Inhibition of the macrophage-induced oxidation of low density lipoprotein by interferon-γ[1]

Loren G. Fong,[2] Timothy S. E. Albert, and Sharon E. Hom

Research Institute, Palo Alto Medical Foundation, 860 Bryant Street, Palo Alto, CA 94301

Abstract The regulation of the macrophage-induced oxidation of low density lipoprotein (LDL) by cytokines was investigated. As an initial source of cytokines, medium from an activated type 2 helper T-cell clone was tested. This cell-free supernatant inhibited the subsequent oxidation of LDL by mouse peritoneal macrophages. The inhibition was concentration- and time-dependent as measured by changes in thiobarbituric acid (TBA) reactive substances. In addition, there were decreases in conjugated diene formation as well as the generation of LDL particles with an increased net negative charge that were recognized by the scavenger receptor. The inhibition was not due to a decrease in cell viability or to nonspecific antioxidant activity, as assessed by measuring phagocytic activity and metal ion-induced oxidation of LDL, respectively. Using antibodies that inactivate specific cytokines, the role of select individual cytokines in this inhibition was investigated. Addition of antibodies against interleukin-3 (IL-3), granulocyte/macrophage-colony stimulating factor (GM-CSF), or tumor necrosis factorα (TNFα) to the media had little or no effect on the ability of the cytokines to affect oxidation by macrophages, whereas anti-interferon-γ (IFN-γ) antibodies completely reversed the inhibition induced by the T-cell supernatant. A role for this cytokine was confirmed using recombinant IFN-γ. A concentration-dependent inhibition was produced with a maximum inhibition to 24% of control cells, whereas smooth muscle cell-dependent LDL oxidation was not affected. To examine the cellular basis for the inhibition, the effect of IFN-γ on oxidant activities (O_2^- production, lipoxygenase activity, and thiol production) were measured. IFN-γ at concentrations that maximally inhibit LDL oxidation stimulated the phorbol myristate acetate (PMA)-induced production of O_2^- 1.4-times greater than control cells after one hour. Similarly, thiol production was increased 29% by IFN-γ pretreatment. In contrast, macrophage lipoxygenase was inhibited approximately 21%. Based on these in vitro findings, the potential regulation of macrophage LDL oxidation by IFN-γ in vivo was also investigated. Macrophages from *Toxoplasma gondii*-infected mice have been shown previously to be activated in situ by an IFN-γ-dependent mechanism. These were tested for their ability to oxidize LDL. Macrophages from these mice oxidized LDL to a much lesser extent than cells from age-matched control mice, demonstrating that the ability of macrophages to oxidize lipoprotein may also be susceptible to regulation possibly also by IFN-γ in vivo. Together these studies demonstrate that the cell-mediated oxidation of LDL can be regulated by cytokines, specifically IFN-γ. This mode of regulation may play a role in regulating this process in the developing atherosclerotic lesion.—**Fong, L. G., T. S. E. Albert, and S. E. Hom.** Inhibition of the macrophage-induced oxidation of low density lipoprotein by interferon-γ. *J. Lipid Res.* 1994. **35:** 893–904.

Supplementary key words atherosclerosis • cytokines • lipid peroxidation • superoxide anion • lipoxygenase • thiol • smooth muscle cells • *T. gondii*

Ultrastructural studies have established that foam cells of the fatty streak lesion are derived primarily from circulating monocytes that have entered the subendothelial space (1, 2); however, the precise mechanism(s) responsible for the conversion of subendothelial monocytes into foam cells are still not clearly understood. One relatively recent model that has received considerable support and is consistent with the ultrastructural studies is the low density lipoprotein (LDL) oxidation hypothesis of foam cell formation (reviewed in ref. 3). This hypothesis proposes that LDL that has entered the artery wall becomes oxidized, producing modified forms of LDL that express a spectrum of biological properties that would affect the atherosclerotic process. Some of these include the stimulation of entry of monocytes into the subendothelium (4, 5), one of the earliest cellular events that is consistently observed in animal models of atherosclerosis, as well as oxidized LDL's avid metabolism by macrophages to lead to cholesterol ester accumulation (6), a feature that is characteristic of foam cells, and the particle's cytotoxic effects (7) that may contribute to endothelial disruption as appears in more advanced lesions. This model is based on studies that have provided support for the hypothesis that

Abbreviations: LDL, low density lipoprotein; O_2^-, superoxide anion; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SOD, superoxide dismutase; HODE, hydroxyoctadecadienoic acid; ETYA, eicosatetraynoic acid; PMA, phorbol myristate acetate; DTNB, 5,5'-dithio*bis*(2-nitrobenzoic acid); IFN-γ, interferon-gamma; IL, interleukin; TNFα, tumor necrosis factor alpha; GM-CSF, granulocyte/macrophage-colony stimulating factor; *T. gondii*, *Toxoplasma gondii*; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; BHT, butylated hydroxytoluene; TBA, thiobarbituric acid; MDA, malondialdehyde; Con A, concanavalin A; TCA, trichloroacetic acid; 15-LO, 15-lipoxygenase.

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[2]To whom reprint requests should be addressed.

there is an oxidation-dependent component to atherosclerosis. This initially came in the form of studies that tested the effectiveness of antioxidants to slow atherogenesis in animal models of atherosclerosis. It was observed that antioxidants or drugs with antioxidant activity were consistently able to reduce the extent of atherosclerosis (8–12). Although the mechanisms that mediate their inhibition have not yet been established, the inhibition of LDL oxidation is a likely possibility as immunohistochemical studies have strongly suggested that oxidized forms of LDL are present within the artery wall (13, 14). Together these support a model that proposes a role for oxidized LDL in atherosclerosis and predicts that processes that operate within the artery wall to regulate the extent of LDL oxidation would affect the extent of atherosclerosis.

The identification of oxidized LDL within localized areas of the artery wall suggests that the oxidation of LDL is not uniform throughout the artery wall. Besides differences in the entry of LDL (15) or its residence time within the artery wall (16), there may be regional differences in the level of oxidative stress; there are localized regulatory mechanisms that modulate the extent of LDL oxidation. Although the precise mechanism(s) by which LDL is oxidized in vivo remains to be established, it is assumed that the oxidation of LDL in the artery wall is cell-dependent. Each of the major cell types that are present in the developing atherosclerotic lesion, smooth muscle cells (17), endothelial cells (18), monocyte/macrophages (19, 20) and lymphocytes (21), have been shown to oxidize LDL in vitro and with the exception of lymphocytes, cell-induced oxidation of LDL has been shown to be susceptible to modulation. The most prominent of these are conditions that affect either superoxide anion (O_2^-) production (17, 19, 22, 23), lipoxygenase enzyme activity (22–24), or thiol production (25), although the contribution of cellular lipoxygenases has recently been questioned (26). Several other agents have also been reported to affect cellular LDL oxidation, including calcium antagonists (27), estrogens (28), and β-blockers (29); however, the basis for their activities is not known. These studies demonstrate that the oxidation of LDL by cells can be modulated; however, the contributions of these agents to the localized regulation of cell-dependent LDL oxidation as it occurs normally in the artery wall are unknown.

One form of regulation that might be of importance is the localized action of cytokines. While a role for cytokines in the regulation of the immune response has clearly been established, a growing body of evidence now supports their potential importance to the atherosclerotic process. Cytokines have been shown to be present within the artery wall (30–32), cytokine mRNA levels are elevated during atherosclerosis (31–33), and they are thought to mediate some aspects of endothelial cell activation in vivo (reviewed in ref. 34). Moreover, cytokines have been shown to affect cellular oxidative systems in cultured cells (35–37). It is thus reasonable to suggest that the synthesis of cytokines by artery wall cells would be most effective as regulators of artery wall cell-mediated oxidation of LDL. To seek evidence for such a regulatory pathway, the oxidation of LDL by macrophages and its susceptibility to modulation by cytokines was investigated in vitro and in vivo.

METHODS

Materials

F-10 medium, RPMI 1640, Dulbecco's Modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from GIBCO Laboratories (Grand Island, NY). FCS was heat-inactivated before use (56°C for 30 min). Cytochrome c (horse heart type IV), L-cysteine, and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO) and superoxide dismutase (SOD; 3500 U/mg) was from CalBiochem (San Diego, CA). Phorbol myristate acetate (PMA) was obtained from LC Laboratories (Woburn, MA) and 5,5'-dithio*bis*(2-nitrobenzoic acid) (DTNB) was from Pierce Chemical Co. (Rockford, IL). [1-^{14}C]linoleic acid (55 Ci/mol) and Na^{125}I were obtained from Amersham Arlington Heights, IL), 9(s)-hydroxyoctadecadienoic acid (9-HODE) and 13(s)-hydroxyoctadecadienoic acid (13-HODE) were from Oxford Biomedical Research Inc. (Oxford, MI), and eicosatetraynoic acid (ETYA) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Rhodamine-labeled latex beads (0.9 μm diameter) were purchased from Polysciences Inc. (Warrington, PA). Recombinant murine interferon-gamma (IFN-γ) was obtained from Genzyme (Cambridge, MA). IFN-γ was diluted with DMEM containing 10% FCS to 20,000 U/ml and aliquots were stored at −80°C. Antibodies that neutralize the bioactivity of IFN-γ, interleukin (IL)-3, tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony stimulating factor (GM-CSF) were purchased from Pharmingen (San Diego, CA).

Animals

Female Swiss Webster mice (27–30 g) were purchased from Simonsen Laboratories (Gilroy, CA) and given standard mouse chow and water ad libitum. A small number of mice were infected with *Toxoplasma gondii (T. gondii)* by the oral administration of 50 ME49 cysts per animal. These mice were maintained under the same conditions as those described for noninfected mice and were used between 3 to 4 weeks after inoculation.

Procedures

Lipoproteins. Human LDL (*d* 1.019–1.063 g/ml) was isolated from EDTA-treated plasma by density gradient

ultracentrifugation (38) and dialyzed against PBS containing 0.01% EDTA. LDL was radiolabeled with $Na^{125}I$ using Iodogen beads (Pierce Chemical Co.) to a specific activity of 50–100 cpm/ng.

Cell lines. Mouse smooth muscle cells were obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained in DMEM containing 20% FCS, 2 mM glutamine, and antibiotics. J774 cells were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, and antibiotics.

Activation of T-cells. HDK-1 and D-10 T-cell clones were resuspended in RPMI 1640 containing 10% FCS (5×10^6 cells/ml) and incubated in the presence and absence of concanavalin A (5 μg/ml) for 24 h at 37°C. The incubation mixtures were centrifuged and the cell-free supernatants were incubated with α-methyl mannoside gel (E-Y Labs; San Mateo, CA) at 4°C overnight with constant rocking. The gel was sedimented by centrifugation and the supernatants were filtered through a 0.45 μm filter. Aliquots were then stored at −80°C until use.

Measurement of LDL oxidation by macrophages. Resident mouse peritoneal macrophages were harvested by peritoneal lavage, resuspended in DMEM supplemented with 10% FCS, 2 mM glutamine, and antibiotics (medium A), and plated in 24-well tissue culture plates (39). The adherent macrophages were preincubated in medium A (0.5 ml) overnight in the presence or absence of additions (i.e., T-cell medium) at 37°C. The cells were rinsed 3 times with F-10 medium and then incubated with LDL (100 μg protein/ml) in F-10 medium (0.5 ml) at 37°C for 8 h. The incubation media were transferred to microfuge tubes containing 2.5 μl 4 mM butylated hydroxytoluene (BHT) and then centrifuged. An aliquot of the supernatant (0.25 ml) was then removed and the amount of thiobarbituric acid reactive substances (TBARS) was measured as described previously (40) using tetramethoxypropane as a standard. The remaining cells were rinsed three times with sterile PBS and the amount of cell protein was measured. The net amount of TBARS generated was calculated after correcting for the amount of TBARS generated in the absence of cells (no cell control) and in the absence of LDL (macrophage control) and the extent of LDL oxidation by macrophages was expressed as the amount of malondialdehyde (MDA) equivalents in nmols per mg cell protein.

Macrophage metabolism of oxidized LDL. The degradation of oxidized LDL by macrophages was measured as described elsewhere (39). Briefly, adherent macrophages in 24-well tissue culture plates were incubated with radiolabeled macrophage oxidized LDL (5 μg/ml) in the presence and absence of unlabeled lipoproteins (40 μg/ml) in DMEM supplemented with 0.5% BSA (pH 7.4) at 37°C for 5 h. The amount of TCA- and silver nitrate-soluble products in the incubation supernatant was then determined and the results were expressed as the amount of oxidized LDL degraded in μg per mg cell protein. The degradation rate of oxidized LDL by control incubated macrophages averaged 5.05 ± 0.55 (mean ± SE of three experiments).

Macrophage superoxide anion production. The production of superoxide anion by macrophages was measured as described by Johnston (41) with slight modifications. Adherent macrophages in 24-well tissue culture plates were preincubated in the presence or absence of IFN-γ (25 U/ml; 18 h) and then washed with Krebs-Ringer phosphate buffer (pH 7.4). One-half of the cells received 0.4 ml of 80 μM cytochrome c in Krebs-Ringer (solution 1) and the other half the same solution plus 40 μg/ml SOD (solution 2). All of the cells were then stimulated by the addition of 0.1 ml of 2.5 μg/ml PMA dissolved in either solution 1 or solution 2 and incubated in a 37°C water bath for 0 to 60 min. Wells containing no cells were included as background controls. After the incubations, the incubation media were transferred to microfuge tubes and centrifuged and their absorbance was read at 550 nm. The cells were washed with PBS and the amount of cell protein was measured. The amount of O_2^- produced was determined by measuring the reduction of cytochrome c that was inhibitable by SOD. The amount of O_2^- produced was calculated using the extinction coefficient of $21.0 \times 10^3\ M^{-1}cm^{-1}$ for reduced cytochrome c and the results were expressed as nmol of O_2^- produced per mg cell protein. The reduction of cytochrome c by both control and IFN-γ-treated cells was inhibited by more than 89% by SOD.

Macrophage lipoxygenase activity. The oxidation of linoleic acid by macrophages was measured exactly as described by Rankin, Parthasarathy, and Steinberg (23) and Sparrow and Olszewski (26). Briefly, adherent macrophages were incubated with 20 μM [1-^{14}C]linoleic acid in F-10 medium (0.5 ml) at 37°C for 20 min. Incubations in the absence of cells were also included as background controls. The incubation medium was transferred to a microfuge tube containing 2.5 μl of 4 mM BHT and then centrifuged. A portion of the supernatant (0.4 ml) was acidified and then extracted with chloroform and methanol by the method of Bligh and Dyer (42). The chloroform phase was dried under nitrogen and the lipid residue was resuspended with chloroform–methanol 1:1. The lipids were separated by thin-layer chromatography on silica gel G plates in diethyl ether–petroleum ether–acetic acid 50:50:1 and radiolabeled lipids were visualized by autoradiography. Based on the migration of lipid standards, oxidized linoleic acid products were identified and quantitated by liquid scintillation spectrometry. The results were expressed as the amount of oxidized linoleate in nmol using the specific activity of linoleate as a standard. In agreement with others (23, 25), the generation of 13-HODE was sensitive to ETYA pretreatment (see Fig. 6).

Macrophage thiol production. The macrophage-dependent

appearance of thiols in the incubation medium was measured as described by Sparrow and Olszewski (25). Briefly, the incubation medium from macrophages was centrifuged and an aliquot of the supernatant (0.45 ml) was mixed with 0.05 ml of 1 mM DTNB dissolved in 0.2 mM sodium phosphate buffer (pH 8). The absorbance was then measured immediately at 412 nm. Medium that was incubated in the absence of cells was included as a background control. The amount of thiol in the medium was calculated using freshly diluted solutions of cysteine (1–20 μM).

Other procedures. Protein was measured by the method of Lowry et al. (43) using albumin as a standard. Macrophage phagocytosis was measured as described previously (40) using rhodamine-labeled latex beads (0.9 μm diameter). Conjugated dienes were estimated by measuring the absorbance at 234 nm (40). The electrophoretic mobility of lipoproteins was measured by agarose gel electrophoresis (44).

Statistics. Statistical analysis was done using non-paired Student's *t*-test.

RESULTS

Inhibition of the macrophage-induced oxidation of LDL by medium from activated HDK-1 cells

Macrophages are able to modify LDL by an oxidation-dependent process (20) and are thought to contribute to the generation of oxidized LDL within the artery wall; however, it is unknown whether this oxidative modification is susceptible to modulation by processes or factors that function within the developing atherosclerotic lesion. One form of modulation that might be relevant is the localized production of cytokines. To examine this possibility, macrophages were pretreated with cytokines and their ability to oxidize LDL was then measured. As an initial source of cytokines, medium from concanavalin A (Con A)-activated HDK-1 cells was tested (hereafter designated activation supernatant). HDK-1 cells are a type 2 T-helper cell clone that synthesize and secrete cytokines after activation with Con A (45). Resident mouse peritoneal macrophages were incubated with this medium at 37°C for 18 h. Incubation with medium from non-activated HDK-1 cells served as a control. The following day the cells were washed, LDL was added and, after an 8-h incubation, the extent of LDL oxidation was measured. Preincubation of the macrophages with the activation supernatant inhibited the subsequent ability of macrophages to oxidize LDL in a concentration-dependent manner, whereas preincubation with medium from non-activated cells was without effect (**Fig. 1**). In five different dose-response experiments, the oxidation of LDL was inhibited maximally to 22.2 ± 5.5% of control incubated cells (mean ± SE). Besides an inhibition of the generation of TBA-reactive substances, there was also a consistent decrease in conjugated diene formation, another measure of lipid peroxidation. Further, the generation of a lipoprotein with an increased net negative charge as measured by agarose gel electrophoresis was also decreased (2.1 ± 0.1 vs. 1.2 ± 0.1; migration relative to native LDL). Preincubation with the activation supernatant also inhibited the generation of biologically modified LDL; testing three different preparations, LDL harvested from macrophages previously treated with the activation supernatant was unable to compete for the degradation of radiolabeled oxidized LDL by macrophages (108.5 ± 5.2% of control LDL incubated in the absence of cells; mean ± SE). Together these studies show that the macrophage-induced oxidation of LDL and the subsequent biological modification of LDL can be modulated by cytokines.

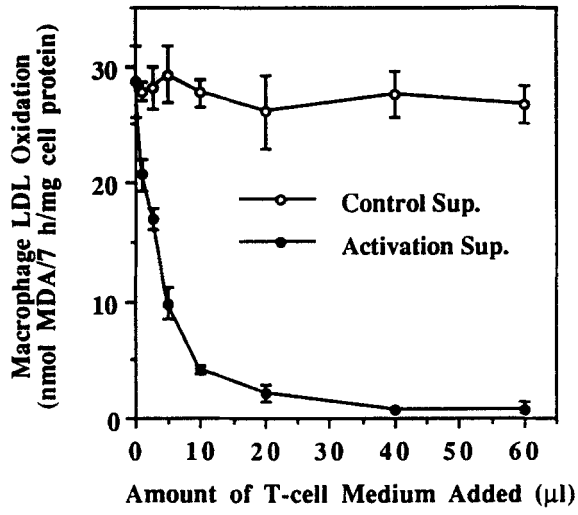

Fig. 1. Inhibition of the macrophage-induced oxidation of LDL by medium from activated HDK-1 T-cells. Macrophages were preincubated with increasing amounts of medium from either non-activated (○) or activated (●) HDK-1 T-cells at 37°C for 18 h. The cells were washed and incubated with LDL (100 μg/ml) in F-10 medium at 37°C for 8 h. The extent of LDL oxidation was then measured as described in the Methods section and the results are expressed as the amount of MDA equivalents in nmol per mg cell protein. The mean ± SD of a representative experiment is shown.

Inhibition induced by HDK-1 medium is not due to cell toxicity or nonspecific antioxidant activity

The inhibition of LDL oxidation by the activation supernatant suggests that a component of the activation supernatant inhibits the ability of macrophages to oxidize LDL in a specific manner; however, there are alternative explanations that could account for the inhibition. The activation supernatant might decrease cell viability or cause a nonspecific inhibition of lipid peroxidation. If the activation supernatant was cytotoxic to the cells, a decreased rate of LDL oxidation would be expected. To test

for cell toxicity, the phagocytosis of fluorescent-labeled beads by control and treated cells was measured. Based on the percentage of cells that were positive for phagocytosis (95% vs. 97%; activation supernatant vs. control supernatant), the activation supernatant did not affect the viability of cells; thus, the inhibition of LDL oxidation cannot be accounted for by cell death. A decrease in LDL oxidation could also be produced if the activation supernatant inhibited the oxidation of LDL by a mechanism independent of its action on the cells, that is if the activation supernatant behaved as a general antioxidant. This possibility is less likely as the macrophages were washed extensively to remove the activation supernatant prior to the measurement of LDL oxidation; however, to examine whether any residual medium that interferes with the oxidation of LDL might remain, the effect of the activation supernatant on the copper ion-induced oxidation of LDL (18) was examined. The oxidation of LDL catalyzed by metal ions mimics the oxidation of LDL induced by cells; thus if the activation supernatant exhibits general antioxidant activity it should also be able to inhibit the oxidation of LDL in this cell-free system. In order to compare the two oxidation systems on an equal basis, the amount of copper ion was titrated until comparable amounts of TBA-reactive substances were generated. Using this amount of copper ion (3 μM), the effect of the activation supernatant on the oxidation of LDL was measured. Empty incubation wells were preincubated overnight with the activation supernatant in an amount shown previously to maximally inhibit macrophage-induced LDL oxidation (60 μl), washed, and then the oxidation of LDL was measured. In contrast to its effect on macrophages, the activation supernatant had no effect on the copper ion-stimulated oxidation of LDL (96.3 ± 2.9% of control; mean ± SE of three experiments). Together these studies show that there is a constituent in the activation supernatant that acts on macrophages to affect their ability to oxidize LDL.

Cytokines in the HDK-1 activation supernatant inhibit macrophage LDL oxidation

There are a number of candidates for the active component; however, cytokines are an obvious possibility. Cytokines are recognized to modulate macrophage functions including oxidative activities (35–37). Activated HDK-1 T-cells secrete several different cytokines (45), any of which might mediate the inhibition. In an attempt to identify the possible cytokines that exhibit inhibitory activity, an activation supernatant from another T-helper cell clone was examined. Activated D-10 cells secrete some cytokines that are in common with HDK-1 cells. By comparing their effects on macrophage LDL oxidation, certain cytokines may be targeted for further study. Similar to activated HDK-1 cells, medium from activated D-10 cells also inhibited the macrophage-induced oxidation of LDL; however, the extent of inhibition was consistently less (37.3 ± 1.4% vs. 78.6 ± 7.3%; mean ± SE of three experiments). Since both were inhibitory, this would suggest that the activity of the HDK-1 activation supernatant is due to a cytokine that is common to both cell types but is present in higher amounts in the HDK-1 supernatant or that a cytokine unique to the HDK-1 supernatant interacts with one of the common cytokines to produce an inhibition. This was examined by testing the contribution of the three cytokines that are common to both T-cell clones (IL-3, GM-CSF, TNFα) and one cytokine that is unique to HDK-1 cells (IFN-γ). This was done by using antibodies that neutralize the bioactivity of these cytokines. The HDK-1 activation supernatant was first preincubated with the specific antibodies and then added to macrophages. Media incubated with antibody alone were included as controls. Antibodies to IL-3 and GM-CSF did not prevent the HDK-1-induced inhibition, while inactivation of TNFα produced a slight but consistent reversal (**Table 1**). In contrast, neutralization of IFN-γ completely blocked the inhibition. In control incubations, neither the anti-IFN-γ nor anti-TNFα antibodies by themselves stimulated the oxidation of LDL by macrophages (data not shown). These studies thus show that both IFN-γ and TNFα contribute to the inhibitory activity of the HDK-1 activation supernatant.

Recombinant IFN-γ inhibits macrophage-induced LDL oxidation

The antibody blocking studies suggest that IFN-γ is a potent inhibitor of macrophage LDL oxidation; however, as the activation supernatant is comprised of a mixture of cytokines, it is possible that two or more cytokines together might be required to produce an inhibition. Interactions between different cytokines that augment or antagonize a cytokine induced effect are common (35). To test whether IFN-γ directly inhibits macrophage-induced

TABLE 1. Identification of cytokines that inhibit macrophage-induced LDL oxidation

Macrophage Preincubation Conditions	LDL Oxidation (n = 3)
Medium	37.1 ± 2.0
Medium + HDK-1AS	2.0 ± 1.6
Medium + HDK-1AS + anti-IL-3	2.0 ± 1.4
Medium + HDK-1AS + anti-GM-CSF	0.2 ± 0.0
Medium + HDK-1AS + anti-TNFα	6.4 ± 1.0
Medium + HDK-1AS + anti-IFNγ	37.5 ± 1.8

The HDK-1 activation supernatant (HDK-1AS) was preincubated in the presence (10 μg protein/ml) or absence of antibodies at 37°C for 2 h prior to their addition to macrophages. The cells were then incubated at 37°C for 18 h and then the oxidation of LDL was measured. The extent of LDL oxidation is expressed as the nmol amount of MDA equivalents per 7 h per mg cell protein. Values are given as means ± SD of a representative experiment.

LDL oxidation, recombinant murine IFN-γ was examined. Macrophages preincubated with IFN-γ inhibited the subsequent oxidation of LDL by macrophages in a concentration-dependent manner (**Fig. 2**) with a maximum suppression to 23.7 ± 4.7% of control at 25 U/ml (mean ± SE of five experiments). These studies show that IFN-γ can directly modulate the macrophage-induced oxidation of LDL and that IFN-γ is a potent inhibitor of this cell function. The inhibition by IFN-γ, though, was not immediate; there was a considerable lag period before an inhibition could be detectable. At least 10–12 h of preincubation with IFN-γ was required before a decrease in LDL oxidation was observed (representative of three experiments). Similar to the HDK-1 activation supernatant, IFN-γ inhibited the generation of a lipoprotein with an increased net negative charge (2.1 ± 0.1 mm vs. 1.2 ± 0.0 mm; migration relative to native LDL), blocked the conversion of the incubated LDL to a form recognized by the scavenger receptor (94.2 ± 8.9% of control LDL incubated in the absence of cells; mean ± SE of three experiments), and did not inhibit the copper ion-induced oxidation of LDL (106.6 ± 4.4% of control; mean ± SE of three experiments).

In vitro studies (46) and more recently studies conducted in vivo (47) have shown that some aspects of macrophage activation by IFN-γ are actually mediated by macrophage-derived factors (i.e., TNFα) that act in an autocrine fashion. As TNFα appears to be a cytokine with inhibitory activity (see Table 1), it is possible that a portion of the inhibition induced by IFN-γ may be mediated by TNFα or another macrophage-derived factor. This would be consistent with the relatively long preincubation

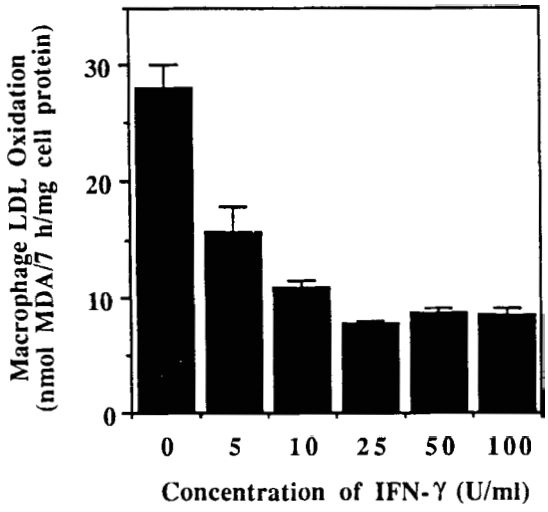

Fig. 2. Inhibition of the macrophage-induced oxidation of LDL by recombinant murine IFN-γ. Macrophages were preincubated in the absence or presence of increasing amounts of IFN-γ at 37°C for 18 h. The cells were washed and the oxidation of LDL was measured as described in the legend to Fig. 1. The mean ± SD of a representative experiment is shown.

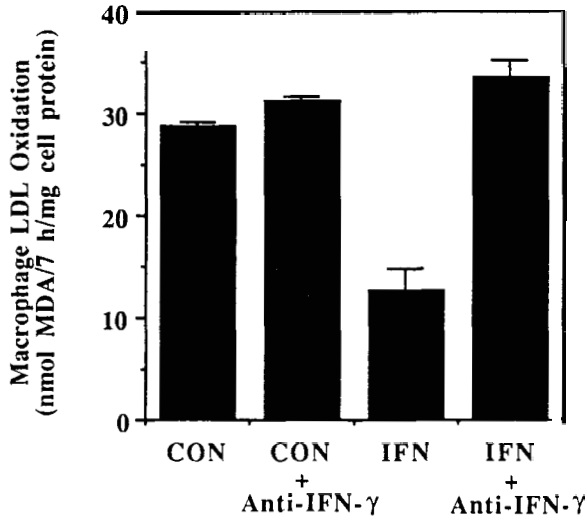

Fig. 3. Determination whether IFN-γ induces an autocrine factor that inhibits macrophage LDL oxidation. Macrophages were preincubated in the absence (control; CON) or presence of IFN-γ (25 U/ml) at 37°C for 18 h. The cell incubation media were harvested and then each was divided into two equal portions. The incubation media were then preincubated in the presence or absence of an anti-IFN-γ antibody (15 μg/ml) at 37°C for 3 h and added to a second set of macrophages. The cells were incubated at 37°C for 18 h after which the oxidation of LDL was measured as described in the legend to Fig. 1. The mean ± SD of a representative experiment is shown.

that is required before IFN-γ produces a decrease in LDL oxidation. To test whether IFN-γ stimulates the synthesis and secretion of a factor(s) that then inhibits LDL oxidation, macrophages were first stimulated with IFN-γ and the incubation medium from these cells was then tested for inhibitory activity on a second set of macrophages. To block the activity of the IFN-γ that is carried over into the second incubation, the anti-IFN-γ antibody was added at a concentration that was determined in preliminary studies to completely block its activity. Using this approach, any factors released by macrophages in response to IFN-γ that inhibit macrophage LDL oxidation would be identified. As shown in **Fig. 3,** the incubation medium from macrophages stimulated with IFN-γ without the addition of the anti-IFN-γ antibody was inhibitory, as expected, due to carryover of IFN-γ. However, when IFN-γ in the incubation medium was inactivated with the antibody, the macrophage incubation medium was no longer inhibitory. This shows that the inhibition induced by IFN-γ is not mediated by a macrophage-derived autoregulatory factor but that IFN-γ directly initiates inhibition of macrophage-induced LDL oxidation.

Inhibition induced by IFN-γ is selective for macrophages

Other cell types besides macrophages have the capacity to oxidize LDL. To examine whether the inhibition of LDL oxidation by IFN-γ is specific for macrophages, the

effects of IFN-γ on the oxidation of LDL by mouse smooth muscle cells and mouse macrophages were compared. Smooth muscle cells were preincubated with IFN-γ at conditions that maximally inhibit macrophage-mediated LDL oxidation (25 U/ml IFN-γ, 18 h) and their ability to oxidize LDL was measured. Unlike macrophages, IFN-γ had no effect on the extent of LDL oxidation stimulated by smooth muscle cells (**Fig. 4**). In three separate experiments, the IFN-γ-treated cells oxidized LDL to the same extent as control incubated cells (103.4 ± 4.6% of control cells; mean ± SE). As a control to ensure that the absence of an inhibition by IFN-γ was not related to differences between a cell line (smooth muscle cell) and a nondividing cell type (peritoneal macrophage), the effect of IFN-γ was also tested using the mouse macrophage-like J774 cell line. This is particularly relevant for IFN-γ as it is known to have antiproliferative activities (48). Confluent monolayers of J774 cells were pretreated with IFN-γ (25 U/ml for 18 h) and their ability to oxidize LDL was measured after 14 h. Similar to its effects on freshly isolated macrophages, IFN-γ also inhibited the oxidation of LDL by J774 cells (42.8 ± 12.2% of control cells; mean ± SE of three experiments). However, it should be noted that even though confluent monolayers were used, a lower amount of cell protein was measured in the IFN-γ-treated cells, which would suggest that some replication is still ongoing in the control incubated cell cultures. Nevertheless, this would not affect the outcome and the inhibition should be considered as a minimum. Together these studies suggest that the inhibition of LDL oxidation by IFN-γ is not a general effect but is cell-selective.

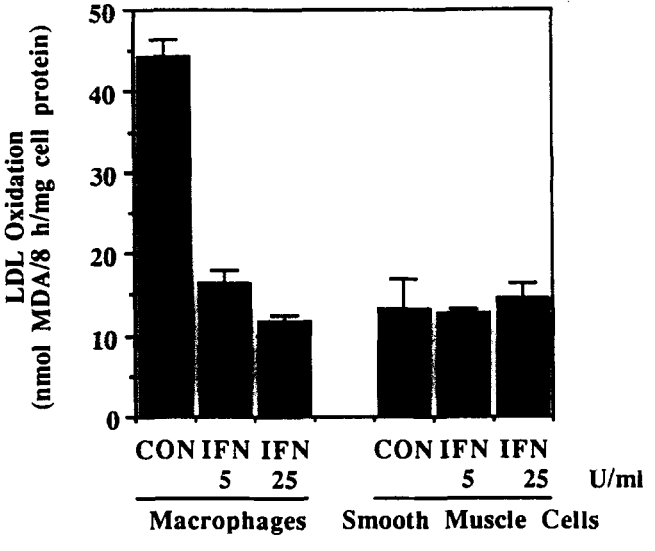

Fig. 4. Effect of IFN-γ on the oxidation of LDL induced by macrophages or smooth muscle cells. Mouse macrophages or murine smooth muscle cells were preincubated with IFN-γ (25 U/ml) at 37°C for 18 h. The cells were washed and the oxidation of LDL was measured after 8 h. The mean ± SD of a representative experiment is shown.

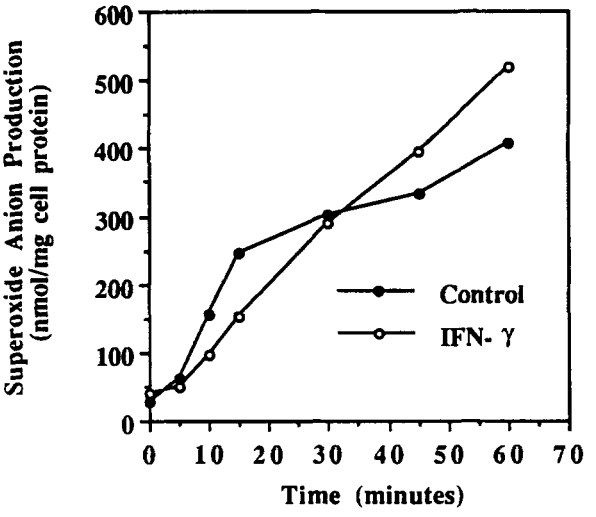

Fig. 5. Effect of IFN-γ on the production of superoxide anion by macrophages stimulated with PMA. Macrophages were preincubated in the absence (●) or presence (○) of IFN-γ (25 U/ml; 18 h) and then stimulated with PMA (0.5 μg/ml). The reduction of cytochrome c that was inhibited by SOD was then determined as described in the Methods section. The results are expressed as the amount of O_2^- produced in nmol per mg cell protein. The mean ± SD of a representative experiment is shown.

IFN-γ does not inhibit superoxide anion production

The capacity of a cell to oxidize LDL most likely depends upon a balance between cellular oxidative and antioxidant systems. This would suggest then that the inhibition induced by IFN-γ is due to either an inactivation of one or more of the oxidative processes that mediate macrophage-stimulated LDL oxidation or to the production of a cell-derived factor that exhibits antioxidant activity.

The oxidation of LDL by macrophages has been shown to be dependent on lipoxygenase activity, O_2^- production and, more recently, thiol production. To examine whether IFN-γ inhibits LDL oxidation by decreasing O_2^- production, the effect of IFN-γ on O_2^- levels was measured. Macrophages were pretreated with IFN-γ at conditions that maximally inhibit LDL oxidation (25 U/ml; 18 h) and then the kinetics of O_2^- production was measured. The levels of O_2^- were determined by measuring the reduction of cytochrome c that is inhibited by SOD after the stimulation of macrophages with PMA (41). Phorbol ester-stimulated cells were studied as we were unable to reliably measure O_2^- production in resident non-stimulated cells (data not shown). In control incubated cells, there was a rapid increase in O_2^- production after the addition of PMA that steadily increased with time but began to slow after about 30 to 45 min (**Fig. 5**). Similarly, PMA also stimulated O_2^- production in IFN-γ pretreated cells; however, the rate of production continued to increase well after the control cell's production rate began to level off. After 1 h, the IFN-γ-treated cells generated

1.4 ± 0.1 times more O_2^- than control cells (mean ± SE of four experiments). These studies show that IFN-γ does not inhibit O_2^- production induced by PMA and, in fact, IFN-γ appears to stimulate the net amount produced. This suggests that other cellular processes are affected.

IFN-γ inhibits macrophage lipoxygenase activity

Cellular lipoxygenase activity has been shown to be susceptible to modulation by cytokines (35). Thus the effect of IFN-γ on this process was examined. To examine whether macrophage lipoxygenase is inhibited by IFN-γ under our experimental conditions, lipoxygenase activity of control and IFN-γ-treated macrophages was measured. Macrophage lipoxygenase activity was assessed by measuring the oxidation of [^{14}C]linoleic acid (23, 26). Control incubated macrophages converted linoleic acid to oxidized forms very efficiently (**Fig. 6**). Two major oxidation products were detected by autoradiography, one that comigrated with 13-HODE and a second minor product of unknown identity. In contrast, very little detectable oxidation products were generated in the absence of cells. The oxidation of linoleic acid was inhibited by pretreatment with ETYA, a known lipoxygenase inhibitor (89.6 ± 2.2% of control cells; mean ± SE of three experiments). Unlike the lipoxygenase inhibitor though, IFN-γ produced only a small decrease. This was the case whether lipoxygenase activity was assessed as the formation of either 13-HODE or the minor oxidized lipid product. Based on three different experiments, IFN-γ inhibited macrophage lipoxygenase activity by 21.3 ± 7.9% (mean ± SE). An inhibition of lipoxygenase activity is consistent with a role for this enzyme in the oxidation of LDL; however, as the magnitude of the inhibition was relatively small this suggested that other mechanisms may also be involved.

IFN-γ does not inhibit macrophage thiol production

Recent studies of Sparrow and Olszewski (25) have demonstrated that the cell-induced oxidation of LDL closely correlated with the appearance of thiols in the incubation medium; an inhibition of this process produced a comparable inhibition of LDL oxidation by both endothelial cells and macrophages. Thus it is possible that IFN-γ inhibits the oxidation of LDL by interfering with the generation of thiols. This was examined by measuring the net amount of thiols present in the medium harvested from macrophages after an 8-h incubation in the absence of LDL. Medium incubated in the absence of cells contained relatively low amounts of thiols (0.6 μM) whereas there was a substantial increase in thiol concentrations in medium harvested from control incubated macrophages (6.9 μM) (**Fig. 7**). In contrast to its effects on LDL oxidation, IFN-γ did not inhibit cellular thiol production but actually produced a slight increase (8.6 μM); in three separate experiments, there was an average increase of 29.3% ± 3.6 (mean ± SE). These data suggest that IFN-γ does not inhibit the ability of macrophages to oxidize LDL by inhibiting the generation of thiols in the medium.

Inhibition of macrophage-induced LDL oxidation in vivo

The above in vitro studies suggest that the oxidation of LDL by macrophages is susceptible to modulation by

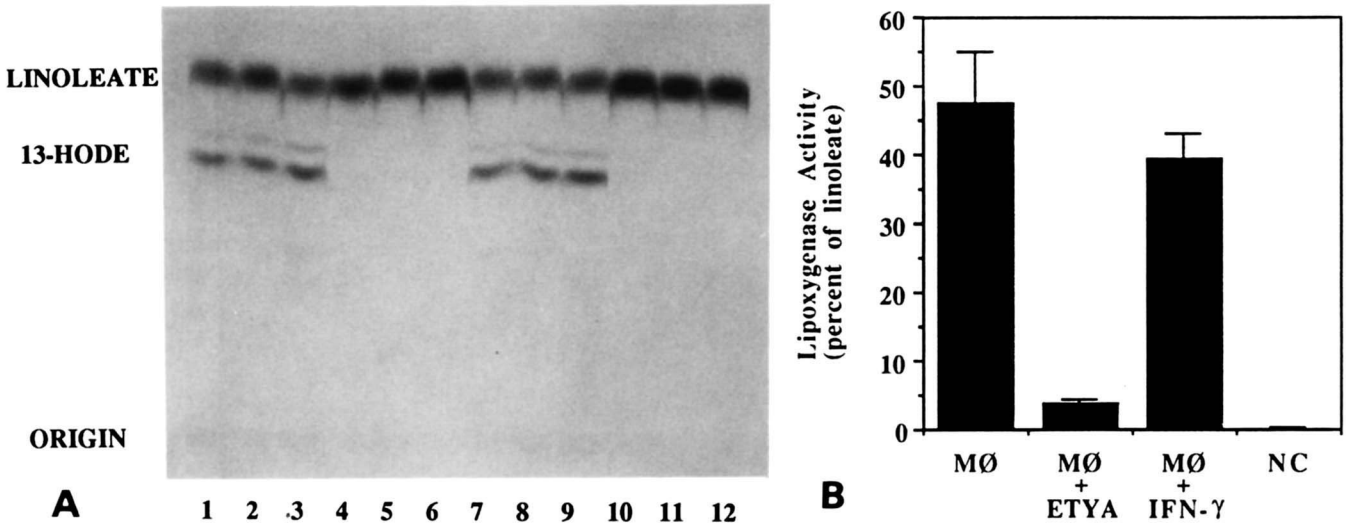

Fig. 6. Effect of IFN-γ on macrophage lipoxygenase activity. Macrophages (MØ) were preincubated in the presence or absence of IFN-γ (25 U/ml; 18 h) and then incubated with 20 μM [^{14}C]linoleate in F-10 medium at 37°C for 20 min. Linoleate that was incubated with macrophages pretreated with 10 μM ETYA for 1 h or incubated in the absence of cells (NC) were also included as controls. The incubation media were then extracted, separated by thin-layer chromatography, and the amount of oxidized linoleate was quantitated by liquid scintillation spectrometry as described in the Methods section. Panel A shows an autoradiogram of TLC analysis of incubation medium from control cells (lanes 1–3), ETYA-treated cells (lanes 4–6), IFN-γ-treated cells (lanes 7–9), and no-cell controls (lanes 10–12). The migration of linoleate and 13-HODE are indicated. Panel B shows the amount of oxidized linoleate in nmol as determined by liquid scintillation spectroscopy. The mean ± SD of a representative experiment is shown.

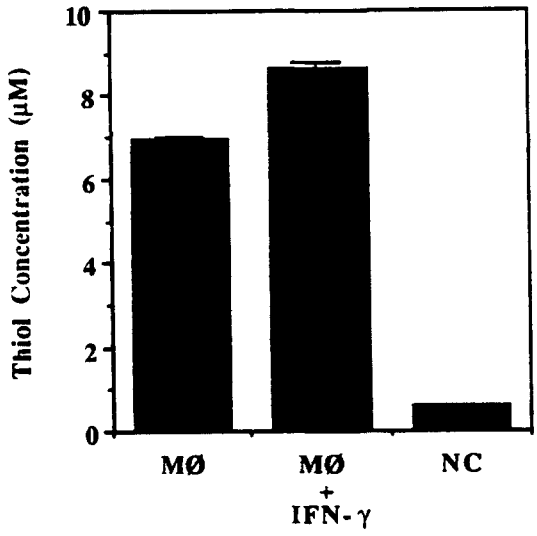

Fig. 7. Effect of IFN-γ on macrophage thiol production. Macrophages (MØ) were preincubated in the presence or absence of IFN-γ (25 U/ml) at 37°C for 18 h. The cells were washed and then incubated in F-10 medium at 37°C for 8 h in the absence of LDL. The concentration of thiols in the medium was then measured as described in the Methods sections. Medium harvested from wells containing no cells (NC) were included as a control. The mean ± SD of a representative experiment is shown. Not shown is that IFN-γ inhibited the macrophage-induced oxidation of LDL by 49%.

cytokines and that IFN-γ is a potent inhibitor of this cellular process. To determine whether the oxidation of LDL can similarly be modulated in vivo, macrophages were harvested from mice that were previously infected with the parasite *Toxoplasma gondii (T. gondii)*. Peritoneal macrophages from mice infected with this organism have been shown to be activated in situ by an IFN-γ-dependent mechanism; pretreatment of mice with an anti-IFN-γ monoclonal antibody prior to infection prevented their activation in vivo (49). Macrophages harvested from age-matched noninfected mice served as controls. The macrophages from the *T. gondii*-infected mice oxidized LDL to a much lower extent than control macrophages (**Table 2**). Although the extent of LDL oxidation varied among individual animals, in each of the three experiments the control macrophages oxidized LDL much more efficiently than cells from the infected mice. These studies demonstrate that the oxidation of LDL by cells is susceptible to modulation in vivo and suggests that cytokines are potentially physiologic relevant regulatory factors of this process.

DISCUSSION

There is now substantial evidence to support the hypothesis that LDL oxidation contributes to atherogenesis. Despite the potential pathologic importance of oxidized LDL to lesion formation, the processes that operate within the artery to control the extent of LDL oxidation are still unclear. Control mechanisms most likely operate as oxidized LDL is not evenly distributed throughout the artery wall. This would be expected as each of the major cell types within the artery wall is able to oxidize LDL without the apparent requirement for an exogenous stimuli other than LDL. One regulatory pathway that could account not only for the focal localization of oxidized LDL in the atherosclerotic lesion but also provide an efficient mechanism of control is the localized production and action of cytokines. These potent modifiers of cellular activities have already been demonstrated to be synthesized within the artery wall and in several cases their level is elevated in areas of atherogenesis (31–33). They have been shown to affect oxidative functions of cells (35–37) and, in fact, during the completion of these studies, Jessup and coworkers (36) have shown that the stimulation of nitric oxide by IFN-γ inhibits the conversion of LDL to a high uptake form and its oxidation by macrophages (37). Thus it is not unreasonable to suggest a role for cytokines in the regulation of cell-induced oxidation of LDL as it occurs within the artery wall. This hypothesis is supported by the in vitro and in vivo studies reported here.

The present studies have documented that the oxidation of LDL by cells is, in fact, susceptible to modulation by cytokines. Upon screening for biological activity with a mixture of cytokines from activated T-cells, an inhibition of macrophage-induced LDL oxidation was produced that was not related either to cell toxicity or nonspecific antioxidant activity (50). The inhibition of LDL oxidation was accompanied by an inhibition of apoB-100 modification, as measured by a smaller increase in net negative charge and a reduced ability to compete for the uptake and degradation of radiolabeled oxidized LDL. These findings are consistent with an inhibition of

TABLE 2. Macrophage from *T. gondii*-infected mice oxidize LDL to a smaller extent

Macrophage Group	LDL Oxidation
Control #1	13.7 ± 0.3
Control #2	33.9 ± 6.0
Control #3	16.9 ± 0.8
T. gondii #1	0.4 ± 0.8
T. gondii #2	7.3 ± 2.9
T. gondii #3	3.0 ± 1.1

Peritoneal macrophages were harvested from mice previously infected with the parasite *T. gondii*. The extent of LDL oxidation was then measured as described in Methods. The results from triplicate wells of macrophages for each of the six different animals tested (three control and three infected) are shown. The extent of LDL oxidation is expressed as the nmol amount of MDA equivalents per 8 h per mg cell protein. Values are the mean ± SD.

the oxidation-dependent modification of LDL. It was surprising, though, that IFN-γ could account for nearly all of the inhibitory activity in the HDK-1 activation supernatant. Interactions among different cytokines are commonplace; however, the inhibition produced did not appear to be dependent upon the activity of multiple cytokines. In contrast, the interaction of cytokines may account for the weaker inhibitory activity of the D-10 activation supernatant; activated D-10 cells secrete large amounts of IL-4 (45), a cytokine that has been shown to induce the synthesis of 15-lipoxygenase (15-LO) in monocytes (35), an enzyme thought to contribute to cell-mediated LDL oxidation.

The balance between prooxidant and antioxidant activities of a cell most likely determines its net ability to oxidize LDL. This predicts that the inhibition of LDL oxidation by IFN-γ could be due to either a direct inhibition of processes that stimulate the oxidation of LDL lipids or that enhance cellular processes that interfere with the oxidative modification of LDL or both. Depending upon the cell type, the cell-dependent oxidation of LDL is associated with either O_2^- production (17, 19, 22, 23), lipoxygenase activity (22–24), and/or thiol production (25). Thus, if IFN-γ were to inhibit LDL oxidation by modulating the prooxidant capability of macrophages, it would be expected that there would be an inhibition of one or more of these processes (19, 22, 23, 25). The activities of these were measured in IFN-γ-treated macrophages. IFN-γ pretreatment increased the rate of production of O_2^- by macrophages. Although basal levels of O_2^- production could not reliably be measured, the studies using phorbol ester-stimulated cells demonstrate that the cellular processes responsible for O_2^- production are intact and not inactivated. Similarly, IFN-γ activation also produced a slight increase in macrophage-dependent thiol production. Although these studies cannot distinguish between an actual stimulation in the release of cellular thiols or an inhibition of the oxidation of thiols present in the medium, the net amount of thiols in the medium was not decreased by IFN-γ, suggesting that other processes were affected. There was an inhibition of the basal activity of macrophage 15-LO. Using linoleic acid as a test substrate, there was an inhibition of its oxidation by 21%. An inhibition was not unexpected as IFN-γ has been shown to inhibit the synthesis of this enzyme in monocytes (35). Although the extent of the inhibition of lipoxygenase activity was not fully concordant with that of the inhibition of LDL oxidation (60%), this does not exclude this as a potential mechanism for the IFN-γ-induced inhibition. It is possible that IFN-γ inhibits the lipoxygenase-dependent pathway at multiple steps. In addition to reducing the activity of the enzyme, which is most likely due to a decrease in its synthesis (35), IFN-γ might also affect cellular processes that are important to the lipoxygenase-dependent oxidation of LDL, analogous to its multiple effects on the scavenger receptor pathway (39). As the individual steps that mediate the oxidation of LDL by 15-LO have not yet been established, it can only be speculated as to the potential sites that are affected. The oxidation of LDL by 15-LO is likely to depend upon either the direct oxidative modification of LDL lipids by 15-LO or to the transfer of cellular lipids oxidized by 15-LO from the cell to the LDL particle. This model suggests that the oxidation of LDL by 15-LO would depend upon the accessibility of the enzyme to the LDL particle or the physical interaction of LDL with cells. Thus, an IFN-γ-induced redistribution of 15-LO to a cellular site that is inaccessible to extracellular LDL or the inhibition of the association of LDL with the cells (i.e., reduced endocytosis or LDL receptor binding) and concomitant slowing of cellular lipid transfer would both produce an inhibition of the extent of LDL oxidation mediated by 15-LO. An inhibition of one of these or other intermediary processes could account for the discrepancy between the extent of inhibition of LDL oxidation and the inhibition of 15-LO activity. An alternative explanation is that IFN-γ inhibits the macrophage-mediated oxidation of LDL by more than one mechanism; besides an inhibition of the lipoxygenase pathway, there may also be a suppression of another prooxidative process or perhaps an enhancement of cellular antioxidant activities. Further studies will be required to investigate these possibilities.

The effects of IFN-γ on LDL oxidation predicts that this cytokine would be a potent inhibitor of this cellular function in vivo. This was tested using macrophages from *T. gondii*-infected mice. Suzuki and colleagues (49) have demonstrated that peritoneal macrophages harvested from mice that were previously infected with this parasite were activated in vivo by a mechanism that was dependent upon IFN-γ. Testing macrophages from mice that were similarly infected consistently showed that there was a reduced capacity to oxidize LDL. Based on these findings, it is attractive to speculate that this inhibition is due to the direct activation of macrophages by IFN-γ. However, as the activation process in vivo most likely proceeds through a series of steps, any of which might be dependent upon IFN-γ, it cannot be established with certainty at which stage IFN-γ is required. Considering the reduced rate of LDL oxidation by these activated macrophages, it is reasonable to suggest that cell-dependent LDL oxidation is susceptible to modulation in vivo and, considering our in vitro observations, that cytokines most likely participate in this regulatory process.

Previous studies have documented that both the activity of cellular oxidative processes and the susceptibility of LDL to oxidative attack (51–53) are important determinants that affect the extent of LDL oxidation. Thus it would be expected that the balance between these two parameters would determine the level of LDL oxidation within the artery wall. We present studies that suggest

that the localized production and action of cytokines might act as a regulatory pathway that might operate in vivo to influence the oxidative status of artery wall cells. Although it may be premature to suggest a role for cytokines, there is evidence to support the hypothesis that cytokines can modulate atherogenesis. Kuo and coworkers (54, 55) have shown that the administration of agents that stimulate interferon production or the administration of interferon directly both suppress the extent of lesion formation in the diet-induced rabbit model of atherosclerosis. The inhibitory activities of IFN-γ on macrophage LDL oxidation reported here and on macrophage scavenger receptor activity (39, 56) together may provide the basis for the anti-atherogenic properties of this cytokine.

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REFERENCES

1. Fowler, S., H. Shio, and N. J. Haley. 1979. Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations. *Lab. Invest.* **41:** 372–378.
2. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103:** 181–190.
3. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
4. Quinn, M. T., S. Parthasarathy, L. G. Fong, and D. Steinberg. 1987. Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc. Natl. Acad. Sci. USA.* **84:** 2995–2998.
5. Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. Gerrity, C. J. Schwartz, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* **87:** 5134–5138.
6. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1983. Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis.* **3:** 149–159.
7. Morel, D. W., P. E. DiCorleto, and G. M. Chisolm. 1984. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis.* **4:** 357–364.
8. Carew, T. E., D. C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA.* **84:** 7725–7729.
9. Kita, K., Y. Nagano, M. Yokode, K. Ishii, N. Kume, A. Ooshima, H. Yoshida, and C. Kawai. 1987. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **84:** 5928–5931.
10. Björkhem, I., A. Henriksson-Freyschuss, O. Breuer, U. Diczfalusy, L. Berglund, and P. Henriksson. 1991. The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arterioscler. Thromb.* **11:** 15–22.
11. Williams, R. J., J. M. Motteram, C. H. Sharp, and P. J. Gallagher. 1992. Dietary vitamin E and the attenuation of early lesion development in modified Watanabe rabbits. *Atherosclerosis.* **94:** 153–159.
12. Sparrow, C. P., T. W. Doebber, J. Olszewski, M. S. Wu, J. Ventre, K. A. Stevens, and Y-S. Chao. 1992. Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant N,N'-diphenyl-phenylenediamine. *J. Clin. Invest.* **89:** 1885–1891.
13. Haberland, M. E., D. Fong, and L. Cheng. 1988. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science.* **241:** 215–218.
14. Rosenfeld, M. E., W. Palinski, S. Ylä-Herttuala, S. Butler, and J. L. Witztum. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis.* **10:** 336–349.
15. Stender, S., and D. B. Zilversmit. 1981. Transfer of plasma lipoprotein components and of plasma proteins into aortas of cholesterol-fed rabbits. Molecular size as a determinant of plasma lipoprotein in flux. *Arteriosclerosis.* **1:** 38–49.
16. Schwenke, D. C., and T. E. Carew. 1989. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. *Arteriosclerosis.* **9:** 908–918.
17. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
18. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
19. Hiramatsu, K., H. Rosen, J. W. Heinecke, G. Wolfbauser, and A. Chait. 1987. Superoxide initiates oxidation of low density lipoprotein by human monocytes. *Arteriosclerosis.* **7:** 55–60.
20. Parthasarathy, S., D. J. Printz, D. Boyd, L. Joy, and D. Steinberg. 1986. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis.* **6:** 505–510.
21. Lamb, D. J., G. M. Wilkins, and D. S. Leake. 1992. The oxidative modification of low density lipoprotein by human lymphocytes. *Atherosclerosis.* **92:** 187–192.
22. Parthasarathy, S., E. Wieland, and D. Steinberg. 1989. A

role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc. Acad. Natl. Sci. USA.* **86:** 1046–1050.
23. Rankin, S. M., S. Parthasarathy, and D. Steinberg. 1991. Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages. *J. Lipid Res.* **32:** 449–456.
24. McNally, A. K., G. M. Chisolm III, D. W. Morel, and M. K. Cathcart. 1990. Activated human monocytes oxidize low-density lipoprotein by a lipoxygenase-dependent pathway. *J. Immunol.* **145:** 254–259.
25. Sparrow, C. P., and J. Olszewski. 1993. Cellular oxidation of low density lipoprotein is caused by thiol production in media containing transition metal ions. *J. Lipid Res.* **34:** 1219–1228.
26. Sparrow, C. P., and J. Olszewski. 1992. Cellular oxidative modification of low density lipoprotein does not require lipoxygenases. *Proc. Natl. Acad. Sci. USA.* **89:** 128–131.
27. Breugnot, C., C. Mazière, M. Auclair, L. Mora, M. F. Ronveaux, S. Salmon, R. Santus, P. Morliere, A. Lenaers, and J. C. Mazière. 1991. Calcium antagonists prevent monocyte and endothelial cell-induced modification of low density lipoproteins. *Free Rad. Res. Commun.* **15:** 91–100.
28. Mazière, C., M. Auclair, M-F. Ronveaux, S. Salmon, R. Santus, and J-C. Mazière. 1991. Estrogens inhibit copper and cell-mediated modification of low density lipoprotein. *Atherosclerosis.* **89:** 175–182.
29. Mazière, C., M. Auclair, and J-C. Mazière. 1992. Lipophilic β-blockers inhibit monocyte and endothelial cell-mediated modification of low density lipoproteins. *Biochim. Biophys. Acta.* **1126:** 314–318.
30. Barath, P., M. C. Fishbein, J. Cao, J. Berenson, R. H. Helfant, and J. S. Forrester. 1990. Tumor necrosis factor gene expression in human vascular intimal smooth muscle cells detected by in situ hybridization. *Am. J. Pathol.* **137:** 503–509.
31. Moyer, C. F., D. Sajuthi, H. Tulli, and J. K. Williams. 1991. Synthesis of IL-1 alpha and IL-1 beta by arterial cells in atherosclerosis. *Am. J. Pathol.* **138:** 951–960.
32. Rosenfeld, M. E., S. Ylä-Herttuala, B. A. Lipton, V. A. Ord, J. L. Witztum, and D. Steinberg. 1992. Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans. *Am. J. Pathol.* **140:** 291–300.
33. Ross, R., J. Masuda, E. W. Raines, A. M. Gown, S. Katsuda, M. Sasahara, T. Malden, H. Masuko, and H. Sato. 1990. Localization of PDGF-B protein in macrophages in all phases of atherogenesis. *Science.* **248:** 1009–1012.
34. Cotran, R. S. 1987. New roles for the endothelium in inflammation and immunity. *Am. J. Pathol.* **129:** 407–412.
35. Conrad, D. J., H. Kuhn, M. Mulkins, E. Highland, and E. Sigai. 1992. Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proc. Natl. Acad. Sci. USA.* **89:** 217–221.
36. Jessup, W., D. Mohr, S. P. Gieseg, R. T. Dean, and R. Stocker. 1992. The participation of nitric oxide in cell free- and its restriction of macrophage-mediated oxidation of low density lipoprotein. *Biochim. Biophys. Acta.* **1180:** 73–82.
37. Jessup, W., and R. T. Dean. 1993. Autoinhibition of murine macrophage-mediated oxidation of low-density lipoprotein by nitric oxide synthesis. *Atherosclerosis.* **101:** 145–155.
38. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
39. Fong, L. G., T. A. T. Fong, and A. D. Cooper. 1990. Inhibition of mouse macrophage degradation of acetyl-low density lipoprotein by interferon-γ. *J. Biol. Chem.* **265:** 11751–11760.
40. Fong, L. G., T. A. T. Fong, and A. D. Cooper. 1991. Inhibition of lipopolysaccharide-induced interleukin-1β mRNA expression in mouse macrophages by oxidized low density lipoprotein. *J. Lipid Res.* **32:** 1899–1910.
41. Johnston, R. B. 1981. Secretion of superoxide anion. *In* Methods for Studying Mononuclear Phagocytes. D. O. Adams, P. J. Edelson, and H. Koren, editors. Academic Press, New York, NY. 489–497.
42. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
43. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
44. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
45. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mossman. 1987. Two types of mouse helper T cell clone III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* **166:** 1229–1244.
46. Langermans, J. A. M., M. E. B. Van der Hulst, P. H. Nibbering, P. S. Hiemstra, L. Fransen, and R. Van Furth. 1992. IFN-γ-induced l-arginine-dependent toxoplasmastatic activity in murine peritoneal macrophages is mediated by endogenous tumor necrosis factor-α. *J. Immunol.* **148:** 568–574.
47. Langermans, J. A. M., M. E. B. Van der Hulst, P. H. Nibbering, and R. Van Furth. 1992. Endogenous tumor necrosis factor alpha is required for enhanced antimicrobial activity against *Toxoplasma gondii* and *Listeria monocytogenes* in recombinant gamma interferon-treated mice. *Infect. Immun.* **60:** 5107–5112.
48. Goldberg, M., L. S. Belkowski, and B. R. Bloom. 1989. Regulation of macrophage growth and antiviral activity by interferon-γ. *J. Cell Biol.* **109:** 1331–1340.
49. Suzuki, Y., M. A. Orellana, R. D. Schreiber, and J. S. Remington. 1988. Interferon-γ: the major mediator of resistance against *Toxoplasma gondii*. *Science.* **240:** 516–518.
50. Jessup, W., V. Darley-Usmar, V. O'Leary, and S. Bedwell. 1991. 5-Lipoxygenase is not essential in macrophage-mediated oxidation of low-density lipoprotein. *Biochem. J.* **278:** 163–169.
51. Parthasarathy, S., J. C. Khoo, E. Miller, J. Barnett, J. L. Witztum, and D. Steinberg. 1990. Low density lipoprotein rich in oleic acid is protected against oxidative modification: implications for dietary prevention of atherosclerosis. *Proc. Natl. Acad. Sci. USA.* **87:** 3894–3898.
52. Jialal, I., and S. M. Grundy. 1992. Effect of dietary supplementation with alpha-tocopherol on the oxidative modification of low density lipoprotein. *J. Lipid Res.* **33:** 899–906.
53. Camejo, G., E. Hurt-Camejo, B. Rosengren, O. Wiklund, F. López, and G. Bondjers. 1991. Modification of copper-catalyzed oxidation of low density lipoprotein by proteoglycans and glycosaminoglycans. *J. Lipid Res.* **32:** 1983–1991.
54. Kuo, P. T., A. C. Wilson, R. C. Goldstein, and R. G. Schaub. 1984. Suppression of experimental atherosclerosis in rabbits by interferon-inducing agents. *J. Am. Coll. Cardiol.* **3:** 129–134.
55. Wilson, A. C., R. G. Schaub, R. C. Goldstein, and P. T. Kuo. 1990. Suppression of aortic atherosclerosis in cholesterol-fed rabbits by purified rabbit interferon. *Arteriosclerosis.* **10:** 208–214.
56. Geng, Y-J., and G. K. Hansson. 1992. Interferon-γ inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. *J. Clin. Invest.* **89:** 1322–1330.