Differentiation of Human Monocytes to Monocyte-Derived Macrophages Is Associated With Increased Lipoprotein Lipase–Induced Tumor Necrosis Factor–α Expression and Production

A Process Involving Cell Surface Proteoglycans and Protein Kinase C

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Abstract—The aim of the present study was to (1) evaluate the responsiveness of human mononuclear cells to lipoprotein lipase (LPL), as assessed by tumor necrosis factor–α (TNFα) production, during the process of differentiation of monocytes to macrophages, and (2) determine the mechanisms by which LPL exerts its effect on these cells. Treatment of human monocytes with purified endotoxin-free bovine LPL (1 μg/mL) resulted in a 161 ± 15% increase in TNFα production over control values (P<0.01). A further increase in TNFα production was observed after treatment of monocyte-derived macrophages (MDMs) with LPL (490 ± 81% over control values, P<0.01). Increased TNFα mRNA expression and protein kinase C activity were also observed in LPL-treated human monocytes and MDMs. These LPL effects were abrogated by the specific protein kinase C inhibitor calphostin C (1 μmol/L). Although heparinase totally abolished LPL-induced TNFα production in human monocytes, this agent did not significantly inhibit LPL effect in human MDMs. In contrast, treatment of MDMs with chondroitinase suppressed LPL-induced TNFα production. Taken together, these data suggest that (1) differentiation of human monocytes to MDMs is associated with increased LPL-induced TNFα mRNA expression and production, (2) a protein kinase C–dependent pathway is involved in the induction of TNFα by LPL in these cells, and (3) LPL effect is mediated by cell surface proteoglycans. As MDMs secrete LPL in the vascular wall, we propose that LPL, by acting as an autocrine activator of MDM function, may contribute to the high level of TNFα found in the atheromatous lesion. (Arterioscler Thromb Vasc Biol. 1999;19:1405-1411.)

Key Words: lipoprotein lipase □ tumor necrosis factor–α □ human mononuclear cells □ proteoglycans □ protein kinase C

Lipoprotein lipase (LPL) is a key enzyme in the catabolism of triglycerides-rich lipoproteins.1 LPL exerts this effect at the vascular endothelium where it is bound to heparan sulfate proteoglycans (HSPGs).2 LPL is synthesized within the atherosclerotic lesion by monocyte-derived macrophages (MDMs) and smooth muscle cells (SMCs).3 It has been suggested that LPL, depending on its location, has a dual role with regard to the atherogenic process.4 Although it may act as an antiatherogenic protein at the periphery where it stimulates the hepatic uptake of atherogenic lipoproteins,4 LPL may exert some atherogenic functions in the arterial wall by favoring lipid accumulation within SMCs and MDMs.5,6 Besides its effect on arterial lipid metabolism, LPL has been shown to enhance human monocyte adhesion to aortic endothelial cells7,8 and to induce tumor necrosis factor–α (TNFα) and nitric oxide production by murine macrophages.9,10 TNFα is a pleiotropic macrophage secretory product involved in the modulation of many immune and extracellular processes.11,12 Accumulating evidences suggest that TNFα exerts potent proatherogenic effects. TNFα induces the expression of cell-adhesion molecules,13,14 and stimulates leukocyte adhesion to endothelial cells15 and chemotaxis.16 TNFα also stimulates new vessel formation17 and induces hemorrhagic necrosis,18 features characteristic of evolving atheroma. This cytokine also promotes tissue factor-like procoagulant activity and suppresses endothelial cell surface anticoagulant activity19 and is synthesized by MDMs and SMCs in human atheroma.20

Migration of blood monocytes and differentiation of these cells to macrophages in the subendothelial space is a cardinal feature of atherosclerosis. Functional changes occurring during monocyte differentiation to lesion macrophages, including enhanced responsiveness to activating factors and increased production of cytokines21 could favor the development and progression of atherosclerosis. We have documented that LPL stimulates TNFα production by murine functions.
macrophages. In addition, others have reported that differentiated macrophages exhibit enhanced ability to secrete and bind LPL. Based on these observations, the present study was conducted to investigate the responsiveness of human mononuclear cells to LPL, as assessed by TNFα production, during the process of differentiation of monocytes to macrophages, as this may be linked to the pathogenesis of human atherosclerosis.

**Methods**

**Reagents**

RPMI 1640 medium, Hanks’ balanced salt solution, TRIZOL reagent, polymyxin B sulfate, and protein kinase C (PKC) assay activity kit were purchased from GibcoBRL. Fetal calf serum was obtained from Hyclone Laboratories Inc. Heparinase III, chondroitinase ABC, 4β-phorbol 12β-myristate 13α-acetate (PMA), and affinity-purified bovine LPL were obtained from Sigma Chemical Co. Before use, LPL was dialyzed against saline, using 10,000 MWCO Slide-A-Lyzer dialysis cassettes purchased from Pierce. Penicillin/streptomycin were purchased from Flow. Calphostin C and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride (H7) were purchased from Calbiochem.

**Determination of Endotoxin Concentration**

LPL preparation was purified by using an endotoxin-removal resin from Associates of Cape Cod. Endotoxin content of the medium and LPL preparation (1 μg/mL) was determined by the Limulus amoebocyte lysate assay (Sigma) and was consistently found to be <6 pg/mL. Treatment of human monocytes and MDMs from all blood donors with LPS 6 pg/mL did not induce any TNFα production or TNFα mRNA expression. Moreover, addition of an LPS inhibitor, polymyxin B sulfate (100 μg/mL), to the culture medium did not inhibit LPL-induced TNFα production by human monocytes and MDMs.

**Isolation of Human Monocytes**

Human monocytes were isolated from 100 mL of anticoagulated (heparin sodium) whole blood collected from nonsmoker healthy male and female donors as previously described. First, peripheral blood mononuclear cells were obtained by density centrifugation, using Ficoll (Nycomed Pharma As). The cells collected from the interface were washed 3 times with Hanks’ balanced salt solution and allowed to aggregate in the presence of fetal calf serum. After further purification by rosetting technique and density centrifugation, recovery of highly purified monocytes (85% to 90%), as assessed by fluorescence-activated cell-sorter analysis, was obtained. Human monocytes were resuspended and cultured in RPMI 1640 medium supplemented with 1% (vol/vol) penicillin/streptomycin, 10% (vol/vol) autologous human serum, and 100 μg/mL polymyxin B sulfate.

**Culture of Human MDMs**

Freshly isolated human monocytes were grown in 24-well culture plates (Falcon, Becton Dickinson) in RPMI 1640 medium containing 1% (vol/vol) penicillin/streptomycin, 10% (vol/vol) human serum, and 100 μg/mL polymyxin B sulfate. The cells were incubated at 37°C in 5% CO2/95% air atmosphere. The medium was replaced every 3 days and the cells were used for experiments within 7 to 10 days of plating.

**Determination of Cell Viability and Total Protein Content**

Cell viability after treatment with PKC inhibitors was assessed by using trypan blue exclusion. Viability was found to be ≥90%. After extensive dialysis against saline and purification, total protein content of the LPL preparation was measured according to the method of Bradford, using a colorimetric assay (Bio-Rad) and BSA as standard.

**Measurement of TNFα Protein**

A double-sandwich ELISA (R & D Systems) was used to determine the quantity of human TNFα secreted by monocytes and MDMs after 24 hours’ exposure to LPL 1 μg/mL alone or in combination with appropriate agents.

**RNA Isolation and cDNA Preparation**

Human monocytes and MDMs (1.5×10⁶) were treated with appropriate agents in 24-well culture plates. The cells were lysed with TRIZol reagent, and cytoplasmic RNA was extracted from the cells by an improvement of the acid–phenol technique of Chomczynski and Sacchi, precipitated and resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating total cellular RNA with 0.1 μg oligo(dT) (Pharmacia) for 5 minutes at 98°C. The mixture was then incubated for 60 minutes at 37°C and for 10 minutes at 99°C in reverse-transcription mixture (Boehringer Mannheim).

**Measurement of TNFα mRNA Expression**

To compare the level of transcription of the TNFα gene in different samples, the levels of TNFα mRNA in untreated and LPL-treated monocytes and MDMs were assessed by PCR. cDNA was amplified by using 2 synthetic primers specific for human TNFα (5’-CAGGGA-GAAAGGTCTCCCCAG-3’ and 5’-CCTTGGTCCTGGTAGGAGAGGACCC-3’) in the PCR reaction mixture (Boehringer Mannheim). Two synthetic primers specific for the housekeeping gene human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5’-CCCTTCACTGACTGACTGGG-3’ and 5’-GTTTGCACTGTTGGTTCG-3’) were used to amplify cDNA. A 325-bp human TNFα cDNA fragment and a 456-bp human GAPDH cDNA fragment were amplified enzymatically in separate tubes by 30 repeated cycles at 98°C for 40 seconds, 60°C for 40 seconds, and 72°C for 90 seconds in a programmable thermal controller (PTC-100, MJ Research Inc.). The reaction product was visualized by electrophoresis on 1% agarose gel containing ethidium bromide.

**Measurement of PKC Activity**

A PKC assay kit (GibcoBRL) was used to measure PKC activity in cytosolic and particulate fractions of human monocytes and MDMs. In brief, adherent human monocytes and MDMs were recovered and homogenized (Dounce; 15 strokes) in 500 μL of ice-cold buffer A (20 mmol/L Tris, pH 7.5, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 25 μg/mL aprotinin, and 25 μg/mL leupeptin). The membrane and cytosolic fractions were separated by ultracentrifugation (100 000g for 30 minutes at 4°C). After recovery of high-speed supernatants containing cytosolic PKC, the corresponding membrane pellets were homogenized in 500 μL of buffer A containing 0.5% Triton X-100. The enzyme from both fractions was partially purified through DE52 chromatography columns. After removal of unbound proteins by washing the columns with buffer B (20 mmol/L Tris, pH 7.5, 0.5 mmol/L EDTA, and 0.5 mmol/L EGTA), fractions containing PKC were eluted with buffer C (20 mmol/L Tris, pH 7.5, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L β-mercaptoethanol, and 0.2 mol/L NaCl). Eluates were analyzed for PKC activity, following the optimum conditions of the assay, by measuring the incorporation of 3P into the synthetic peptide Ac-mycelin basic protein.

**Statistical Analyses**

Data were analyzed by Student’s t test for single comparisons and by Student–Newman–Keuls test for multiple comparisons. Results are expressed as mean±SEM values.
Results

Effect of LPL on TNFα Production by Human Monocytes and MDMs

Incubation of freshly isolated human monocytes with LPL (1 μg/mL) for 24 hours at 37°C resulted in a significant increase in TNFα production (161 ± 15% over control values, P < 0.01) (Figure 1A). A further increase in TNFα production was observed after treatment of human MDMs with LPL (490 ± 81% over control values, P < 0.01) (Figure 1B).

To determine whether LPL-induced lipolysis of lipoproteins is involved in the increase in TNFα production, human monocytes were also incubated, in some experiments, with LPL (1 μg/mL) in the absence of exogenous lipoproteins for 24 hours at 37°C. Culture of these cells in serum-free medium led to a similar 2-fold increase in LPL-induced TNFα production (data not shown).

Effect of PKC Inhibitors on LPL-Induced TNFα Production by Human Monocytes and MDMs

To determine the intracellular signaling involved in LPL-induced TNFα production, human monocytes and MDMs were pretreated with PKC inhibitors for 1 hour before being exposed to LPL for an additional 24-hour period. Exposure of monocytes and MDMs to the specific inhibitor calphostin C (1 μmol/L) completely abolished LPL-induced TNFα production (Figure 1A and 1B). A similar inhibitory effect of the PKC inhibitor H7 (20 μmol/L) on LPL-induced TNFα secretion by monocytes and MDMs was also observed (Figure 2A and 2B).

Figure 1. Effect of LPL on TNFα production by human monocytes and MDMs; inhibitory effect of calphostin C. Freshly isolated human monocytes (A) or cultured MDMs (B) (1×10^6 cells/mL) were treated for 24 hours at 37°C with or without LPL (1 μg/mL) in the presence or absence of 1 μmol/L calphostin C (Calp.C). At the end of the incubation period, the culture media were collected, centrifuged, and stored at −20°C. TNFα levels in the culture media were determined by ELISA. Data are mean ± SEM values of 4 separate experiments. **P < 0.01 versus medium (Med); ααP < 0.01 versus LPL; αααP < 0.001 versus LPL.

Effect of H7 on LPL-induced TNFα production by human monocytes and MDMs. Freshly isolated human monocytes (A) or cultured MDMs (1×10^6/mL) (B) were treated or not with H7 (20 μmol/L) for 1 hour at 37°C. The cells were then incubated for 24 hours in the presence or absence of LPL (1 μg/mL) for 24 hours. At the end of the incubation period, the culture media were collected, centrifuged, and stored at −20°C. TNFα levels in the culture media were determined by ELISA. Data are mean ± SEM values of 4 separate experiments. *P < 0.05 versus medium (Med); **P < 0.01 versus medium; αP < 0.05 versus LPL; ααP < 0.01 versus LPL.

Figure 2. Effect of H7 on LPL-induced TNFα production by human monocytes and MDMs. Freshly isolated human monocytes (A) or cultured MDMs (1×10^6/mL) (B) were treated or not with H7 (20 μmol/L) for 1 hour at 37°C. The cells were then incubated for 24 hours in the presence or absence of LPL (1 μg/mL) for 24 hours. At the end of the incubation period, the culture media were collected, centrifuged, and stored at −20°C. TNFα levels in the culture media were determined by ELISA. Data are mean ± SEM values of 4 separate experiments. *P < 0.05 versus medium (Med); **P < 0.01 versus medium; αP < 0.05 versus LPL; ααP < 0.01 versus LPL.

Effect of LPL on Human Monocyte and MDM TNFα mRNA Expression

To investigate the molecular mechanisms responsible for LPL-induced TNFα production by human monocytes and MDMs, the levels of TNFα mRNA in LPL-treated and untreated cells were assessed by PCR. LPL (1 μg/mL) induced a significant 1.4 ± 0.1-fold increase in TNFα mRNA expression over control values in human monocytes (P < 0.05) (Figure 3A). A further increase in TNFα mRNA expression (2.5 ± 0.2-fold increase over control values, P < 0.01) was observed after treatment of human MDMs with LPL (Figure 3B). Pretreatment of both human monocytes and MDMs with calphostin C (1 μmol/L) inhibited LPL-induced TNFα mRNA expression (Figure 3A and 3B).

Effect of LPL on Human Monocyte and MDM PKC Activation

Because LPL-induced TNFα expression appeared to require an active PKC, we next examined the direct effect of LPL on PKC activity. A maximal increase in PKC activity in the membrane fraction of human monocytes (1.3 ± 0.1-fold increase over control values, P < 0.05) and MDMs (2.1 ± 0.4-fold increase over control values, P < 0.05) was observed after a 10-minute exposure of human mononuclear cells to LPL (1 μg/mL) (Figure 4A and 4B). The increase in PKC activity in the membrane fractions of these cells was associated with a significant decrease in the PKC activity in the cytosol. Treatment of human monocytes and MDMs with calphostin C (1 μmol/L) totally suppressed LPL-induced membrane PKC activity (Figure 4A and 4B).
To further establish the involvement of PKC in LPL-stimulated TNFα production, we evaluated the effect of PKC depletion by PMA on the induction of TNFα production by LPL. Overnight treatment of human monocytes with 100 ng/mL of PMA dramatically reduced both membrane PKC activity (percentages over control values: medium, 100 ± 2%; LPL, 130 ± 7%; PMA, 91 ± 4%; and PMA + LPL, 85 ± 3%) and TNFα secretion (percentages over control values: medium, 100 ± 4%; LPL, 156 ± 12%; PMA, 85 ± 9%; and PMA + LPL, 90 ± 7%).

**Effect of Heparinase and Chondroitinase on LPL-Induced TNFα Production by Human Monocytes and MDMs**

To investigate the role of cell surface proteoglycans in the stimulatory effect of LPL on TNFα production by human monocytes and MDMs, these cells were pretreated or not for 1 hour at 37°C with heparinase (1 U/mL) or chondroitinase ABC (1 U/mL), washed 3 times with PBS, and then exposed to LPL (1 μg/mL) for 24 hours at 37°C. Although heparinase treatment resulted in a complete abrogation of LPL-induced TNFα production by human monocytes (Figure 5A), it did not significantly reduce LPL-induced TNFα secretion by MDMs. (Figure 5B). In contrast, treatment of MDMs with chondroitinase totally suppressed LPL-induced TNFα production (Figure 5C).

**Discussion**

Accumulating evidences support the concept that LPL acts in the vascular wall as an atherogenic protein. The proatherogenic properties of LPL have been attributed mainly to its ability to favor the retention of lipoproteins in the vascular wall26,27 and to promote lipid accumulation within lesion macrophages.28,29 We previously suggested that LPL may contribute to the development of human atherosclerosis by a new mechanism, that of inducing the release of macrophage TNFα in the arterial wall.9 To further test this possibility, we determined in the present study the responsiveness of human mononuclear cells to LPL, as assessed by TNFα production, during the process of differentiation of monocytes to macrophages.

The present study demonstrates that incubation of human monocytes and MDMs with LPL leads to enhanced TNFα production. These results, together with our previous observations in murine macrophages, further establish the role of LPL in the control of mononuclear cell activation. Our observation that human monocyte differentiation to macrophages is associated with increased responsiveness to LPL, as assessed by TNFα production, supports the hypothesis that LPL, by activating lesion macrophage function, may promote atherogenesis. Our
finding that LPL stimulates TNFα production both in the presence and absence of exogenous lipoproteins, also suggests that the lipolytic properties of LPL are not essential for the modulatory effect of LPL on monocyte cell function.

It is well established that TNFα gene expression is tightly regulated both at the transcriptional and posttranscriptional levels.\(^\text{9,30,31}\) Determination of TNFα mRNA levels in LPL-treated human monocytes and MDMs clearly demonstrates an induction of TNFα gene expression in these cells. Although the limited amount of biological material extracted from human cells did not allow us either to perform run-on experiments or to evaluate TNFα mRNA stability, our previous finding that LPL increases the steady-state levels of TNFα mRNA in murine macrophages by both transcriptional and posttranscriptional mechanisms\(^\text{9}\) suggests that similar mechanisms may be responsible for LPL-induced TNFα mRNA expression in human mononuclear cells.

Available evidence indicates that triggering transmembrane signaling leads to cytokine secretion and monocyte/macrophage activation. In particular, PKC activation has been shown to play a central role in the regulation of monocyte/macrophage TNFα expression and production in response to various stimuli.\(^\text{32–34}\) Because each of the methods used for studying PKC presents specific limitations, we assessed in the present study the involvement of PKC activation in LPL-treated human monocytes and MDMs by complementary methods. In accordance with our previous data obtained with murine macrophages,\(^\text{9}\) our results demonstrate that LPL induces, in both human monocytes and MDMs, PKC translocation from the cytosol to the membrane. Our finding that PKC redistribution to the particulate cell fraction is greater in LPL-treated human MDMs than in monocytes, and parallels that of LPL-induced TNFα secretion in these cells, suggests that LPL-induced PKC translocation in monocytes/macrophages correlates with PKC activation and cell activation. Further evidence for a role of PKC in mediating LPL-induced TNFα production in human monocytes is provided by our experiments showing that PKC depletion by PMA and pharmacological inhibition of PKC by calphostin C and H7 totally inhibit LPL-induced monocyte and MDM TNFα production. Because these two PKC inhibitors interact with the calcium-dependent regulatory domain of PKC, this latter observation suggests that classic, calcium-dependent PKC isoforms could be preferentially activated in LPL-treated monocytes and MDMs. This possibility is further supported by the observation that PKC-α and -β, which require Ca\(^{2+}\) binding for activation, are the two predominant isoforms found in human monocytes.\(^\text{32}\) In addition to its role in macrophage function, PKC activation also regulates several vascular functions such as vascular permeability,\(^\text{35}\) contractility,\(^\text{36}\) cellular proliferation,\(^\text{37}\) basement membrane synthesis,\(^\text{38}\) and signal transduction mechanisms for hormones\(^\text{39}\) and growth factors.\(^\text{40}\) Whether LPL, by inducing PKC activation, may regulate some of these functions is presently unknown and will be the subject of future studies.

Two plasma membrane molecules that behave as LPL-binding proteins have been identified in human monocytes and MDMs. These include cell surface proteoglycans, HSPGs, and chondroitin sulfate proteoglycans (CSPGs).\(^\text{22}\) Differentiation of human monocytes into macrophages has been shown to be associated with a dramatic increase in cell surface HSPGs and by the production of CSPGs. These differentiation-related changes have been linked to the greater ability of differentiated macrophages to bind LPL, compared with monocytes.\(^\text{22}\) Our results, which show that heparinase treatment of human monocytes totally inhibits LPL-induced TNFα production, demonstrate that LPL binding to the monocytic cell surface HSPG is required for its effect on TNFα production. In contrast, our observations that chondroitinase but not heparinase effectively suppresses LPL-induced TNFα pro-
duction by MDMs clearly indicate that CSPGs expressed on MDM cell surface mediate the induction of TNFα production by LPL. Because expression of CSPGs on the differentiated macrophage cell surface is associated with enhanced LPL binding,23 our results suggest that differentiation-associated changes in LPL binding capacity could be responsible for the increased human MDM responsiveness to LPL that we reported in the present study. In addition, our observation that LPL induces PKC activation in MDMs also supports the possibility that CSPGs expressed on human MDMs may play a role in the modulation of second messengers.

Overall, this study demonstrates that differentiation of human monocytes into macrophages is associated with increased responsiveness to LPL, as assessed by TNFα production. Although the in vivo relevance of our observations remains uncertain, one may postulate that during the atherosclerotic process, the massive accumulation of MDMs and SMCs, which both produce LPL, and the local expression in the arterial wall of LPL-stimulatory factors could generate sufficiently high local LPL concentrations to modulate macrophage function. LPL secreted in the vascular wall may, by activating lesion macrophage function or even newly migrating human monocytes, contribute to the high level of TNFα found in the athromatous lesion. Such an LPL effect may contribute to the development and progression of the atherogenic process.

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References


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