Oncostatin M Activates Low Density Lipoprotein Receptor Gene Transcription in Sterol-repressed Liver Cells

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Abstract
Oncostatin M (OM), a cytokine produced by macrophages and activated T cells, has been shown to be a potent inducer of liver low density lipoprotein receptor (LDLR) activity by increasing LDL uptake and cell surface LDLR number in HepG2 cells. To investigate whether OM regulates the transcription of the LDLR gene and if the effect is independent of the sterol pathway, we examined the effects of OM on the promoter activity of the LDLR gene and the expression of LDLR mRNA. HepG2 cells were transfected with hybrid genes containing three different lengths of DNA fragments from the 5′ flanking region of the LDLR gene that were fused to the coding region of the chloramphenicol acetyltransferase (CAT) gene. OM induced an approximately 3-fold increase in CAT activities in pLDLR-CAT vector-transfected cells that were incubated in lipoprotein-depleted medium and a 6-fold increase in CAT activities when the transfected cells were treated with sterols. OM stimulated similar increases in CAT activities in HepG2 cells transfected with pLDLR-CAT 234, pLDLR-CAT 1563, and pLDLR-CAT 6500, suggesting that the essential cis-acting element that mediates the OM effect is located within the 177 base pairs upstream of the transcription start site of the LDLR gene. Examination of the regulation of the endogenous LDLR mRNA expression by OM gave results similar to those in transfected cells. OM increased the levels of mRNA of LDLR, regardless of the presence or absence of lipoprotein and sterols. These data suggest that the up-regulation of the LDLR by OM is at the transcriptional level through a nonsterol-mediated mechanism.

Introduction
Many studies have established a correlation between elevated plasma LDL cholesterol levels and the occurrence of coronary heart disease. This correlation suggests that elevated LDL cholesterol in plasma is a major risk factor for the development of atherosclerosis (1, 2). A wealth of information indicates that most of the LDL cholesterol eliminated from the body is removed from the plasma by LDLR-mediated uptake in the liver (3, 4). Thus, the expression level of hepatic LDLRs directly influences plasma cholesterol levels and, therefore, regulation of liver LDLRs represents a key mechanism by which various agents could affect the development of atherosclerosis.

A well-understood mechanism of LDLR regulation is the sterol negative feedback pathway, through which increasing cellular cholesterol causes a decrease in the number of cell surface LDLRs by inhibiting LDLR gene transcription. Involved in this pathway is a 16-bp sequence extending from −68 to −53 in the 5′ flanking region of the LDLR gene designated as the sterol regulatory element (SRE-1) (5). Very recently, two SREBP1s (SREBP-1 and SREBP-2) have been purified and cloned (6, 7). SREBP-1 and SREBP-2 are highly homologous, and both proteins contain a basic helix-loop-helix leucine zipper domain. SREBP-1 is shown to bind to SRE-1 sequence and control the transcription of the LDLR gene. Analysis of the promoter structure revealed that the SRE is one of three imperfect, tandem-arranged repeats (8). In vitro studies have demonstrated that the other two repeats bind the positive transcription factor SP1 (9).

In addition to the sterol negative feedback pathway, LDLRs are also subject to nonsterol mediated regulation in human liver cells (10–13). This sterol-independent regulation pathway could play an important role in vivo when cholesterol levels in the blood are high and the LDLRs are down-regulated. In the last few years, there has been a growing number of reports that cytokines and growth factors modulate LDLR activity in hepatic cell types. However, in contrast to the detailed understanding of the regulatory mechanism of the LDLR gene by sterol, very little is known about the regulation of the LDLR gene by sterol-independent factors.

Recently, OM, a cytokine produced by activated macrophages and T cells (14, 15), has been shown to be a strong inducer for the liver LDLR (16). It increases LDL uptake and cell surface LDLR number in HepG2 cells, a hepatoma-derived cell line that resembles normal hepatocytes. The mechanism for up-regulation appears to be novel in that: (a) OM induced a similar increase in the numbers of LDLRs, regardless of the lipoprotein content in the incubation medium of HepG2 cells; (b) OM did not stimulate HepG2 cells to proliferate; (c) the effect was dependent on a rapid stimulation of cellular protein tyrosine phosphorylation; and  

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3 The abbreviations used are: LDL, low density lipoprotein; LDLR, LDL receptor; bp, base pairs; SRE, sterol regulatory element; SREBP, SRE binding protein; OM, oncostatin M; CAT, chloramphenicol acetyltransferase; FBS, fetal bovine serum; MEV, mevalonic lactone; sgi130-Rg, recombinant soluble gp130 immunoglobulin fusion protein; Leu8-Rg, recombinant Leu-8 immunoglobulin fusion protein; LPDS, lipoprotein-depleted serum; CHO, 10 μg/ml cholesterol +1 μg/ml 25-hydroxycholesterol; TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin; IL-6R, interleukin 6 response element; Egr-1, early growth response gene-1; LIF, leukemia inhibitory factor; PMA, phorbol myristic acid; cDNA, complementary DNA; ATCC, American Type Culture Collection.
(d) induction of the Egr-1 gene precedes LDLR up-regulation (17).

In order to examine whether OM regulates the transcription of the LDLR gene and if the effect is independent of the sterol pathway, we transfected HepG2 cells with LDLR promoter reporter gene constructs. These vectors contain three different lengths of fragments of DNA from the 5' flanking region of the LDLR gene that have been fused to the coding region of the bacterial CAT gene. Here, we report that OM regulates liver LDL expression by activating LDLR gene transcription in a sterol-independent pathway.

Results

In order to examine whether OM regulates the transcription of the LDLR gene and if the effect is independent of the sterol pathway, we transfected HepG2 cells with hybrid genes containing three different lengths of fragments of 5' flanking DNA from the LDLR gene fused to the coding region of the CAT gene (8). Using these reporter gene constructs, we determined the promoter activity of LDLR upon OM stimulation in HepG2 cells cultured in medium containing 10% FBS plus or minus 10 μM 25-hydroxycholesterol. In the presence of 25-hydroxycholesterol (10 μM) and MEV (12 mM), the CAT activity in cells transfected with pLDLR-CAT vectors were reduced by 70 to 80% compared to cells cultured in the absence of sterol, which indicated that the LDLR promoter is subject to the normal sterol negative feedback regulation in our transient expression system. Following down-regulation of LDLRs by 25-hydroxycholesterol, incubation with OM (100 ng/ml) for 15 h stimulated 164 and 290% increases in CAT activities in pLDLR-CAT 1563 and pLDLR-CAT 6500 transfected cells, respectively, whereas incubation of the sterol-treated cells with PMA did not alter the CAT activity (Fig. 1A). The effects of sterol and OM on the expression of CAT activity in the transfected cells were specific to the LDLR promoter, because under the identical conditions, the expression of pSV2CAT was not significantly altered (Fig. 1B). Similarly, no significant changes of β-galactosidase activity or total cellular protein content were observed in these cells (data not shown), confirming that cell growth was not affected.

In order to define the minimum region of the LDLR promoter through which OM acts, we examined the CAT activity in pLDLR-CAT 234 transfected cells. pLDLR-CAT 234 contains a 177-bp fragment of the 5' flanking sequence of the LDLR promoter (~234 to ~58). This 177-bp fragment has been shown to contain the most important sequences for expression and sterol regulation of the LDLR gene, including the SRE, two SP1 binding sites, the transcription initiation sites, and two TATA-like sequences (8). Similar to the effect of OM on the other two constructs, pLDLR-CAT 1563 and pLDLR-CAT 6500, OM stimulated a 210% increase in CAT activity in the presence of 25-hydroxycholesterol and MEV (Fig. 1C) in pLDLRCAT-234-transfected cells. Fig. 2 shows the average results from several independent transfection experiments. At 15 h incubation, OM induced similar increases in CAT activities in sterol-treated cells transfected with pLDLR-CAT 234 (205%) or pLDLR-CAT 1563 (210%). The effect of OM on pLDLR-CAT 6500 was slightly higher (289%).

To determine whether the OM effect is dose dependent, the cells transfected with pLDLR-CAT 6500 were exposed to 25-hydroxycholesterol for 48 h. OM at the indicated concentrations was added and incubated for the last 15 h. As shown in Fig. 3, the concentration of OM required to give a half-maximal increase was 3 to 5 ng/ml. At a concentration of 50 ng/ml or higher, a maximum stimulation of 400% was achieved. This dose-dependent effect on the promoter activity is consistent with the dose range required for OM to regulate LDL protein as reported previously (16, 17).

To investigate whether OM stimulation of the LDLR promoter was initiated after ligand-receptor interaction, we investigated the effect of the OM soluble receptor, Sgp130-Rg, in transfected HepG2 cells. Sgp130-Rg is a chimeric
fusion protein composed of the extracellular domain of gp130 and the constant region of human immunoglobulin (18). Sgp130-Rg was shown to inhibit the OM-elicited biological activity by preventing OM binding to its cell surface receptor (19). At the 100 ng/ml concentration, OM caused an approximately 3-fold increase in CAT activity in sterol-repressed HepG2 cells that were transfected with pLDLR-CAT 6500. The presence of sgp130-Rg (10 μg/ml) abolished this effect, whereas the irrelevant control chimeric protein Leu8-Rg (60 μg/ml) had no effect (Table 1).

To ensure that the OM activity on LDLR gene transcription is independent of the cellular cholesterol level, we conducted similar transfection experiments in LPDS (10%) and in LPDS medium containing 10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol (LPDS + CHO). This composition has been widely used in sterol regulation. Fig. 4 shows the mean result from three independent transfection experiments. The promoter activity of LDLR-CAT 1563 was increased 2.7-fold in LPDS medium and 6.3-fold in LPDS + CHO medium after 24 h of OM stimulation. The higher activity of OM in sterol-repressed cells is likely due to the lower transcriptional basal activity of the LDLR gene.

To further study the transcriptional regulation of the LDLR gene, we examined the effect of OM on endogenous LDLR mRNA of HepG2 cells. Total RNA was isolated from cells cultured in 10% LPDS RPMI 1640 alone or supplemented with 100 ng/ml OM for various lengths of time. As shown in Fig. 5A, incubation of HepG2 cells with OM for 3 h resulted in a 3-fold increase in the level of mRNA for LDLR, and this level remained elevated for the remainder of the 24 h treatment period. The induction of expression of LDLR mRNA was also detected in sterol-repressed HepG2 cells. As shown in Fig. 5B, treatment of cells with the sterol greatly diminished the level of mRNA for the LDLR (Fig. 5B, Lane 2). Stimulation of these cells with OM for 2 h reversed the sterol suppression effect (Fig. 5B, Lane 3). The levels of mRNA for γ-actin were not changed significantly by the treatment.

By using pLDLR-CAT constructs, we compared OM activity with several other cytokines including TNF-α, IL-1, and TGF-β, all which have been reported to increase LDLR activity. As shown in Fig. 6, in sterol-treated HepG2 cells, TNF-α, IL-1, and TGF-β did not increase the pLDLR-CAT CAT activity, but incubation of these cells with OM stimulated an approximately 3-fold increase in CAT activity.

Finally, because OM is functionally related to IL-6 and two types of IL-6REs have been identified in several IL-6-inducible genes (20), we analyzed the sequence of the 5'
flanking region of the LDLR gene upstream from the initiator methionine codon (+1 to −234) to determine whether the IL-6REs are present in this region. A computer-aided sequence analysis shows that neither the type 1 IL-6REs, characterized by the consensus sequence T(T/G)NNG-NAA(T/G), nor the type 2 IL-6REs, characterized by the hexanucleotide motif CTGGGA, are present in the promoter region of the LDLR gene.

Discussion
In this report, we demonstrate that OM, a peptide uniquely derived from activated mononuclear leukocytes, regulates the transcription of the LDLR gene in liver-derived cells. OM induced an approximately 2- to 3-fold increase in CAT activity in pLDLR-CAT-transfected cells incubated in LPDS medium and 6- to 7-fold increase in CAT activity when the transfected cells were cultured in LPDS+CHO medium. Under the similar culture conditions, the degree of induction in promoter activity by OM is similar to the extent of the increase in LDLR protein and mRNA. The effective concentration range of 0.1 to 1 nM for OM to activate the promoter of LDLR closely matches the concentration range for up-regulation of LDL protein and activity. These results suggest that the up-regulation effect of OM on the LDLR is largely at the transcriptional level. PMA was reported to increase LDLR expression in HepG2 cells (17). The results from this study indicate that PMA does not activate the LDLR promoter in 25-hydroxycholesterol-treated cells, regardless of which pLDLR-CAT vector was transfected. This finding is consistent with our earlier study characterizing the signaling pathway linked to LDLR up-regulation by OM, which suggested that protein kinase C is not involved (17), and further suggests that OM regulates LDLR expression through a novel mechanism.

The mechanism involved in the activation of LDLR gene transcription by OM appears to be independent of sterol-mediated regulation. Cholesterol and 25-hydroxycholesterol, a cholesterol metabolite, are potent inhibitors of LDLR gene expression and work primarily via the SRE. The transcriptional activity of the LDLR promoter in HepG2 cells was reduced to a level of 10–20% of control by treatment with these sterols. However, OM increased the transcription of the LDLR gene, regardless of the state of the sterol repression. This was shown by the increased level of endogenous mRNA for LDLR and CAT activities, both of which are under the control of the LDLR promoter.

The unique and important aspect of nonsterol-mediated regulation of LDLR gene expression by OM can be recognized by comparing the effects of OM with other cytokines such as TNF-α, IL-1, and TGF-β. In our transient expression system, OM is the only cytokine that increased the transcriptional activity of LDLR in sterol-repressed cells, whereas the other tested factors have no effect. These data are consistent with a previous observation reported by Stopeck et al. (21) who showed that TNF-α and IL-1 increased the transcription of LDLR of HepG2 cells in the absence of LDL and cholesterol. The presence of LDL and cholesterol in the incubation medium abolished the activity of these cytokines. In contrast to TNF and IL-1, OM has no effect on the growth of HepG2 cells, and the activation of LDLR gene expression cannot be prevented by sterol.

Fig. 6. Comparison of OM activity on pLDLR-CAT with other cytokines. HepG2 cells were transfected with pLDLR-CAT 1563 for 3 h. After transfection, cells were incubated in medium containing 10% LPDS plus sterols (10 μg/ml cholesterol+1 μg/ml 25-hydroxycholesterol for 48 h. Cytokines were added to the indicated dishes for the last 24 h before harvesting. The cytokine concentrations used were those recommended by the vendors to achieve maximum biological activities (OM, 100 ng/ml; TNF-α, 100 ng/ml; IL-1β, 50 ng/ml; TGF-β, 100 ng/ml). The biological activity of each factor was confirmed by a growth assay conducted in HepG2 cells. Twenty μl of cell extract per sample was used for CAT assay, which was carried out at 37°C for 1 h. The reaction products were separated by thin layer chromatography plate and detected by autoradiography. The autoradiograms were exposed for 2 days. The radioactivity spots were cut out and counted in a scintillation counter. The CAT activity in transfected cells cultured in the medium containing LPDS plus sterols alone is presented as 100% activity (two experiments).
Our earlier work shows that the effect of OM on LDLR expression is proceeded by a rapid and strong induction of the Egr-1 gene (17). Furthermore, a closely matched Egr-1 binding motif (GAGGGCCGCG) was discovered in the 5' flanking region of the LDLR gene (~328 to ~320). That location is separated from the sterol regulatory sequence (~234 to ~58). We speculated that OM may cause a greater response in cells transfected with pLDLR-CAT 1563, which contains the Egr-1 binding site, compared with cells transfected with pLDLR-CAT 234, which lacks this regulatory sequence. However, results from multiple transfection experiments did not support this hypothesis. OM has similar effects in pLDLR-CAT 234- and pLDLR-CAT 1563-transfected cells. Furthermore, cotransfection of pCMV-Egr1, an Egr-1 expression vector, with pLDLR-CAT 1563 did not increase the transcriptional activity of pLDLR-CAT 1563 (preliminary result). These data together suggest that Egr-1 induction by OM is not part of the signal cascade leading to LDLR gene transcription.

It is observed that OM belongs to a cytokine family which includes IL-6, LIF, ciliary neurotrophic factor, and IL-11. These cytokines possess certain overlapping biological functions. In HepG2 cells, OM, IL-6, and LIF all strongly induce acute-phase protein genes through the IL-6REs present in the promoter regions of the target genes (20). However, the expression of the LDLR gene is only slightly increased by IL-6 and LIF (15, 17). Furthermore, the promoter region (~234 to +1) of the LDLR gene does not contain any IL-6REs. This suggests that the effect of OM on the LDLR gene may be mediated through a unique, unidentified regulatory element.

In our study, OM induced similar increases in CAT activities in the cells transfected with the vectors pLDLR-CAT 234, pLDLR-CAT 1563, and pLDLR-CAT 6500. This suggests that the essential regulatory elements which mediate OM effects are located within the 177-base pair fragment. Experiments designed to identify the cis and transacting elements that modulate OM activation of LDLR transcription are currently in progress.

There have been a number of reports on the regulation of LDLR expression by sterol-independent factors. For example, insulin has been shown to increase LDLR density and the mRNA level (10, 11), and growth hormone, which is a factor structurally related to OM, has been shown to increase the liver LDLR expression in humans and in animals (12). However, the molecular mechanisms underlying the actions of insulin and growth hormone are unknown. Therefore, it is important to understand how OM regulates the expression of the LDLR at the molecular level. The mechanisms by which OM up-regulates the LDLR expression may also be used by other factors such as growth hormone. Our data clearly demonstrated that OM activates the transcription of the LDLR gene, regardless of the state of sterol repression. This suggests that OM regulates the transcription of LDLR gene by a novel mechanism which is independent of the classical sterol pathway.

Materials and Methods

Cells and Reagents. The human hepatoma cell line HepG2 was obtained from ATCC (Bethesda, MD) and cultured in RPMI 1640 supplemented with 10% FBS. Cell culture reagents were obtained from Gibco. PMA and 25-hydroxycholesterol were purchased from Sigma. MEV was purchased from Aldrich Chemical Corp. Human recombinant OM was expressed in Chinese hamster ovary cell supernatants and purified by reverse phase high performance liquid chromatography (22). Human recombinant TNF-α, IL-1β, and TGF-β were purchased from R&D Systems (Minneapolis, MN). The soluble OM receptor immunoglobulin chimera protein sgp130-Rg and the control chimera protein Leu8-Rg were purified from supernatants of COS-M6 cells transfected with the respective plasmid vectors by protein A-Sepharose chromatography as described previously (18).

Plasmid Expression Vectors and cDNA Probes. The plasmid vectors pLDLR-CAT 234, pLDLR-CAT 1563, and pLDLR-CAT 6500 were constructed by insertion of three fragments of the human LDLR promoter with a common 3' end into pSV-CAT as described previously (8). These vectors were generously provided to us by Dr. David Russell (University of Texas, Southwestern Medical Center, Dallas, TX). The promoterless plasmid, pSV-CAT, and pSV2CAT (Promega, Madison, WI) were used as negative and positive controls. pSV-α-galactosidase (Promega, Madison, WI) was used to control transfection efficiency.

A 1.2-kilobase fragment of human LDL cDNA was obtained by digestion of pLDR3 (ATCC) plasmid with BamHI and purified from low melting point agarose. A 1.0-kilobase insert of human γ actin cDNA was obtained by digestion of plasmid HHC176 (ATCC) with EcoRI. The purified cDNA fragments were radiolabeled to a specific activity of 10⁶ cpm/μg by [32P]dATP by the random priming method. The [32P]-labeled cDNAs were purified by G-50 Sephadex columns (Pharmacia).

Transient Expression Assays. HepG2 cells were transfected with plasmid DNA by the method of calcium phosphate coprecipitation. Briefly, cells were plated at a density of 1.5 x 10⁵ cells per 6-cm culture plates in 3 ml RPMI 1640 supplemented with 10% FBS the day before transfection. One h before transfection, the media was changed, and 5 ml fresh medium was added to each plate. Plasmid DNAs (30 μg/ml pLDLR-CAT and 10 μg/ml pSV-α-galactosidase) were dissolved in TE (10 mM Tris, 0.1 mM EDTA) buffer containing 0.25 mM CaCl₂ and mixed with an equal volume of 2X HBS (1.5 mM Na₂HPO₄, 0.28 mM NaCl, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.12). Forty-five min later, 0.5 ml of the DNA/CaPO₄ precipitates was added to each culture dish. After 3 to 4 h, media was removed, and cells were washed with phosphate-buffered saline and treated with 15% glycerol in 1X HBS for 1 min. Subsequent to glycerol shock, cells were washed with phosphate-buffered saline and fed with fresh media (10% FBS-RPMI). In order to down-regulate LDLR, transfected HepG2 cells were fed with media containing 25-hydroxycholesterol (10 μM, dissolved in ethanol) and MEV (12 μM solubilized in water). The final concentration of ethanol in the culture media was 0.1%. In some experiments, the repression of LDLR was achieved by incubating cells with 10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol. The MEV was included to provide a precursor for endogenous cholesterol synthesis and to provide a substrate for the synthesis of nonsterol mevalonic acid derivatives required for normal cell growth (23). After 24 h, the cells were fed with fresh media with 25-hydroxycholesterol plus MEV, and OM was added to the plates for the indicated times during the sterol treatment. All the plates were harvested together 48 h after transfection by scraping, followed by three cycles of freeze-thawing.
Protein measurements were performed according to Lowry (24) using bovine serum albumin as the standard. Cell extracts (20 μl) from each plate were assayed for β-galactosidase activity as described (25). For the CAT assay (26), the cell extracts were normalized for β-galactosidase activity in order to correct for transfection efficiency. The percentage of chloramphenicol substrate acetylated for each cell extract was determined by scintillation counting. Duplicate plates were used for transfection and subsequent CAT assays for each data point. The results represent the mean values. For some experiments, the transfection efficiency was normalized by equal amount of cellular protein, which gave the same results as correction with β-galactosidase activity.

RNA isolation and Northern Blot Analysis. Cells were lysed in guanidinium isothiocyanate, and total RNA was isolated by ultracentrifugation through a cesium chloride cushion (27). Total RNA (20 μg/lane) was separated by electrophoresis in 1% agarose gels containing 6% formaldehyde and transferred onto nitrocellulose membrane. Gels were routinely stained with ethidium bromide prior to blotting to confirm that equal amounts of RNA were assayed. The prehybridization, hybridization, and washing conditions were conducted as described previously (28). The filters were exposed to Kodak XAR film with an intensifying screen at −70°C for 1 to 3 days. Where indicated, the blots were stripped and reprobed with γ-actin to ensure that equivalent amounts of RNA were being assayed. Differences in hybridization signals were quantitated using an LKB Laser Densitometer (2202 Ultroscan).

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References