that affect hepatocyte cultures. Transforming growth factor beta 1 has been shown to inhibit proliferation of primary hepatocytes (10). Interleukin 6 (hepatocyte-stimulating factor) increased the expression of acute phase proteins in human hepatoma (HepG2) cells (11). Finally, endotoxin-stimulated macrophage conditioned medium increased the production of fibronectin in primary rat hepatocytes (12).

These findings suggest that macrophages can influence liver function and lipoprotein metabolism. Therefore, we investigated the ability of macrophage conditioned media or purified macrophage secretory proteins to affect LDLR in the human hepatoma cell line, HepG2.

MATERIALS AND METHODS

Reagents

The culture media and certified fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand

Abbreviations: LDL, low density lipoprotein; LDLR, LDL receptor; LPDS, lipoprotein-deficient serum; FBS, fetal bovine serum; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; CSF, colony stimulating factor; DiI-LDL, fluorescent LDL; PMA, phorbol myristate acetate; CCM, control macrophage conditioned medium; SCM, stimulated macrophage conditioned medium; TNF, tumor necrosis factor.

Island, NY). Brewers Modified Thioglycollate Medium (4%) came from BBL Microbiology System (Cockeysville, MD). DiI-LDL (human) and lipoprotein-deficient serum (LPDS, bovine) came from Biomedical Technologies, Inc. (Boston, MA). Transforming growth factor-beta 1 (recombinant human), oncostatin M (human), and monoclonal antibodies were from Oncogen (Seattle, WA). The OM1, OM2, and OM3 monoclonal antibodies were directed against epitopes on native oncostatin M; OM1 did not neutralize the effects of oncostatin M in a growth inhibition assay while OM2 completely abbrogated oncostatin M-induced activity in a concentration-dependent fashion (Radka, S. F., J. Kallestad, P. S. Linsley, and M. Shoyab, unpublished results). The other cytokines and growth factors were recombinant human proteins and were from Genzyme (Boston, MA). All other reagents were from Sigma, Inc. (St. Louis, MO).

Macrophage culture

Peritoneal macrophages were obtained from outbred male Swiss ICR mice (20–35 g) from Harlan Sprague-Dawley. Four percent sterile Brewers Modified Thioglycollate medium (2 ml) was injected into the peritoneal cavity; 5 days later macrophages were collected by peritoneal lavage with 10 ml phosphate-buffered saline containing 3 mM EDTA (pH 7.4). Freshly collected macrophages were pelleted by centrifugation (400 *g* for 5 min). Red blood cells were removed by hypotonic lysis (water for 30 sec followed by addition of 10-fold excess phosphate-buffered saline). The cells were collected by centrifugation and resuspended in Dulbecco's Modified Eagle's Medium (high glucose; DMEM) with 10% fetal calf serum. The cells were 95% pure as determined by differential staining. The macrophages were plated into 12-well dishes (Costar, Cambridge, MA) at 1×10^6/ml per well and allowed to adhere for 2 h in a 37°C humidified 5% CO_2 incubator. Nonadherent cells were removed with 2 washes of DMEM. Macrophage monolayers were incubated for 20 h in the presence of the indicated stimulators in 1 ml DMEM.

Human monocytes (THP-1 monocytic cells; ATCC, Bethesda, MD) maintained in RPMI 1640 and 10% FBS were induced to differentiate by changing the medium to RPMI 1640 containing 50 nM phorbol myristate acetate for 24 h. The mouse macrophage cell line (RAW) was obtained from ATCC.

LDL receptor assays

HepG2 hepatoma cells were obtained from American Type Culture Collection (Bethesda, MD). Twenty four hours after passage into 12-well plates (2×10^5 cells/well), the culture media (RPMI + 10% FBS) were removed and replaced with conditioned media from macrophage cultures (final composition was 50% conditioned medium, 45% RPMI, and 5% LPDS). After 18 h, LDL uptake in HepG2 cells was assayed by the fluorescent LDL (DiI-LDL) technique (13, 14), modified as described below. DiI-LDL (2 μg/ml) was added to the HepG2 monolayers. Two hours later (uptake was linear for 3 h) the monolayers were washed 3 times to remove free DiI-LDL and fixed with 4% formalin in phosphate-buffered saline. Accumulated fluorescence was measured with a fluorescence microscope-computer combination and JAVA image analysis software (Jandel Scientific, CA) by quantitating the average intensity of an area of interest. Each area of interest included approximately 30 cells. Three different areas in each of duplicate or triplicate monolayers were measured and averaged. The amount of LDL protein taken up was determined as follows. Known protein amounts of DiI-LDL were extracted into chloroform and the fluorescence intensities of the chloroform solutions were measured with a fluorescence spectrophotometer (Perkin-Elmer: excitation 520 nm, emission 578 nm). A standard curve of μg protein versus fluorescence intensity was generated. Unfixed cell monolayers that had taken up DiI-LDL were solubilized with 0.1% sodium dodecyl sulfate and the lipids (and DiI) were extracted into chloroform. The fluorescence intensity of the extracted DiI-LDL from the monolayers was spectrophotometrically quantitated and compared with the standard curve. In some cases uptake of DiI-LDL into unfixed monolayers was measured by image analysis and then the DiI was extracted and measured with the spectrophotometer in order to ensure accuracy of the image analysis technique. Cell protein concentrations were measured by Coomassie blue dye staining (Pierce).

Surface LDLR were quantitated in fixed (4% formalin) HepG2 monolayers using standard ELISA assay protocols. Briefly, the monolayers were blocked for 2 h with a 3% solution of bovine serum albumin in PBS and then incubated with the anti-LDLR antibody, C-7 (15). The secondary antibody was a goat anti-mouse-peroxidase conjugate (Cooper Biochemical). After removal of excess antibody the colorimetric assay solution (0.1 M citrate phosphate (pH 5), 4 mg/ml O-diphenylene diamine, and 0.00012% hydrogen peroxide) was added for 8–10 min. Color development was stopped by the addition of HCl (2.5 N final concentration) and the absorbances (490 nm) were measured on a microtiter plate reader (Molecular Devices).

Results obtained from the DiI-LDL uptake and ELISA measurements of LDLR in HepG2 monolayers cultured in standard inducing media conditions (LPDS) were similar or identical to data obtained from ^{125}I-labeled LDL binding studies in HepG2 cells cultured in LPDS (compare the 38% increase in receptors in Table 3 to the 35% increase measured in reference 16). To establish the validity of the fluorescent LDL assay for HepG2 cells, we measured uptake and binding of ^{125}I-labeled LDL in cells that were down-regulated with media containing LDL (FBS),

TABLE 1. Validity of the fluorescent LDL assay for HepG2 cells

Experiment	ng LDL/mg Cell Protein	Percent of Control
^{125}I-labeled LDL binding		
FBS control	4.8 ± 1.2	
LPDS control	8.0 ± 0.7	167[a]
LPDS + oncostatin M	15.0 ± 1.0	188[b]
^{125}I-labeled LDL uptake		
FBS control	85.0 ± 5.0	
LPDS control	142.0 ± 2.0	167[a]
LPDS + oncostatin M	213.0 ± 24.0	150[b]
DiI-LDL uptake		
FBS control	120.0 ± 7.0	
LPDS control	190.0 ± 7.0	158[a]
LPDS + oncostatin M	280.0 ± 15.0	147[b]

Values are presented as means ± SEM.
[a]Relative to FBS control.
[b]Relative to LPDS control.

up-regulated with media lacking LDL (LPDS), or in cells up-regulated with LPDS and treated with oncostatin M. Radiolabeled LDL (0.164 μCi/μg) uptake and binding studies were performed according to the techniques of Semenkovich and Ostlund (16), while DiI-LDL assays were performed on parallel cultures as outlined above (**Table 1**). The data in Table 1 confirm that similar results are obtained from the DiI-LDL and ^{125}I-labeled LDL methods for assaying LDLR in HepG2 cells.

Western blots

Samples of macrophage conditioned media were concentrated 100-fold, dried in vacuo, and separated by 15% SDS-PAGE. After transfer to nitrocellulose and blocking with 5% nonfat milk in PBS, the blots were incubated with spent supernatants from the OM3 hybridoma, which has been shown previously to contain antioncostatin M IgG (Radka, S. F., J. Kallestad, P. S. Linsley, and M. Shoyab, unpublished results). Peroxidase-conjugated F(ab)'$_2$ goat anti-mouse Ig (Pel-Freeze, Rogers, AK) was added and, after washing, the reaction was developed by Enhanced Chemoluminescence (Amersham).

Sterol synthesis assay

Cholesterol synthesis was determined by the method of Beg, Reznikov, and Avigan (17). Briefly, 2 μCi/ml [^{14}C]acetate (15 Ci/mmol) was incubated with HepG2 cultures for 2 h. After washing the monolayers to remove free radiolabel, the cells were lysed with 1.5 N NaOH for 10 min, and the lysate was saponified at 70°C for 1.5 h. The unsaponified lipids were extracted with petroleum ether and dried under a stream of nitrogen. In some cases the lipids were resuspended in ethanol–acetone 1:1 and the sterols were precipitated with 1% digitonin. Radioactivity was measured with a scintillation counter.

Column chromatography

Macrophage media were concentrated 500-fold on an Amicon filter (YM-5). One ml was applied to a GF 250XL column (Dupont Zorbax Bioseries, 21.1 mm × 25 cm) on a System Gold HPLC (Bechman), eluted with a flow rate of 1 ml/min, and collected in 1-ml fractions. The mobile phase was phosphate-buffered saline (pH 7.2). Molecular weight standards were B-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and myoglobin (17 kDa).

TABLE 2. Macrophage conditioned media stimulates LDL uptake in HepG2 cells

Macrophage Stimulant	Stimulation of HepG2 LDL Uptake by Macrophage Conditioned Media: n	Stimulation of HepG2 LDL Uptake by Macrophage Conditioned Media: Percent Stimulation
Mouse macrophage conditioned media		
Control (unstimulated conditioned medium)	4	0
LPS control (200 ng/ml LPS was added directly to HepG2 cultures)	2	0
LPS (200 ng/ml)	4	67 ± 6
Acetylated LDL (20 μg/ml)	3	−16 ± 9
Zymosan (20 μg/ml)	2	3 ± 2
GM-CSF (1000 U/ml)	2	−3 ± 3
M-CSF (1000 U/ml)	2	−1 ± 14
Human macrophage conditioned media		
Undifferentiated + LPS (200 ng/ml)	2	0
PMA differentiated control	2	0
PMA differentiated + LPS	2	31 ± 4

Mouse peritoneal macrophages or human macrophages (THP-1) were cultured as outlined in Methods. Serum-free media (DMEM or RPMI 1640) were removed from macrophage cultures stimulated with the indicated factors for 20 h, centrifuged (400 *g*), and mixed with equal amounts of RPMI 1640 containing 10% lipoprotein-deficient fetal bovine serum (LPDS). The 50% conditioned medium (1 ml) was added to HepG2 cells cultured in 12-well plates and LDL uptake was assayed as in Methods. Control uptake was 205 ng LDL/mg cell protein. The results are presented as means ± ½ the range between the two averages (n = 2 experiments) or means ± SEM (n = more than 2 experiments).

RESULTS

Conditioned media from mouse peritoneal macrophages treated with one of several activating factors were added to HepG2 cultures. Endotoxin (LPS)-stimulated macrophage medium induced an increase in HepG2 LDL uptake while LPS added directly to HepG2 cultures had no effect (**Table 2**). Addition of unstimulatd macrophage conditioned medium or conditioned medium from macrophages treated with other activators (GM-CSF, M-CSF, zymosan, acetyl LDL) did not increase LDL uptake (Table 2). The human monocytic cell line, THP-1, acquires some of the characteristics of differentiated macrophages in the presence of phorbol ester stimulators of protein kinase C (18). Conditioned media from phorbol ester-differentiated THP-1 cultures activated with LPS also stimulated LDL uptake in liver cells (Table 2) indicating that human macrophages have the ability to increase HepG2 LDL uptake.

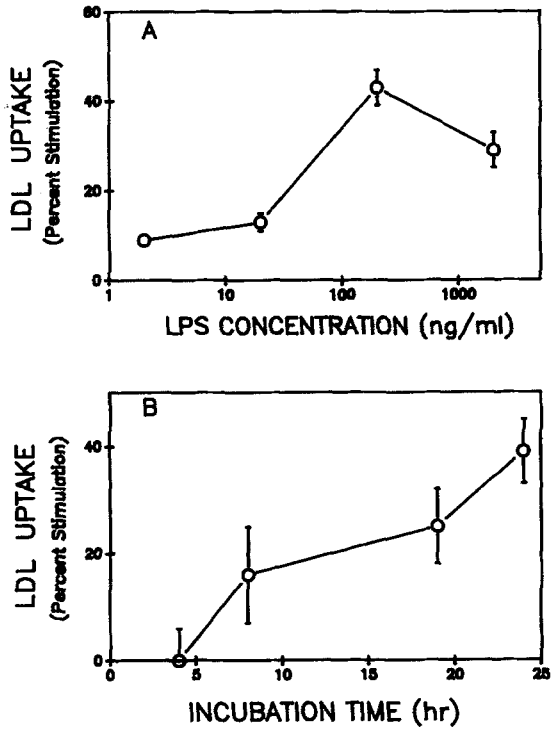


Fig. 1. Endotoxin (LPS)-stimulated macrophage conditioned medium and HepG2 LDL uptake. The experimental conditions are as described in Materials and Methods. A: Dependence of HepG2 LDL uptake stimulation on LPS concentration in macrophage cultures. The data are means ± SEM. For the 200 ng/ml value, n = 5 different experiments. The remaining values were from three experiments. B: Dependence of LDL uptake stimulation on LPS incubation time in macrophage cultures. LPS (200 ng/ml) was added to macrophage cultures and incubated for the various times indicated before adding the macrophage conditioned media to HepG2 monolayers. The data are from one experiment (means ± SD for n = 6 different determinations) and are qualitatively similar to the results from two other experiments. Control uptake was 213 ng LDL/mg cell protein for both A and B.

TABLE 3. Effect of macrophage conditioned medium on HepG2 LDLR immunoreactivity

Medium Condition	LDLR Immunoreactivity (OD-490/mg cell protein)
5% FBS control	3.4 ± 0.1
5% LPDS control	4.7 ± 0.2
CCM	4.35 ± 0.2
SCM	5.6 ± 0.3

After incubation for 20 h under the conditions shown, HepG2 monolayers were analyzed for LDLR by the ELISA as described in Methods. CCM: HepG2 cells were incubated with equal parts of 10% LPDS and control macrophage conditioned medium. SCM: cells were incubated with equal parts of 10% LPDS and stimulated macrophage conditioned medium. The data represent means ± SEM for three different experiments. SCM values are significantly different from CCM and LPDS controls ($P < 0.05$, Student's paired t test). Average proteins (μg ± SEM) were: FBS, 292 ± 25; LPDS, 297 ± 30; CCM, 298 ± 32; SCM, 272 ± 31.

The ability of LPS to stimulate release of factors from murine macrophages that gave rise to increases in LDL uptake was concentration dependent. At 200 ng/ml, LPS gave the maximum response of about a 40% increase (**Fig. 1A**). Although LDL uptake was always stimulated by conditioned media from LPS-treated macrophages, the magnitude of stimulation varied between experiments. The macrophage-induced increase in LDL uptake was dependent on LPS incubation time, with the maximum stimulation occurring after 24 h of incubation (Fig. 1B).

To determine whether the macrophage effect on LDL uptake was due to a stimulation of receptor cycling or an increase in receptor number, we developed an ELISA assay consisting of an antibody (C7) directed against the LDL receptor (15). Using this assay, we compared receptor immunoreactivity in HepG2 cultures treated with media from unstimulated or stimulated macrophages. As illustrated in **Table 3,** stimulated macrophage conditioned medium (SCM) induced an increase in LDL surface receptor protein of approximately 30%.

The effects of macrophages on LDL receptors were compared to the up-regulation of LDL receptors that is induced by the absence of cholesterol in the medium. In HepG2 cells deprived of cholesterol (incubated in lipoprotein-deficient serum), LDL uptake increased approximately 60% compared to control cells cultured in the presence of cholesterol (uptake was 280 ng and 175 ng LDL per mg cell protein, respectively; **Fig. 2A**). This stimulation is similar in magnitude to values reported by others for HepG2 cells grown in cholesterol-free media (16). Conditioned medium from LPS-stimulated macrophages (SCM) induced a 42% increase (Fig. 2B) in LDL uptake compared to conditioned medium from unstimulated macrophages (CCM). It is important to note that this up-regulation by SCM occurred in HepG2 cells incubated in LPDS and therefore already up-regulated by the absence of media cholesterol.

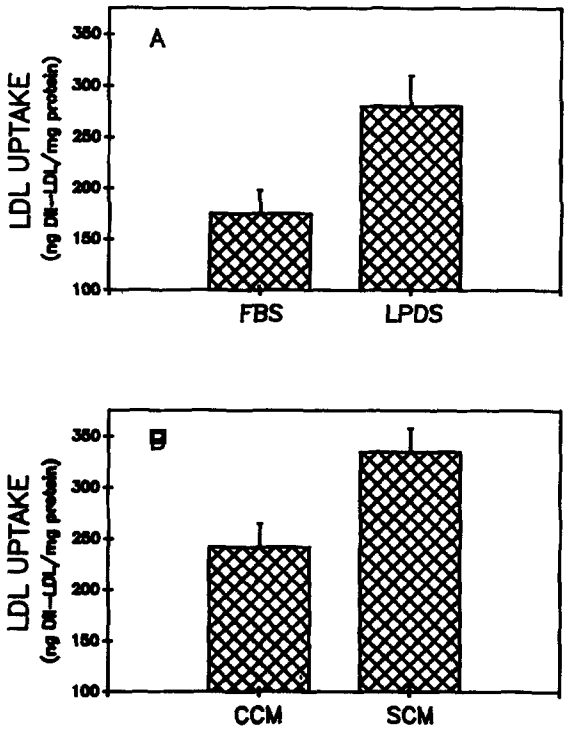


Fig. 2. Comparison of LDL uptake stimulation induced by cholesterol-free media (A) and by macrophages (B). LDL uptake was measured in cultures of HepG2 cells incubated with down-regulating medium containing cholesterol (5% FBS in RPMI 1640), up-regulating lipoprotein depleted medium (LPDS), control unstimulated macrophage conditioned medium (CCM), and conditioned medium from macrophages stimulated with 200 ng/ml LPS (SCM). Note that the media components of LPDS, CCM, and SCM are 50% DMEM, 45% RPMI 1640, and 5% LPDS. LDL uptake was quantitated using the fluorescent LDL technique and normalized to amount of cell protein. The data represent the means ± SEM from four different experiments. The SCM value is significantly different from the CCM value ($P < 0.05$, Student's paired t test). Average proteins (μg ± SEM) are, FBS: 292 ± 25; LPDS: 297 ± 30; CCM: 298 ± 32; SCM: 272 ± 31.

In HepG2 control cells incubated in the absence of cholesterol (LPDS) there was a 38% increase in LDL receptor protein compared to controls incubated in the presence of cholesterol (Table 3). The magnitude of this induction agrees very well with results obtained by others using HepG2 cells cultured in cholesterol-free media (35% increase in LDL binding ability based on ^{125}I-labeled LDL binding techniques, ref 16).

In order to investigate the mechanism by which macrophages induced HepG2 LDL receptors, we studied the effect of macrophage conditioned medium on cholesterol synthesis. In lipoprotein-deficient medium, both control and stimulated conditioned media inhibited the incorporation of radiolabeled acetate into HepG2 sterols (28% and 41% respectively, **Table 4**). Interestingly, although control conditioned media decreased cholesterol synthesis by 28%, it was not able to stimulate LDL uptake (compare LPDS control and CCM in Fig. 2).

Activated macrophages are known to secrete growth factors (PDGF or MDGF) which can increase cell growth (19). To determine whether the macrophage effect on LDL receptors could be explained by an increase in cell number, we assayed total cell protein. There was no significant difference in total cell protein in any of the treatments. Average proteins (μg ± SEM) from one series of experiments were FBS: 292 ± 25; LPDS: 297 ± 30; CCM: 298 ± 32; SCM: 272 ± 31. In addition, macrophage conditioned media did not increase DNA synthesis after 20 h of incubation, as measured by [^{3}H]thymidine incorporation in HepG2 cells (data not shown).

The identity of the LDL receptor stimulating activity from mouse macrophages was investigated. The activity was sensitive to heating (100°C for 10 min) and to proteolysis, but not to freeze/thaw cycling (**Table 5**). In addition, the activity eluted in the void volume from G-25 Sephadex column chromatography. These findings suggested that the up-regulating factor was a protein with a molecular mass of at least 5 kDa. We further fractionated the conditioned media on a GF 250XL gel filtration column and analyzed the fractions for ability to stimulate LDL uptake in HepG2 cell cultures. As shown in **Fig. 3,** the largest peak of activity eluted from the column at approximately 20 kDa. Tumor necrosis factor eluted as a tight peak at fraction 47 (54 kDa, arrow) presumably as a trimer.

A number of proteins with molecular masses in the range of 20 kDa are secreted by macrophages. Therefore we investigated the LDL uptake stimulation abilities of several known macrophage secretory proteins (**Table 6**). Tumor necrosis factor-alpha inhibited LDL uptake slightly while platelet-derived growth factor (BB) had no effect. Interleukin-1, transforming growth factor-beta 1, and interleukin-6 induced relatively minor increases (25–30%) while oncostatin M, a protein with an apparent molecular mass of 18 kDa (as determined by gel filtration) and first isolated from a human macrophage cell line (21), gave a strong stimulation.

To determine whether oncostatin M is present in stimulated macrophage conditioned media, we preincubated the media with a neutralizing monoclonal antibody

TABLE 4. Inhibition of sterol synthesis by macrophage conditioned media

Medium	Percent Inhibition of HepG2 Sterol Synthesis
Control macrophage conditioned medium	28 ± 6
Stimulated macrophage conditioned medium	41 ± 6

Sterol synthesis was assayed in HepG2 cells treated with the indicated medium as outlined in Methods. The data represent means ± SEM of three separate experiments, each done in duplicate. The average for HepG2 controls grown in DMEM + 5% LPDS was 10,300 (± 486) cpm. Percent inhibition was calculated by dividing cpm values for the conditioned media-treated macrophages by cpm from HepG2 control monolayers, subtracting the resulting number from 1, and multiplying by 100.

TABLE 5. Effects of various treatments on the LDL receptor up-regulating ability of macrophage conditioned medium

Condition	LDL Uptake (ng LDL/mg cell protein)	Percent Inhibition
Control conditioned medium	245	
LPS-stimulated conditioned medium	375 ± 16	0
100°C for 10 min	250 ± 14	96
Freeze/thaw (3 cycles)	363 ± 26	8
Proteolysis	323 ± 2	40
G-25 filtration	353 ± 9	17

HepG2 cells were incubated for 20 h with stimulated macrophage conditioned medium that had been treated as shown and LDL uptake was measured. Proteolysis was achieved by adding macrophage conditioned medium to Immobilized Pronase-CB (3 U/ml; Pierce) and incubation at room temperature for 1 h. The pronase-agarose beads were removed by centrifugation and the media were added to the cells. For column chromatography, a G-25 Sephadex column (0.5 × 10 cm) was equilibrated with phosphate-buffered saline (pH 7.4). The conditioned medium was applied to the column and the void volume was analyzed in HepG2 cultures for stimulation of LDL uptake. The percent inhibition data reflect the ability of the indicated treatment to reduce only the portion of the LDL uptake induced by LPS-stimulated conditioned media. Each treatment was done at least twice, and the data represent the mean LDL uptake ± either SEM (when the data are from more than two experiments) or ½ the differences between the averages (when the data are from two experiments).

directed against oncostatin M (OM2). OM2 inhibited the up-regulation of the LDL receptor induced by macrophage conditioned media while neither a non-neutralizing oncostatin M antibody (OM1) nor an unrelated antibody (L6) was able to prevent the effect (**Fig. 4, left**). More direct results from an immunoblot study confirmed that a peptide of approximately 28 kDa is present in macrophage media and is recognized by anti-oncostatin M monoclonal antibodies (Fig. 4, right). The differences in oncostatin M molecular mass (18 kDa by gel filtration vs. 28 kDa by gel electrophoresis) has been documented (21) and may be due to nonspecific interaction of the peptide with the solid phase during gel filtration or to three-dimensional folding properties of the protein.

DISCUSSION

Low density lipoprotein receptor (LDLR) expression is thought to be largely controlled at the level of transcription and has been linked inversely to intracellular cholesterol levels (1–5). The disadvantage of this kind of regulation is that when circulating cholesterol is elevated because of the diet, LDL cholesterol taken into the hepatocyte causes LDLR down-regulation. The result is that the ability of the liver to further lower circulating levels of LDL cholesterol is compromised. Therefore a cholesterol-independent mechanism for up-regulation of liver LDLR would be desirable. The possible existence of up-regulatory mechanisms for LDLR that are distinct from cholesterol-

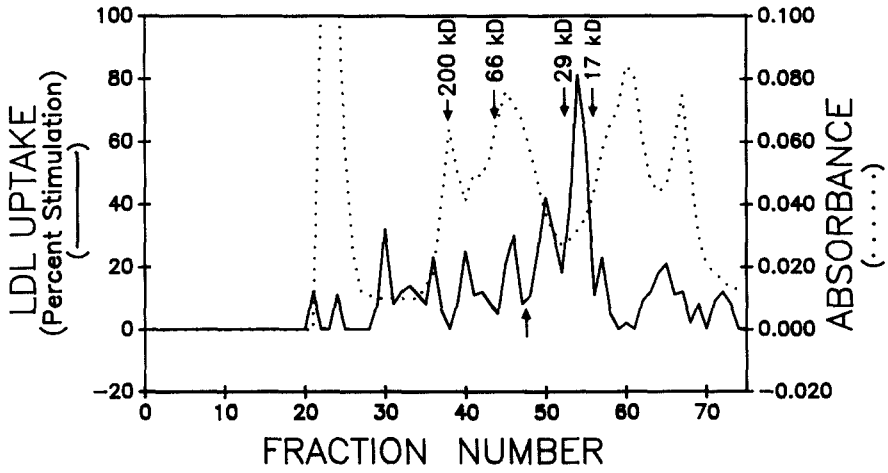


Fig. 3. Fractionation of LDL receptor up-regulatory activity in macrophage conditioned medium. Fractions of macrophage conditioned media, separated on the basis of size as described in Materials and Methods, were added to HepG2 cultures (20%, v/v) in LPDS medium and LDL uptake was analyzed. Proteins were monitored by measuring absorbance at 280 nm. The presence of tumor necrosis factor-alpha in its trimeric form was detected in fraction 47 (arrow) using the L929 cytotoxicity assay (20). These data represent the results from one experiment and are essentially identical to results obtained from two other experiments.

TABLE 6. Effects of selected factors on HepG2 LDL uptake

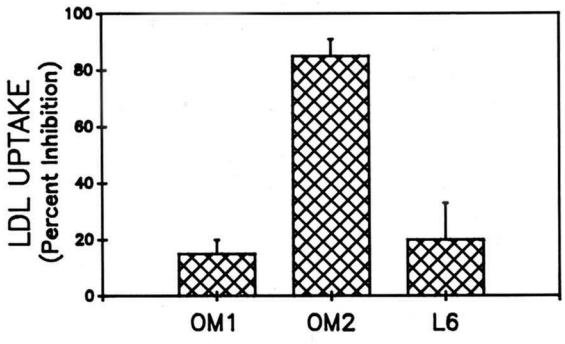
Factor	LDL Uptake (ng/mg cell protein)	Percent Change
Control uptake	180	
Tumor necrosis factor-alpha (100 ng/ml)	171 ± 11	−5
Interleukin 1-alpha (25 ng/ml)	229 ± 7	27
Interleukin 6 (100 ng/ml)	230 ± 6	28
Transforming growth factor-beta (200 ng/ml)	223 ± 12	24
Platelet-derived growth factor-BB (10 ng/ml)	180 ± 9	0
Oncostatin M (100 ng/ml)	315 ± 15	75

HepG2 cells were cultured for 20 h in the presence of RPMI containing 5% LPDS and the indicated factor. LDL uptake was assayed as described in Methods. The data represent the means ± SEM of at least three different experiments.

mediated pathways has not been fully explored. The findings in the present study are novel and indicate that a macrophage secretory factor(s) has the ability to up-regulate LDLR in cultured human liver (HepG2) cells by a mechanism that appears to be distinct from cholesterol-related LDLR regulation.

The macrophage-induced increase in both LDL uptake and in LDLR occurred in cells already up-regulated by incubation in LPDS (cholesterol-free media). As agents that inhibit cholesterol synthesis can also induce further LDLR up-regulation in HepG2 cells cultured in LPDS (R. I. Grove, unpublished observations), we investigated the effects of macrophages on cholesterol synthesis in HepG2 cells. We found that media from both unstimulated and stimulated macrophages inhibited incorporation of radiolabel into cholesterol. However, only the conditioned media from stimulated macrophage cultures up-regulated LDLR. The simplest explanation for these findings is that the macrophage up-regulatory effect includes a novel mechanism.

Proteins secreted by macrophages with known abilities to alter hepatocyte protein expression include IL-6, TGF-B, and IL-1 (9–12, 22). All three gave minor increases in LDL uptake in HepG2 cells. Oncostatin M, another protein secreted by macrophages (21), consistently stimulated strong increases in LDL uptake (as high as 2.5-fold in some experiments). The ability of the neutralizing oncostatin M antibody (OM2) to inhibit the up-regulation induced by the macrophages indicates that oncostatin M is present in the conditioned media and plays a major role in the LDLR response. However, the discovery that several macrophage secretory proteins have the ability to affect LDLR regulation raises the possibility that the up-regulation induced by the conditioned media is the sum of both positive and negative influences on LDLR. The presence of TNF in LPS-stimulated macrophage conditioned media has been documented (20). Although the inhibitory effects of TNF alone on LDL uptake in HepG2 cells are minor, significant suppression of the stimulation induced by oncostatin M might occur when both factors



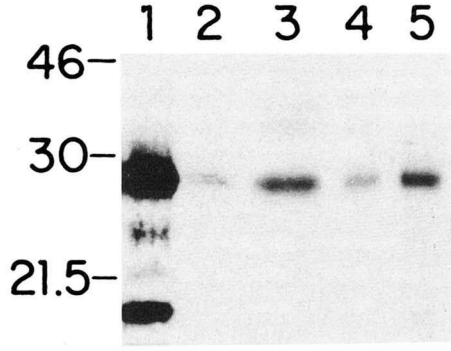


Fig. 4. Left: Inhibition of the macrophage effect on LDL uptake with an anti-oncostatin M antibody. Stimulated macrophage conditioned medium was incubated with 5 μg/ml antibody for 60 min and then was added to HepG2 monolayers. The antibody identities are as follows: OM1, the non-neutralizing oncostatin M antibody; OM2, the neutralizing oncostatin M antibody; L6, the unrelated control antibody. LDL uptake was determined after 18 h of incubation and was 265 ng LDL/mg cell protein for HepG2 monolayers treated with SCM. Percent inhibition was calculated as the percent decrease in only the stimulated portion of LDL uptake. The data are presented as means ± SEM for three experiments, each done in triplicate. Right: Detection of mouse oncostatin M by Western blot analysis. Stimulated mouse macrophage conditioned media were tested for immunoreactivity with the anti-human oncostatin M monoclonal antibody OM3. Lanes 1 and 2: purified human recombinant oncostatin M (10 ng and 1 ng, respectively). Lanes 3–5: fractions from HPLC separations of LPS-stimulated macrophage conditioned media which eluted between the 17 and 29 kDa markers and possessed the highest LDLR up-regulatory activity (fraction 52 usually, see Fig. 3). Lane 3 is from mouse peritoneal macrophages and lanes 4–5 are from a mouse macrophage cell line (RAW cells). The concentration of mouse oncostatin M as estimated from the immunoblot (lane 3) using the antihuman monoclonal antibody was between 3 and 5 ng/ml.

are present. On the other hand, minor up-regulators like IL-1 may add to, or synergize with the oncostatin M effects on LDL receptor activity. Variation of the levels of different factors in conditioned media between experiments may help explain the variability observed in the LDLR response.

The finding that oncostatin M gave the strongest up-regulation of HepG2 LDLR raises interesting questions about the role of oncostatin M in vivo. Oncostatin M, a protein secreted by macrophages, was originally discovered because of its ability to inhibit proliferation in several tumor cell lines (21). It was also shown to stimulate proliferation in some normal fibroblasts but to have no effect on other normal and transformed cell types (21). Further investigation revealed that oncostatin M was a novel peptide (23) with a molecular mass of approximately 18 kDa when analyzed by gel filtration and 28 kDa when analyzed by gel electrophoresis (21), and was recognized by a selective cell surface receptor (24). The existence of a selective receptor for oncostatin M suggests that the factor may exert its effects on LDLR via activation of a surface receptor on HepG2 cells.

Very little is known about surface membrane receptor-mediated LDLR up-regulation in hepatocytes. It has been reported that in primary hepatocyte cultures, insulin increases expression of LDLR (25) presumably by a mechanism related to its interaction with the cell surface insulin receptor. The mechanism by which estrogen derivatives induce up-regulation of the LDLR in HepG2 cells (16) most likely involves a nuclear estrogen binding protein. Growth factors up-regulate the LDLR in fibroblasts (26), but the increase in LDLR expression may be associated with the coordinate up-regulation of cellular proteins required for growth. The finding that oncostatin M increases LDLR without stimulating HepG2 proliferation raises the possibility that this factor may have selective effects on LDLR expression.

The up-regulation of the HepG2 LDLR in response to either macrophage media or incubation in LPDS is relatively small in comparison to the up-regulation observed in fibroblast LDLR upon incubation in cholesterol-free media (16). The difference between these cell types may be due to the reported inability of the HepG2 LDLR to down-regulate appreciably in the presence of cholesterol (16). Whether this is due to basic differences in LDLR regulation in various cell types or peculiar to the HepG2 cells is not known. However, this characteristic may explain the relatively small up-regulations (50–75%) induced in HepG2 cells by oncostatin M.

Our data clearly indicate that mouse macrophages and human premonocytic leukemia cells have the ability to produce a factor that acts on cultured liver cells to increase LDLR uptake. Oncostatin M mRNA has also been found in activated human macrophage cultures derived from peripheral monocytes (23). Perhaps the most interesting question remaining is whether tissue macrophages in the liver (Kupffer cells) can produce the peptide. Although oncostatin M has been detected in human serum (S. Radka, unpublished observations), production of oncostatin M in the liver would likely result in more effective peptide concentrations at the hepatocyte membrane. Certain abnormal conditions (hypercholesterolemia, for example) might induce Kupffer cells to release oncostatin M, which then would increase the ability of the liver to remove LDL cholesterol from the plasma. If so, oncostatin M may represent an important new pathway that influences cholesterol homeostasis.

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