Autoregulation of the Modified Low Density Lipoprotein Receptor in Human Monocyte-Derived Macrophages

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Regulation of the macrophage receptor for modified low density lipoprotein (LDL) was evaluated using human monocyte-derived macrophages and acetyl LDL. Factors that regulate native LDL receptor activity in other cell types, such as the cholesterol content of the incubation medium, insulin, and platelet-derived growth factor had no effect on acetyl LDL degradation. Conditioned medium from mature macrophages significantly stimulated acetyl LDL degradation and enhanced cholesterol esterification by freshly isolated monocytes. Time course studies indicated that increasing time in culture was associated with increasing potency of the stimulating activity of macrophage-conditioned medium. These data suggest that a macrophage secretory product may be a prime modulator of modified LDL receptor activity on monocyte-macrophages, thus exerting an autocrine regulatory effect. The positive autoregulation of the human macrophage receptor for modified LDL could accelerate cellular cholesteryl ester accumulation and macrophage-derived foam cell formation.


Foam cells that characterize the atherosclerotic plaque are believed to be derived from both macrophages and arterial smooth muscle cells. The origin of the macrophage-derived foam cell is probably the blood-borne monocyte which migrates through the endothelial cell layer into the vascular wall where it accumulates large amounts of cholesteryl ester.

There are at least two receptor-mediated systems by which cholesterol can enter human macrophages. First, native low density lipoprotein (N-LDL) is bound, taken up, and degraded by a saturable and high affinity process consistent with the classic LDL receptor pathway described by Brown and Goldstein in fibroblasts. Second, a distinct receptor system has been described which is unique to macrophage-type cells and endothelial cells. This receptor binds and degrades LDL that has been structurally modified to produce a more negatively charged particle. Acetylation and malondialdehyde (MDA) treatment are two chemical modifications that lead to specific high affinity, saturable binding at this site.

Degradation of modified LDL by cultured macrophages appears to occur to a much greater extent than does degradation of N-LDL, thereby causing a larger intracellular accumulation of cholesterol ester. Macrophages incubated in long-term culture with modified LDL take on the morphologic characteristics of foam cells, a transformation which cannot be accomplished by incubation with N-LDL. Importantly, two possible mechanisms by which modified LDL could be produced in vivo at sites of incipient plaque formation have recently been reported. The aggregation of platelets can lead to MDA modification of LDL, and the interaction of LDL with endothelial cells also alters LDL in such a way that it is recognized by the modified-LDL receptor on macrophages.

In this study we have investigated the regulation of the modified LDL receptor using human monocyte-derived macrophages and acetyl LDL (A-LDL) as a model for chemically modified lipoprotein.
Methods

Materials

Sodium $^{125}$-iodine (carrier-free in 0.1 M NaOH) was purchased from Amersham (Arlington Heights, Illinois). $^{14}$C-oleic acid was obtained from New England Nuclear (Boston, Massachusetts). Plasmagel was obtained from Roger Bellon Laboratories (Neuilly, France). Ficoll-Hypaque from Pharmacia Fine Chemicals (Piscataway, New Jersey), and RPMI 1640 medium from Grand Island Biological Company (Grand Island, New York). Bovine serum albumin (BSA) was obtained from Pentax Chemicals (Elkhart, Indiana). Pure pork regular insulin was a gift of Eli Lilly (Indianapolis, Indiana). Disposable tissue culture dishes and filters were purchased from Corning Glassworks, Scientific Products (Corning, New York) and disposable pipettes from Falcon Labware, Division of Becton, Dickinson and Company (Oxnard, California).

Cell Preparation and Culture

Blood was obtained from healthy normal volunteers after a 14-hour fast. Monocytes were separated from whole blood by method B of Fogelman et al. as described in detail elsewhere. In brief, red cells were removed by sedimentation in Plasmagel. The white cells in the plasma fraction were separated by counterflow centrifugation in a Beckman J6 Elutriator rotor. Cells in the monocyte-enriched fractions were centrifuged through Ficoll-Hypaque, yielding a preparation of monocytes which was approximately 95% pure.

Cells were plated in RPMI 1640 medium supplemented with autologous serum (AS) or BSA depending on the experiment. Cells maintained in long-term culture were grown in 20% AS and were fed twice weekly. Macrophage-conditioned media (MCM) was collected in 20% AS or in 0.2% BSA. MCM was centrifuged to remove cell debris and stored at 4°C until use. At the end of some experiments, the cells were washed three times with cold PBS and dissolved in 0.1 N NaOH for the measurement of protein content.

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Low density lipoprotein ($d = 1.019 - 1.063$ g/ml) was prepared by preparative ultracentrifugation and acetylated by the method of Brown and Goldstein. Native and acetylated LDL were iodinated with $^{125}$I by the iodine monochloride method as modified for proteins at a concentration within the high affinity binding range, was used as an integrated measure of receptor activity. At the end of the incubation, the medium was removed and the TCA soluble radioactivity (noniodide) was extracted as previously described and non-cell-associated degradation was subtracted from total degradation to give a measure of cell-associated N or A-LDL degradation.

For measurement of cholesterol esterification rate, cells were incubated with $^{14}$C-oleate acid for 24 hours at 37°C by the method of Brown and Goldstein. At the end of the incubation, the cells were washed five times in phosphate-buffered saline, harvested, and the lipids extracted by the method of Stein et al. Lipids were separated by thin layer chromatography in hexane/diethyl ether/methanol/acetic acid (90:20:3:2 vol/vol). The cholesterol ester spots were visualized using iodine and counted for $^{14}$C radioactivity. Cellular protein measurement was performed by the method of Lowry.

Partially purified platelet-derived growth factor prepared by previously described methods was kindly provided by Russell Ross.

Results

The development of A-LDL receptor activity was examined as a function of time in culture and was compared to N-LDL receptor activity. Cells were grown in 20% AS for various intervals, at the end of which they were washed twice with serum-free medium and placed in 20% AS containing $^{125}$I-A-LDL (7.5 μg/ml) for measurement of their respective degradation rates over the ensuing 4 hours. A-LDL degradation

![Graph](http://atvb.ahajournals.org/figure1.png)

Figure 1. Effect of lipoprotein concentration of the incubation medium on native LDL receptor activity in human macrophages. Mature human monocyte-derived macrophages were washed and placed in 0.2% BSA which was supplemented with unlabeled N-LDL (■) or A-LDL (○) for 24 hours at the concentrations indicated. The cells then were washed, and $^{125}$I-N-LDL (7.5 μg/ml) was added and a 4-hour degradation assay was performed.
was much greater than N-LDL degradation immediately after isolation (56 ± 3 vs 9 ± 1 ng/culture·hr⁻¹; p < 0.001). Furthermore, A-LDL degradation increased as a function of time in culture, increasing to 761 ± 32 and to 1536 ± 81 ng/culture·hr⁻¹ at 5 and 12 days, respectively, while N-LDL degradation increased to a much lesser extent (70 ± 6 and 54 ± 6 ng/culture·hr⁻¹ at 5 and 12 days, respectively).

The effect of lipoprotein cholesterol content of the medium on A- and N-LDL receptor activity was examined in mature macrophages. We changed the medium of mature cells that had been in culture for 10–14 days to serum-free medium containing 0.2% BSA with or without unlabeled A-LDL or N-LDL. After a 24-hour exposure to these conditions, the media were removed and the cells washed twice in serum-free medium. Then either ¹²⁵I-A-LDL or ¹²⁵I-N-LDL was added at concentrations of 7.5 μg/ml for a 4-hour degradation assay. ¹²⁵I-A-LDL degradation was not influenced by the 24-hour preincubation with either 200 μg/ml unlabeled N- or A-LDL (data not shown). However, ¹²⁵I-N-LDL degradation was depressed in a concentration-dependent fashion by preincubation with either lipoprotein. Unlabeled A-LDL was more effective than unlabeled N-LDL in suppressing subsequent ¹²⁵I-N-LDL degradation at every concentration tested, presumably due to greater intracellular delivery of cholesterol by A-LDL than by N-LDL (figure 1).

Insulin has been shown to increase N-LDL degradation by cultured human fibroblasts¹⁷ and has been claimed to be atherogenic in its own right.¹⁹ We therefore studied the influence of insulin on A- and N-LDL receptor activity in human macrophages. Mature macrophages (10–14 days in culture) were washed twice with serum-free medium and then placed in 0.2% BSA with increasing concentrations of insulin. After a 24-hour exposure to the hormone, ¹²⁵I-A or N-LDL (7.5 μg/ml) was added for measurement of the respective receptor activities. Insulin had no significant effect on A or N-LDL receptor activities in either physiologic or pharmacologic concentrations (50–10,000 μU/ml). Similar results also were obtained in freshly isolated cells and when incubation with insulin was carried out in serum-containing medium (data not shown).

Another factor thought to be important in the pathogenesis of atherosclerosis is the platelet-derived growth factor (PDGF).¹ To test whether PDGF affected A or N-LDL receptors on macrophages, mature cells (10–14 days in culture) were washed twice in serum-free medium and placed in 0.2% BSA. After 24 hours, PDGF (with mitogenic equivalent of 5% whole blood serum) was added in 50 μl of Ringer’s solution alone was added to the cells. After a further 24 hours, ¹²⁵I-A-LDL or ¹²⁵I-N-LDL (7.5 μg/ml) was added for a 4-hour degradation assay. PDGF did not significantly influence either A- or N-LDL degradation by macrophages, while N-LDL degradation by arterial smooth muscle cells was stimulated as previously described¹⁶ (figure 2).

We previously demonstrated that a macrophage secretory product stimulates LDL catabolism by vascular smooth muscle cells.²⁰ We therefore examined the effect of MCM from 5- and 6-day old macrophages on ¹²⁵I-A-LDL degradation by freshly isolated

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Lack of effect of platelet-derived growth factor (PDGF) on native and modified LDL receptor activity in human macrophages. Mature macrophages were washed and placed in 0.2% BSA for 24 hours. Partially purified PDGF was then added for another 24 hours. Then either ¹²⁵I-A-LDL or ¹²⁵I-N-LDL (7.5 μg/ml) was added and a 4-hour degradation assay was performed. C = control; P = PDGF.
monocytes. To test the effect of MCM collected in 20% serum, freshly isolated monocytes were plated in 0.5 ml of 20% AS plus either 0.5 ml of MCM in 20% serum or 20% serum that had been incubated in the absence of cells. To test the effect of MCM collected in 0.2% BSA, freshly isolated monocytes were plated in 0.5 ml 20% AS plus either 0.5 ml MCM in 0.2% BSA or 0.2% BSA that had been incubated in the absence of cells. The cells then were incubated for 24 hours, after which \(^{125}\text{I}-\text{A-LDL} \) (7.5 \( \mu \text{g/ml} \)) was added for measurement of its degradation over the next 4 hours. MCM enhanced A-LDL receptor activity in 10 experiments using serum-containing or serum-free MCM (figure 3). MCM had no effect on the degradation of \(^{125}\text{I}-\text{A-LDL} \) in cell-free dishes. The effect in the presence of cells was due to stimulation of specific degradation (as measured by subtracting the degradation which occurred in the presence of a 20-fold excess of unlabeled A-LDL). Stimulation of specific degradation averaged 50% in serum-containing MCM and 35% in serum-free MCM. Human arterial smooth muscle cell-conditioned media had no effect on \(^{125}\text{I}-\text{A-LDL} \) degradation in freshly isolated monocytes (data not shown).

The physiologic significance of this enhancement of modified LDL receptor activity would rest on its ability to increase intracellular cholesterol delivery. Therefore, the effect of MCM on esterification of cholesterol was tested. When fresh monocytes were incubated with 25 \( \mu \text{g/ml} \) of A-LDL, MCM resulted in an approximate doubling of the incorporation of \(^{14}\text{C}-\text{oleic acid} \) into cholesterol ester (figure 4).

The ability of MCM to enhance A-LDL degradation did not require specific macrophage stimulation. Zymosan or bacterial endotoxin activation of macrophage cultures during the collection of the MCM did not lead to enhanced potency (data not shown). The ability of macrophages to produce the factor that stimulated the degradation of A-LDL was, however, dependent on the duration of time in culture (figure 5). MCM in 20% AS was collected over the periods
indicated in the legend to figure 5. MCM collected during the first 3 hours decreased the amount of 125I-A-LDL degraded by fresh monocytes from 3400 ± 486 to 1987 ± 405 ng/mg-4hr⁻¹. Thereafter, a time-dependent increase in A-LDL degradation was observed; the most potent MCM was secreted by Day 7-9 macrophages which stimulated A-LDL degradation to 6285 ± 292 ng/mg-4hr⁻¹ (figure 5).

Discussion

Human monocyte-derived macrophages possess distinct high affinity binding sites for native and chemically-modified LDL. To study the regulation of the binding site for modified LDL, factors known to influence N-LDL degradation in other cell types or thought to be important in the pathogenesis of atherosclerosis were evaluated with respect to their effects on the activity of the modified LDL receptor.

It has been postulated that platelet-derived growth factor has a significant role in the development of the atherosclerotic plaque. It is released by aggregating platelets and may accumulate at sites of injury to the vascular endothelium. It is a potent mitogen that stimulates the proliferation of vascular smooth muscle cells. In these cells it also increases N-LDL binding and degradation. We therefore examined the effect of PDGF in mature macrophages but found no effect on either N-LDL or A-LDL degradation rates. This suggests that this important mitogen is not involved in the cholesterol ester accumulation that leads to macrophages becoming foam cells. In studies using cultured human fibroblasts, Chait et al. demonstrated that insulin in physiologic concentrations enhanced N-LDL degradation. Stout has suggested that the increased incidence of atherosclerotic complications in diabetics results from insulin excess rather than insulin deficiency. In cultured macrophages, insulin had no effect on either N- or modified LDL receptor activity. Both of these compounds, i.e., insulin and PDGF, may exert their effect on the LDL receptor to provide the cell with a supply of cholesterol to be used for cell division. Since macrophages are nondividing cells and hence may not require this regulatory mechanism, the lack of effect of PDGF or insulin on N-LDL degradation in macrophages is not surprising.

Macrophages secrete a multitude of products. Recent reports have described a macrophage-derived growth factor and a factor, possibly the same as the macrophage-derived growth factor, that stimulates N-LDL uptake by vascular smooth muscle cells. We now report that a macrophage secretory product present in macrophage-conditioned medium also stimulates A-LDL degradation by freshly isolated monocytes. Furthermore, this factor produces a substantial increase in the rate of cellular cholesterol esterification that could potentially be of importance in the formation of atherosclerotic foam cells. The decline in A-LDL degradation caused by 0-3 hours MCM suggests secretion of an inhibitor of A-LDL receptor activity immediately after cells are put into culture. This may explain the observations of Fogelman et al. that when fresh cells are cultured on a surface to which they adhere, there is an immediate decrease in modified LDL receptor activity relative to cells left in suspension. In addition, detailed time course studies of modified LDL receptor activity showed that receptor activity remained stable or declined slightly during the first 3 days of culture and sharply increased thereafter. This time frame coincides with our observations on the release of MCM stimulatory activity and suggests that these changes in receptor activity are secondary to changes in amount or character of a macrophage secretory product. Another cellular secretory product has very recently been implicated in the regulation of the modified-LDL receptor. Human lymphocyte-conditioned medium depresses the activity of this receptor system and lymphokines may therefore also be important in its regulation.

The notion that cells can secrete substances that affect their own metabolism is well known and the process has been termed autocrine regulation. An area of emerging interest involves investigating whether secretory products of macrophages can modulate other aspects of macrophage function in a positive or negative feedback manner. Such autocrine regulatory mechanisms may involve arachidonic acid metabolites, interferon, or α-2-macroglobulin. The identity of the secretory product which regulates A-LDL receptor activity is presently under investigation.
Massive accumulation of cholesterol ester can be observed in macrophages cultured for prolonged periods in the presence of modified LDL.\(^4,6\) Prolonged incubation with N-LDL produces no such effect.\(^7\) Our data are consistent with these observations. A-LDL receptor activity is greater than N-LDL receptor activity in freshly isolated monocytes and increases with time in culture. A-LDL activity remains high despite preincubation of cells with high concentrations of N- or modified lipoproteins, while N-LDL degradation is dramatically suppressed by preincubation with as little as 15 \(\mu g/ml\) of N- or modified LDL. More importantly, however, the positive autoregulation of modified LDL receptor activity by a macrophage secretory product would lead to an increasing capacity of this receptor system to deliver cholesterol to the interior of the cell for subsequent esterification and storage. If the rate of uptake exceeded that of cholesterol efflux, this could eventually lead to foam cell formation. We have used freshly isolated monocytes as our assay system for the modified LDL receptor activity because baseline A-LDL degradation is lowest in freshly isolated cells and more mature macrophages secrete high levels of this factor, making enhancement by the addition of MCM difficult to demonstrate. Because enhanced A-LDL receptor activity is associated with monocyte maturation to macrophage, there is an attractive possibility that macrophage secretory products enhance monocyte maturation and that this is reflected in enhanced A-LDL receptor activity. This question is currently being examined using other markers of monocyte maturation, such as lysozyme secretion.

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