Interleukin 8 Is Induced by Cholesterol Loading of Macrophages and Expressed by Macrophage Foam Cells in Human Atheroma*

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In order to identify novel genes expressed in macrophage-derived foam cells, we used a multigene assay to examine the expression of genes in control versus cholesterol-loaded macrophages. We compared THP-1 macrophages incubated with or without acetylated LDL (acLDL) ± acyl-CoA:cholesterol O-acyltransferase (ACAT) inhibitor (compound 58035) for 20 h and assessed changes in mRNA of chemokines, growth factors, interleukins, and adhesion molecules. Among 49 genes examined, an increase in mRNA was observed only for interleukin 8 (IL-8) in THP-1 macrophages. Northern analysis confirmed a 3- to 4-fold increase of IL-8 mRNA and an enzyme-linked immunosorbent assay (ELISA) revealed a corresponding increase in IL-8 in conditioned medium. Oxidized LDL (oxLDL) also induced IL-8 mRNA, but native LDL had no effect. 58035 had a moderate effect on IL-8 induction by acLDL. AcLDL-induced IL-8 expression was concentration- and time-dependent. The time course of IL-8 induction paralleled that of cholesterol loading. MCP-1, a chemokine implicated in recruiting monocytes in atherogenesis, was also induced by acLDL. The induction of MCP-1, however, peaked at 1 h after addition of acLDL and returned to basal level by 20 h while IL-8 induction peaked at 8 h and was still 2-fold higher than basal level at 20 h. IL-8 induction was also observed in fresh human monocyte-derived macrophage cells treated with acLDL. Finally, immunohistochemistry and in situ hybridization studies using specimens of human coronary atheromas showed expression of IL-8 mRNA in a macrophage-rich area. We conclude that IL-8 is induced in macrophage foam cells as a response to cholesterol loading. The chemotaxiant and/or mitogenic effects of IL-8 on neutrophils, T cells, smooth muscle, or vascular endothelial cells may contribute to the progression and complications of atherosclerosis.

Cholesterol-loaded macrophages are one of the hallmarks of atherosclerosis (1-3). Recent evidence indicates that foam cells play a major role in the initiation and clinical complications of atherosclerotic lesions (4, 5). Macrophages respond to cholesterol loading by altering the cellular metabolism and uptake of cholesterol (6, 7), increasing synthesis of phospholipids (8), increasing synthesis and secretion of apolipoprotein E (9), and enhancing lipoprotein (a) and apoprotein (a) internalization and degradation (10). However, the altered regulation of other genes not involved in the regulation of cellular lipid content has also been observed. Macrophage foam cells show increased expression of tissue factor (11), matrix metalloproteinases (12, 13), monocyte chemoattractant protein 1 (MCP-1) (14, 15), lipoxygenase (15), and other molecules (16). Although the physiological relevance of these alterations to foam cell formation is not completely understood, they probably contribute to different diseases in the progression of atherosclerosis (11-15).

Atherosclerosis is a multifactorial, complex pathological process. Cell-cell and cell-matrix interactions and communications involving macrophages, vascular smooth muscle cells, vascular endothelial cells, and lymphocytes are likely to be involved. The identification and characterization of novel genes involved in this cross-talk could be crucial to deciphering the mechanisms of atherogenesis. As an approach to identifying new genes involved in atherogenesis, we have assayed multiple genes for altered levels of their mRNAs in responses to cholesterol loading of foam cells. We have identified human IL-8 as an inducible factor produced by cholesterol-loaded macrophages and have found that the regulation of its production is correlated with cholesterol loading. IL-8 is a potent chemokine, but has received little attention as a potential contributor to atherogenesis.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media and reagents were obtained from Life Technologies, Inc. Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). Compound 58035 (3-(decyldimethylsilyl)-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide), an inhibitor of acyl-CoA:cholesterol O-acyltransferase (ACAT) (17), was prepared in dimethyl sulfoxide as a stock solution (10 mg/ml); the final dimethyl sulfoxide concentration in both treated and control cells was 0.025% All other chemicals were from Sigma.

Cells—Human THP-1 cells (from the American Type Culture Collection) were grown and maintained in suspension culture in RPMI 1640 medium containing 10% fetal bovine serum (v/v), penicillin (100 units/ml), streptomycin (100 μg/ml), and 5 × 10⁻⁵ M β-mercaptoethanol. For each experiment, the THP-1 cells were plated in 16 × 22 mm dishes at a density of 3 × 10⁵ cells/dish in RPMI 1640 medium, 1 × HU-Nutridoma (Boehringer Mannheim), penicillin (100 units/ml), streptomycin (100 μg/ml), and 1 × 10⁻⁵ M PMA. After 20 h, the medium was replaced with fresh medium and acLDL, oxLDL, or 58035 was added and incubated for the indicated period of time.

Human monocyte-derived macrophages (18) were maintained for 13 days in RPMI 1540, 10% human serum; 1 ng/ml granulocyte-macrophage-colony stimulating factor was added on days 1, 4, and 11 to up-regulate the scavenger receptor and thus facilitate cholesterol loading (19). On day 14, the cells were incubated with RPMI 1640, 1 × Hu-Nutridoma, and 1 ng/ml granulocyte-macrophage-colony stimulat-

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‡ The abbreviations used are: MCP-1, monocyte chemoattractant protein 1; IL-8, interleukin 8; LDL, low density lipoprotein; acLDL, acetylated low density lipoprotein; oxLDL, oxidized low density lipoprotein; PMA, phorbol 12-myristate 13-acetate; ACAT, acyl-CoA:cholesterol O-acyltransferase; ELISA, enzyme-linked immunosorbent assay; SMA, smooth muscle actin.
ing factor. On day 15, the cells were incubated with fresh medium; acLDL and/or 58035 was added, and the experiments were conducted.

Lipoproteins—LDL and HDL from fresh human plasma were isolated by preparative ultracentrifugation (20). AcLDL was prepared by reaction with acetic anhydride as described by Goldstein and co-workers (21) and butylated hydroxytoluene was used in preparation to minimize any oxidation of acLDL. OxLDL was prepared by exposing LDL to 10 μmol/liter cupric sulfate in phosphate-buffered saline for 20 h. The oxidation was terminated by addition of butylated hydroxytoluene to a final concentration of 40 μmol/liter. Dialysis against phosphate-buffered saline with several changes was used to remove dialyzable factors.

Northern Blot Analysis and Multigene Assay—Total cellular RNA was isolated from the macrophages using the guanidine thiocyanate lysis method with RNazol (Tel-Test, Inc.) according to the manufacturer’s protocol. Blotting and hybridization were performed as described previously (22). The human IL-8 cDNA probe, from a 1.2-kilobase pair EcoRI fragment of human IL-8 cDNA, and the human MCP-1 probe, from a 0.7-kilobase pair EcoRI fragment of human MCP-1 cDNA, were radiolabeled with α-32PdCTP using a random priming method (23).

The initial multigene assay was performed as described previously (24). Briefly, the total RNA isolated from the cultured cells was reverse-transcribed to generate first strand cDNA. The second strand of cDNA was synthesized in the presence of α-32PdCTP. This pool of labeled cDNA fragments was hybridized to a nylon sheet on which a known amount of plasmid DNA from genes of interest had been individually dotted. The relative mRNA level of each gene was normalized against the signal of β-actin.

Immunohistochemistry and in Situ Hybridization—The cRNA probes were produced from a 270-base pair human IL-8 cDNA subcloned into pBluescript. T7 and T3 promoters flanking the cDNA fragment were used to make the sense and antisense cRNA probe by in vitro transcription. Riboprobes were labeled with digoxigenin 11-UTP according to the protocol of Boehringer Mannheim. Nonisotopic in situ hybridization was performed using a modification of the manufacturer’s protocol. Paraffin-embedded sections were incubated in xylene for 10 min to remove paraffin, and then the sections were subjected to dehybridization/rehybridization cycles in solutions of various ethanol concentrations. The sections were digested with 10 μg/ml proteinase K at 37 °C for 30 min. After postfixing, prehybridization was for 3 h at 50 °C in buffer containing 50% formamide, 250 μg/ml yeast tRNA, 200 μg/ml salmon sperm DNA, 1.5 mM NaCl, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.2% bovine serum albumin, 4 mM EDTA, 20 mM Tris, pH 7.5. For hybridization, dextran sulfate was added to prehybridization buffer to a final concentration of 20%. Sense or antisense riboprobe was added at 10 ng/μl. Sections were incubated overnight at 50 °C in humidified conditions. The sections were washed at room temperature in excess 2 × SSC for 5 min, and then digested with 50 μg/ml RNase A in 2 × SSC at 37 °C for 30 min. After washing, the sections were blocked by incubation in blocking buffer consisting 5% normal horse serum and 0.3% Triton X-100 in 100 mM Tris, pH 7.5, 150 mM NaCl. The sections were then sequentially incubated and washed with anti-digoxigenin-alkaline phosphatase antibody. After washing, the alkaline phosphatase binding was detected using the substrates nitro blue tetrazolium5-bromo-4-chloro-3-indolyl phosphate. The sections were counterstained with hematoxylin. Immunohistochemistry using antibodies against MAC-1 and mouse serum albumin was performed as described (25).

RESULTS

Induction of IL-8 mRNA in Macrophages by AcLDL—In order to identify novel genes expressed in cholesterol-loaded macrophages, a multigene assay was used. This assay can be used to survey the cellular mRNA level of several dozen genes of interest in a single experiment. The human monocytic leukemia cell line, THP-1, was chosen because this cell line is highly differentiated and, when stimulated with phorbol ester, demonstrates macrophage-like characteristics including expression of scavenger receptors (26–28). Further, incubation of phorbol ester-stimulated THP-1 cells with acLDL results in increased levels of cholesterol (29), implying that the cell line is useful as a model for studying foam cell formation in vitro. The genes selected for the assay included a variety of chemokines, growth factors, interleukins, adhesion molecules, and proteases (see legend to Fig. 1), all of which are known to be expressed in macrophages in various conditions (24). The expression of these genes is important for regulation of the functions of macrophages. In the experiment shown in Fig. 1, PMA-pretreated THP-1 cells were cultured for 20 h in RPMI medium (control, panel A), with added LDL (panel B) or acLDL (panel C) or acLDL plus the ACAT inhibitor 58035 (panel D). Total RNA was isolated from the cells, reverse-transcribed and radiolabeled, and hybridized to filters containing arrays of cDNAs of the genes of interest.

Among 49 genes examined, an increase in mRNA was observed only for IL-8 in acLDL-treated THP-1 cells (Fig. 1). Scanning the filter revealed no significant alteration in mRNA levels of any other genes tested. After normalization to β-actin, the increase in IL-8 mRNA was estimated to be 2.1-fold for acLDL-treated cells versus control macrophages. 58035 alone had no effect (data not shown) but increased IL-8 mRNA by ~5-fold when used with acLDL. Native LDL had a marginal effect on IL-8 mRNA levels, causing increases of 1.2- or 1.4-fold, with or without 58035, respectively. Since phorbol esters up-regulate scavenger receptors while down-regulating LDL receptors (27, 28), these results suggest that IL-8 mRNA is induced as a result of uptake of acLDL by the scavenger receptors. The synergy between ACAT inhibitor and acLDL could indicate that accumulation of unesterified cholesterol provides the signal leading to IL-8 induction.
Bacterial endotoxin has been demonstrated to be a potent stimulator of IL-8 expression in macrophages (31, 32). Therefore, the possibility that these results reflected bacterial endotoxin contamination of the lipoprotein preparations needed to be addressed. Several approaches were used to exclude this possibility. First, the bacterial endotoxin content of the lipoprotein preparations needed to be estimated. The endotoxin content was estimated to be −0.3 pg/µg acLDL protein. No detectable IL-8 mRNA induction was observed by Northern blot analysis when THP-1 cells were treated with up to 100 µg/ml LPS alone (data not shown). Second, polymyxin B, an inhibitor of LPS action on many types of cells, blocked the effect of LPS (10 µg/ml) on IL-8 induction in THP-1 cells, but had no effect on IL-8 induction by acLDL (data not shown). Finally, as shown above, native LDL had no effect on IL-8 mRNA levels, even though the same batch of LDL was used to prepare the acLDL, which did induce the response.

Dose Response and Time Course of IL-8 mRNA Induction by AcLDL—To explore further the relationship between acLDL treatment and IL-8 induction, dose-response and time course experiments were performed. IL-8 protein in medium increased with increasing acLDL concentration to a level three times control at 150 µg/ml acLDL (Fig. 4). Although 58035 did not produce a significant augmentation of the IL-8 response at high concentrations of acLDL, at lower concentrations of acLDL (≤100 µg/ml), 58035 and acLDL had synergistic effects on IL-8 accumulation. A dose-response experiment carried out with oxLDL showed an effect similar to acLDL (data not shown).

For the time course experiment, we compared the response of IL-8 and MCP-1. MCP-1, a member of β chemokine, has been demonstrated to be an inducible gene in vascular endothelial cells, smooth muscle cells, and monocytes upon various stimuli (33, 34) and is expressed in human atheroma (13, 35). The acLDL time course experiments were performed in the presence of 75 µg/ml acLDL and 5 µg/ml 58035. Northern blot analysis was used to follow the alteration of IL-8 and MCP-1 mRNA content of the cells. The results are shown in Fig. 5A, and the mRNA content of IL-8 and MCP-1 (normalized against β-actin mRNA) is quantitated in Fig. 5B. There were marked differences in the kinetics of the response of IL-8 and MCP-1 to cholesterol loading. IL-8 mRNA increased gradually and peaked after 8 h of incubation at −2.5-fold higher than basal level. Then the IL-8 mRNA decreased and at 20 h of incubation the level was −2.5-fold higher than the basal level. In contrast, the increase in MCP-1 mRNA could be observed as early as 10 min after incubation, and the peak effect occurred after 1 h incubation at 3.5-fold basal level. Subsequently, MCP-1 mRNA content gradually decreased and returned to basal level 20 h after incubation. The time course for MCP-1 mRNA explains why the increase of MCP-1 mRNA was not observed in the multigene assay, since the RNA samples used in that assay were collected from cells after 20 h of incubation.

To examine the relationship between altered IL-8 mRNA content and accumulation of cellular cholesterol, cellular free cholesterol and cholesteryl ester were determined. As shown in Fig. 5C, the cellular free cholesterol content increased rapidly during the first 8 h of incubation, paralleling the induction of IL-8 mRNA (Fig. 5B). Subsequently, cellular cholesterol content changed little and IL-8 mRNA decreased. In the presence...
of 58035, the cellular content of cholesteryl ester did not change, reflecting inhibition of ACAT (Fig. 5C).

The time course data suggested that the free cholesterol content of the cell may be related directly to IL-8 mRNA induction in acLDL-treated cells. To further test this hypothesis, THP-1 cells were treated either with free cholesterol or cholesteryl ester incorporated into liposomes in the presence or absence of 58035 in a manner which has been shown to increase the free cholesterol content of macrophages (36). The IL-8 mRNA level, however, did not show any significant alteration even though THP-1 cellular free cholesterol content was increased up to 3.1-fold over control levels. A possible explanation is that acLDL and liposome-mediated cholesterol loading of macrophages may result in accumulation of free cholesterol in different cellular compartments and induce different cellular responses. Mechanisms governing cellular cholesterol levels and compartmentation, however, are still largely unknown (see Ref. 37 for review).

Next, the effects of a known competitor of scavenger receptor-mediated acLDL binding were assessed. As shown in Fig. 6, IL-8 mRNA induction by acLDL (lane 3) was blocked completely by polyinosinic acid (lane 4), while the competitor alone (lane 2) had no effect, indicating that IL-8 mRNA induction requires scavenger receptor-mediated uptake of acLDL.

Characterization of IL-8 Expression in Fresh Human Monocyte Macrophages and Coronary Atheroma—All of the experiments described thus far have utilized macrophage-like cells derived from human THP-1 monocytes, a dividing human cell line. To assess the potential physiological significance of these findings, we determined if modified LDL induces similar responses in macrophages derived from human peripheral blood monocytes. These cells were differentiated into macrophage-like cells in vitro by culturing in media containing human serum and granulocyte macrophage/colony-stimulating factor for 2 weeks. The media were replaced by fresh serum-free medium containing Nutridoma (Boehringer Mannheim), and the cells were then incubated with LDL or acLDL in the presence or absence of 58035. Northern analysis, as shown in Fig. 7A, reveals a ~3.1-fold increase in IL-8 mRNA content of the cells incubated with acLDL and ~3.3-fold increase with acLDL plus 58035 as compared with control or 58035 alone. A parallel increase of IL-8 protein content in the corresponding cell culture media was also detected with the ELISA assay (Fig. 7B).

Thus, IL-8 mRNA is induced by acLDL-mediated cholesterol loading of macrophages in vitro. Nothing, however, is known about the expression of IL-8 mRNA in human atheromatous lesions. To address this question, IL-8 in situ hybridization experiments were carried out using human coronary atheroma...
tomy specimens and IL-8 cRNA probes. Monoclonal antibody against smooth muscle actin (SMA) and an antibody against MAC-1, a marker antigen of macrophages, were used to mark cell types in serial sections. Fig. 8 shows the results of IL-8 in situ hybridization and cell-specific immunostaining of sequential sections from one specimen. An area with strong hybridization with the antisense cRNA probe against IL-8 mRNA (purple stain, Fig. 8, A and B) also showed heavy staining with a monoclonal antibody against the MAC-1 antigen (brown stain, Fig. 8, C and D), indicating the presence of macrophages and cells expressing IL-8 mRNA in the same area. By contrast, this area showed no signal with the IL-8 sense cRNA probe (data not shown) and did not react with monoclonal antibody against SMA (Fig. 8, E and F) even though an adjacent area was heavily stained by the smooth muscle cell antibody (Fig. 8E). Similar results were obtained on two different atherectomy specimens. These results indicate that IL-8 mRNA is expressed in a macrophage-rich area of the lesion, consistent with expression in macrophage foam cells.

**DISCUSSION**

This study reveals that cholesterol loading of macrophages by scavenger receptor-mediated endocytosis of acLDL results in increased IL-8 mRNA and protein production. IL-8 appears to be expressed in foam cells in human atheromas. A role of IL-8 in disease processes such as reperfusion injury and adult respiratory distress syndrome has been documented (38). The present finding suggests the possibility of a role of IL-8 in plaque progression and complications.

Two lines of evidence suggest that IL-8 mRNA induction by acLDL is mediated, at least in part, by an increase in the content of cellular free cholesterol. The effect of acLDL and compound 58035, an ACAT inhibitor, indicates that cellular accumulation of free cholesterol rather than cholesteryl ester is related to IL-8 induction. Further, the time course of cellular free cholesterol accumulation indicates a rapid increase in cellular free cholesterol content which precedes the increase in IL-8 mRNA, also suggesting a close relationship between cellular free cholesterol content and IL-8 mRNA level. Recently, Terkeltaub et al. (30) reported increased production of IL-8 by THP-1 cells when incubated with oxLDL but not with acLDL. The discrepancy is probably due to the fact that nondifferentiated monocytic THP-1 cells were used in that study. Without phorbol ester treatment, these cells have few acLDL receptors (27–29). Terkeltaub et al. (30) suggested that oxidativel lipid or protein degradation end products or lysophospholipids may contribute to IL-8 induction by oxLDL. We have exercised caution to minimize any oxidation of acLDL preparations used in this study, and acLDL showed a potency similar to that of oxLDL in dose-response experiments. Therefore, oxidative degradation end products cannot account for IL-8 induction by acLDL.

An important finding in this study is the identification of IL-8 mRNA in human coronary atheromatous lesions. The in situ signals indicate a localized expression of IL-8 in the lesions. Due to the nature of the specimen (atherectomy), it is difficult to discern the microtopography of the sections. Nonetheless, immunohistochemical processing of sequential sections with specific monoclonal antibody against macrophage and smooth muscle antigens reveals that the IL-8 mRNA is localized in a distinct macrophage-rich area. It is likely that those cells expressing IL-8 mRNA are macrophages, although double staining would be desirable in future studies for further documentation. Little work has been done beyond the current study to investigate the correlation between IL-8 generation and atherogenesis. In one other study, an increased content of IL-8 protein has been observed in atherosclerotic human abdominal aortic aneurysms compared with normal aortic tissues (39), although the original source of IL-8 is not addressed in
IL-8 Expression in Macrophage Foam Cells

This study.

The multigene assay used in this study to identify IL-8 mRNA induction among multiple genes tested demonstrates the usefulness of this method in detecting altered mRNA levels of genes of interest. As compared with polymerase chain reaction-based differential display, another method commonly used to identify novel genes with altered mRNA levels (40), this assay has the advantage of simplicity and reproducibility. However, it is evident that changes in unknown genes or low abundance mRNAs would not be detected. Recent studies using expressed sequence tags of PC12 cells before and after treatment with nerve growth factors illustrate that a multitude of cDNAs may be altered when cells are induced to change state (41). Thus, it is likely that the pattern of altered gene expression in macrophage foam cells is much more complex than documented to date.

Both IL-8 and MCP-1 belong to the chemokine superfamily that has two major branches based on the structure of the first pair of cysteine residues. IL-8 has the CC structure and is a member of the α chemokine family, while MCP-1 maintains a CC sequence and belongs to the β interferon, a major chemokine family (42). Due to its monocyte chemotactic activity, MCP-1 has attracted attention for its potential role in recruiting monocyte/macrophage cells in atheroma. OxLDL was recently shown to induce expression by endothelial cells and smooth muscle cells of MCP-1 (33), and MCP-1 has been identified within macrophage-derived foam cells in atherosclerotic lesions (14, 35). No comparable attention has been given to IL-8 in atherosclerosis studies, probably because IL-8 was originally identified as a potent chemotactic factor for neutrophils, a cell type thought to be relatively uncommon in human atherosclerotic lesions. However, recent studies demonstrate that IL-8 is a multifunctional chemokine involved in many biological processes that could potentially play important roles in atherogenesis. For instance, IL-8 has been shown to be a potent chemotactic factor for T lymphocytes (43). T cells have been identified in human atherosclerotic lesions although their function is not clear (44, 45). Recent studies reveal the presence of γ-interferon, a major product of T lymphocytes, in human atheromatous lesions (46) and suggest its involvement in local intercellular communications which may be critical in atherogenesis (47). IL-8 also has mitogenic and chemotactic activities toward vascular smooth muscle cells (48, 49). A recent study has shown that IL-8 induces proliferation and chemotaxis of human umbilical vein endothelial cells and is a potent angiogenic agent (50). Migration and proliferation of smooth muscle cells are another hallmark of atherosclerosis, and neovascularization is also a commonly observed feature of atherosclerotic lesions (see Refs. 51–53 for review). Smooth muscle cell migration and proliferation is believed to be a major contributor to atherosclerotic plaque formation, and angiogenesis in atherosclerotic plaques may predispose to intramural hemorrhage, thrombosis, and plaque rupture. It is not clear what role, if any, IL-8 plays since many other protein and peptide factors have been also implicated in regulation of these processes (51–54). However, findings made through our study and that of Terkeltaub et al. (50) strongly support a role of IL-8 in atherogenesis. The potential contribution of IL-8 to the development and complications of atherosclerosis warrants further investigation.

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