Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase

(interleukin 4/interferon γ /airway epithelium/eosinophil/atherosclerosis)

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ABSTRACT Arachidonate 15-lipoxygenase (arachidonate:oxygen 15-oxidoreductase, EC 1.13.11.33) is a lipid-peroxidating enzyme that is implicated in oxidizing low density lipoprotein to its atherogenic form. Monocyte/macrophage 15-lipoxygenase is present in human atherosclerotic lesions. To pursue a basis for induction of the enzyme, which is not present in blood monocytes, the ability of relevant cytokines to regulate its expression was investigated. Interleukin 4 (IL-4), among 16 factors tested, specifically induced 15-lipoxygenase mRNA and protein in cultured human monocytes. Interferon γ and hydrocortisone inhibited this induction. High-performance liquid chromatography analysis of lipid extracts from IL-4-treated monocytes detected 15-lipoxygenase products esterified to the cellular membrane lipids, indicating enzymatic action on endogenous substrates. Stimulation of IL-4-treated monocytes with calcium ionophore or opsonized zymosan A enhanced the formation of 15-lipoxygenase products. These data identify IL-4 and interferon γ as physiological regulators of lipoxygenase expression and suggest an important link between 15-lipoxygenase function and the immune/inflammatory response in atherosclerosis as well as other diseases.

Lipoxygenases are lipid-peroxidating enzymes that are implicated in the pathogenesis of a variety of inflammatory disorders such as arthritis, psoriasis, and asthma (1). The 5-lipoxygenase catalyzes the oxygenation of free arachidonic acid to its hydroperoxy derivative, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid—the first step in the biosynthesis of the potent inflammatory mediators leukotrienes (1, 2). The 12- and 15-lipoxygenases are involved in the biosynthesis of other bioactive metabolites from free arachidonic acid, such as lipoxins (1). However, 15-lipoxygenase is unique among the human lipoxygenases in that it is capable of oxygenating polyenoic fatty acids esterified to membrane lipids (3) or lipoproteins (H.K., unpublished observation) and hence it may have biological roles distinct from its action on free arachidonic acid.

15-Lipoxygenase has been implicated in the pathogenesis of atherosclerosis. Purified lipoxygenases can convert low density lipoprotein (LDL) to its atherogenic, oxidized form *in vitro* (4), and the oxidation of LDL by endothelial cells and macrophages can be prevented by lipoxygenase inhibitors (5, 6). Moreover, 15-lipoxygenase colocalizes with oxidized LDL in human and rabbit atherosclerotic lesions (7, 8). The finding that 15-lipoxygenase is expressed in macrophages of human atherosclerotic lesions but not in the precursor cell, the monocyte, nor in related cells such as alveolar macrophages (9, 10) prompted us to search for relevant molecules that may account for the disease-specific induction of the enzyme. Because cytokines have been implicated in the cellular response seen in atherosclerosis (11, 12), we exam-

ined the effect on 15-lipoxygenase expression of different cytokines produced by cells present in atherosclerotic lesions.

In this report, we identify interleukin 4 (IL-4) and interferon γ (IFN- γ) as two inflammatory cytokines that potently and specifically regulate the expression of human monocyte 15-lipoxygenase. In addition, we document that the induction of this enzyme coincides with the appearance of lipoxygenase products in the membrane ester lipids. These results have implications for the role of the 15-lipoxygenase in a variety of inflammatory disorders.

MATERIALS AND METHODS

Materials. Cytokines were obtained from Upstate Biotechnical, Lake Placid, NY [IL-4, IFN- γ , and tumor necrosis factor α (TNF- α)], Genzyme [IL-3, IL-5, IL-6, granulocyte/macrophage colony-stimulating factor (GM-CSF), and monocyte colony-stimulating factor (M-CSF)], the Biological Mediator Response Program: (IL-1 α , IL-1 β , IL-2), Boehringer Mannheim [platelet-derived growth factor BB (PDGF-BB)], and Sigma (transforming growth factor β_1 (TGF- β_1)). All cytokines were of human origin except IL-5, which was available only in murine form. Cu²⁺-treated oxidized LDL was kindly provided by Sampath Parthasarathy, University of California, San Diego.

Cell Purification and Culture. Mononuclear cells were isolated from human buffy coats by density centrifugation using Ficoll/Hypaque cushions. The mononuclear cell band was removed; washed by centrifugation with Hanks' balanced salt solution; resuspended (20×10^6 cells per ml) in RPMI 1640 medium with 10% human AB serum, L-glutamine, and antibiotics; and allowed to adhere to 150-mm polystyrene tissue culture dishes for 2 hr at 37°C. After the removal of nonadherent cells, the adherent monocytes were harvested by gentle scraping, washed, and resuspended (2×10^6 cells per ml) in the culture medium (RPMI 1640 with 10% fetal bovine serum, L-glutamine, and antibiotics). More than 90% of the cells stained positive for nonspecific esterase and the typical preparation had a viability of >90% by trypan blue exclusion. The monocytes were incubated for 3 days with or without cytokines as indicated. In some experiments, cells were further purified by fluorescence-activated cell sorting techniques. Briefly, the monocytes were incubated at 4°C for 30 min with a fluorescein-conjugated mouse monoclonal antibody, anti-CD14 (anti-Leu-M3, Becton Dickinson) diluted 1:10 with RPMI 1640 containing 10% fetal bovine serum. The monocytes were then washed once in the same

Abbreviations: 15-HETE, (5Z,8Z,11Z,13E)-15-hydroxy-5,8,11,13-eicosatetraenoic acid; 13-HODE, (9Z,11E)-13-hydroxy-9,11-octadecadienoic acid; 12-HETE, (5Z,8Z,10E,14Z)-12-hydroxy-5,8,10,14-eicosatetraenoic acid; LDL, low density lipoprotein; IL-4, interleukin 4; IFN- γ , interferon γ ; PMA, phorbol 12-myristate 13-acetate.

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medium and resuspended at a concentration of 10^6 cells per ml in phosphate-buffered saline containing propidium iodide (50 μ g/ml) for 5 min. Cells were sorted using a FACStar flow cytometer (Becton Dickinson) on the basis of forward and side light scatter, propidium exclusion, and expression of CD14. Of the cells collected, 97% were positive for CD14 expression. After 3 days of IL-4 treatment, the monocytes were analyzed by the immunohistochemical protocol described below using a phycoerythrin-conjugated mouse anti-rabbit second antibody (Tago).

Immunofluorescence. Expression of 15-lipoxygenase was assessed by indirect immunofluorescence (13) using a rabbit polyclonal antibody (1:100 dilution) to human recombinant 15-lipoxygenase (14). Immunofluorescence preparations were examined after applying a drop of Dabco (1,4-diazabicyclo[2.2.2]octane, Aldrich) fluorescence enhancer.

Cell Stimulation and Lipid Extraction. Cellular capacity for oxygenation of exogenous substrate was tested by incubating cells with arachidonic acid dissolved in ethanol (0.5%). Cells were harvested by gentle scraping, resuspended in phosphate-buffered saline (2×10^6 cells per ml), and incubated with arachidonic acid (160 μ M) at 37°C for 15 min. Cell suspensions were extracted with 1 volume of 2-propanol containing 1.2% (vol/vol) acetic acid (pH 3.5) and 1 volume of chloroform. Prostaglandin B₂ was used as an internal standard for all extractions.

To investigate the oxygenation of endogenous substrates by 15-lipoxygenase, IL-4-treated monocytes were incubated overnight with 37 MBq of [14 C]linoleic acid and 37 MBq of [14 C]arachidonic acid in RPMI 1640 supplemented with 1% fetal bovine serum, L-glutamine, 25 mM Hepes, and antibiotics. Typically >95% of the cell-associated radioactivity was incorporated into cellular ester lipids as assessed by HPLC analysis of polyenoic fatty acids before and after alkaline hydrolysis. The radiolabeled cells were then washed and replated in the same medium but containing IL-4 (700 pM). After 3 days, cells were either harvested without stimulation or stimulated by addition of opsonized zymosan A (2 mg/ml) or calcium ionophore A23187 (2–10 μ M) to the culture dish and incubated for 30 min at 37°C. The cells were scraped and resuspended in 1 ml of phosphate-buffered saline, and then triphenyl phosphite was added to reduce hydroperoxy lipids to their corresponding hydroxy compounds. After acidification to pH 3, prostaglandin B₂ was added as an internal standard. The lipids were extracted either from the cell suspension or from the cell pellet and supernatant separately and analyzed for lipoxygenase products before and after alkaline hydrolysis in methanolic 1 M KOH (30 min at 60°C under argon atmosphere). The recovery of the internal standard varied between 60% and 80%. Without stimulation, the cell viability assessed by trypan blue exclusion was >90% ($n = 3$). After stimulation by zymosan A (2 mg/ml) or ionophore A23187, the viability varied between 77% and 90% ($n = 2$) or between 86% and 93% ($n = 3$), respectively.

HPLC of 15-Lipoxygenase Products. Extracts were reconstituted in chromatography solvent and analyzed on a Waters 840 HPLC system connected to a Waters 490 multiwavelength detector. Reverse-phase HPLC was performed as described (14). For chiral-phase HPLC, products that were coeluted with hydroxy fatty acid standards were methylated with diazomethane and repurified by reversed-phase HPLC. Chiral-phase HPLC was performed with a Rexchrom Pirkle type A column [250 mm $\times$ 5 mm; ionically linked (*R*)-dinitrobenzoyl phenylglycine as chiral phase; 5- μ m particle size; Regis, Morton Grove, IL] using a solvent system of hexane/2-propanol (100:0.5, vol/vol) and a flow rate of 0.7 ml/min. Racemic standards of (5Z,8Z,10E,14Z)-12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and (5Z,8Z,11Z,13E)-15-hydroxy-5,8,11,13-eicosatetraenoic

acid (15-HETE) were prepared by vitamin E-controlled autoxidation of methyl arachidonate (15). Straight-phase HPLC was performed on a Mikrosorb SI 80 column (120 $\times$ 4.6 mm; 3- μ m particle size; Rainin, Woburn, MA) using an isocratic solvent system of hexane/2-propanol/acetic acid (100:0.5:0.05) with a flow rate of 1 ml/min. The retention times for the standards in straight-phase HPLC are indicated by arrows in Fig. 4B.

RNA Blots. Monocytes were purified as above and cultured with and without IL-4 and with both IL-4 and IFN- γ . After 3 days, the cells were harvested and poly(A)⁺ RNA was prepared (16) and analyzed by Northern hybridization (17). Rabbit reticulocyte total RNA was used as a standard (18). The RNA blot was probed with 32 P-labeled human 15-lipoxygenase cDNA (19) or human γ -actin cDNA. Both probes were labeled to 10^8 cpm/ μ g of DNA. The RNA blot was hybridized in 50% formamide at 42°C, washed in 0.15 M NaCl/0.015 M sodium citrate, pH 7/0.1% SDS for 30 min at 65°C and exposed to Kodak XAR film for 21 hr at -70°C.

RESULTS

Freshly isolated human monocytes (>90% pure) were incubated with various cytokines for 3 days and screened for specific immunofluorescence with an antibody to human recombinant 15-lipoxygenase. These cytokines (Table 1) were selected because of their relevance to atherogenesis and monocyte/macrophage function. Monocytes cultured with IL-4 for 3 days reproducibly exhibited specific immunofluorescence, whereas freshly isolated monocytes and monocytes cultured without IL-4 exhibited no fluorescence (Fig. 1). The positive staining of IL-4-treated monocytes could be specifically inhibited by preincubating the 15-lipoxygenase antibody with recombinant human 15-lipoxygenase prior to immunostaining, and nonimmune antiserum yielded no immunofluorescence. The induction of 15-lipoxygenase was detected with IL-4 concentrations as low as 60 pM and was near maximal at 700 pM. In time-course experiments, 15-lipoxygenase immunofluorescence was detected at 12 hr and was near maximal at 2 days of culture. To determine whether

Table 1. Regulation of monocyte 15-lipoxygenase expression

Factor	Immunofluorescence	
	Alone	With IL-4
Cytokines		
IL-1 α (130 pM)	—	+
IL-1 β (110 pM)	—	+
IL-2 (2.5 nM)	—	+
IL-3 (1000 units/ml)	—	+
IL-4 (60 pM)	+	+
IL-5 (200 pM)	—	+
IL-6 (1000 units/ml)	—	+
IFN- γ (100 pM)	—	—
GM-CSF (30 units/ml)	—	+
M-CSF (100 units/ml)	—	+
PDGF-BB (3.5 nM)	—	+
TNF- α (100 nM)	—	+
TGF- β ₁ (100 pM)	—	+
Oxidized LDL (10 μ g/ml)	—	+
PMA (50 nM)	—	—
Hydrocortisone (100 nM)	—	—

Regulation of monocyte 15-lipoxygenase by specific cytokines (abbreviations defined in *Materials*), oxidized LDL, phorbol 12-myristate 13-acetate (PMA), and hydrocortisone. Monocytes were cultured for 3 days and stimulated by each factor listed either alone or with IL-4 (700 pM). The doses were selected for their ability to induce relevant biologic effects in other cell systems. Cultures were assessed by indirect immunofluorescence. The results summarize at least two independent cultures per factor.

FIG. 1. Induction of 15-lipoxygenase in IL-4-treated human monocytes/macrophages. Human monocytes were cultured for 3 days in the presence (Right) or absence (Left) of IL-4 (700 pM) and assayed for 15-lipoxygenase expression by indirect immunofluorescence. No immunofluorescence was detected in freshly isolated monocytes (data not shown).

the induction was a direct effect of IL-4 on monocytes, cells were purified by fluorescence-activated cell sorting using an antibody to the monocyte surface marker CD14 and then was incubated with IL-4. These highly purified CD14-positive monocytes (97% pure) exhibited similar levels of IL-4-dependent 15-lipoxygenase immunofluorescence.

The IL-4 induction of 15-lipoxygenase was specific among 16 factors tested including 13 cytokines, Cu^{2+} -oxidized LDL, hydrocortisone, and PMA (Table 1). This specificity was also demonstrated in monocytes allowed to differentiate in culture for 7 days prior to treatment. Because preliminary experiments using a mixture of cytokines suggested that some compounds may have been inhibiting the IL-4-dependent induction of 15-lipoxygenase, individual agents were re-screened in the presence of IL-4. IFN- γ was identified as the only cytokine capable of inhibiting the induction of 15-lipoxygenase (Table 1). This inhibition was dose-dependent in the range of IFN- γ concentrations tested (10–700 pM) and was maximal at 100 pM. PMA (50 nM) and hydrocortisone (100 nM) also inhibited the IL-4 response.

Immunoblots of whole cell extracts of IL-4-treated monocytes exhibited a single immunoreactive band at ≈ 70 kDa that comigrated with authentic 15-lipoxygenase prepared from human leukocytes (data not shown). Incubation of IL-4-treated monocytes with exogenous arachidonic acid led to the formation of the expected products, 15(*S*)-HETE and 12(*S*)-HETE, in a ratio of 10:1 as determined by reverse- and chiral-phase HPLC (Fig. 2) (14). Untreated monocytes and monocytes treated with both IL-4 and IFN- γ yielded no lipoxygenase products. RNA blots hybridized with a radio-labeled, full-length cDNA for 15-lipoxygenase detected the typical RNA hybridization signal for human 15-lipoxygenase in poly(A)⁺ RNA prepared from IL-4-treated monocytes (Fig. 3). The upper band in lane 3 is of unknown significance but is characteristic of RNA blots of human leukocyte 15-lipoxygenase (data not shown). No hybridization was seen in RNA prepared from freshly isolated monocytes, untreated monocytes, or monocytes treated with both IL-4 and IFN- γ , suggesting that IL-4 induces transcriptional activation of the 15-lipoxygenase gene and that IFN- γ inhibits this activation.

Further experiments were performed to investigate the intracellular action of the induced monocyte 15-lipoxygenase. Freshly isolated monocytes were prelabeled with [^{14}C]arachidonic and [^{14}C]linoleic acid and the extracts of the cellular lipids were analyzed (before and after hydrolysis)

FIG. 2. Metabolism of exogenous arachidonic acid by IL-4-treated monocytes. 15-Lipoxygenase activity was assessed by measuring the formation of 15-HETE from exogenous arachidonic acid (160 μM) by reverse-phase HPLC. Chromatograms are shown for assays of cultured monocytes, monocytes cultured in the presence of IL-4 (7.00 pM), and monocytes cultured in the presence of both IL-4 (700 pM) and IFN- γ (100 pM). Each incubation contained 2×10^6 cells. The IL-4-treated cells formed ≈ 500 ng of 15-HETE per 10^6 cells. Retention times of authentic standards are indicated. (Inset) Chiral-phase HPLC of the enantiomers of 12- and 15-HETE. Upper trace shows racemic standards and lower trace shows products formed by IL-4-treated monocytes.

for 15-lipoxygenase metabolites, such as 15(*S*)-HETE and 13(*S*)-hydroxy-9,11-octadecadienoic acid [13(*S*)-HODE]. 15-Lipoxygenase products were not detected in the absence of IL-4 (data not shown) or in the nonhydrolyzed lipid extracts

FIG. 3. Northern blot analysis of monocyte 15-lipoxygenase induction by IL-4. (A) Poly(A)⁺ RNA was prepared from freshly isolated monocytes, cultured monocytes, monocytes cultured in the presence of IL-4 (700 pM), and monocytes cultured in the presence of both IL-4 (700 pM) and IFN- γ (100 pM). Each lane contained 1 μg of poly(A)⁺ RNA except the standard lane, which contained rabbit reticulocyte total RNA (8 μg). Positions of 28S and 18S rRNA are indicated at left and 15-lipoxygenase transcripts are indicated at right. (B) The same blot was probed with γ -actin cDNA.

of IL-4-treated monocytes (Fig. 4A). In contrast, the hydrolyzed lipid extracts of IL-4-treated cells contained two major radiolabeled compounds that migrated in the hydroxy fatty acid region (Fig. 4A). The early eluted product (retention time, 36 min) was identified as 15(*S*)-HETE by straight-phase and chiral-phase HPLC (Fig. 4B). In some experiments a second compound (retention time, 40 min) was detected. Although this peak migrated in the region of 5-HETE on reverse-phase HPLC, straight-phase HPLC analysis did not confirm the presence of 5-HETE, and thus its chemical structure remains unclear.

The amount of 15-HETE formed by IL-4-treated cells varied from 0.1% to 2% of the radioactivity eluted with arachidonic acid ($n = 9$). In each case, however, stimulation of IL-4-treated cells with either ionophore A23187 (2 μ M) or opsonized zymosan A (2 mg/ml) significantly increased the amount of 15-HETE and 13-HODE esterified to the cellular membranes. With these stimuli, 15-HETE was also detected in the nonhydrolyzed extracts, indicating release and metabolism of free arachidonic acid. Finally, the predominant lipoxygenase product formed under all conditions tested was 15(*S*)-HETE. Only small amounts of 13-HODE were recovered despite the fact that equal amounts of [1- 14 C]arachidonic acid and [1- 14 C]linoleic acid were used for labeling the cells. However, HPLC analysis of the 14 C-labeled polyenoic fatty acids indicated an arachidonic acid/linoleic acid ratio of about 5:1, suggesting that arachidonic acid was more efficiently incorporated into the cells. In addition, it might be possible that exogenous linoleic acid is metabolized via alternative pathways or converted to arachidonic acid.

DISCUSSION

Our results associate specific inflammatory cytokines with the regulation of the lipid-peroxidating enzyme 15-lipoxygenase in the human monocyte/macrophage. The induction of monocyte 15-lipoxygenase by IL-4 coincides with the appearance of lipoxygenase products such as 15(*S*)-HETE in the membrane lipids. Because the precursor to 15-HETE is a highly reactive hydroperoxy compound that

may rapidly decompose via radical-mediated reactions, these experiments define an enzymatic pathway of radical formation with implications for host defense. In addition, these data document changes in membrane composition that occur due to 15-lipoxygenase.

While the significance of the observed membrane remodeling is not entirely clear, it has been shown that exogenous 15-HETE can be reincorporated into the membrane (20, 21) in such a way that it structurally alters the second messenger diacylglycerol, which is released during cell signaling (21). Alternatively, mammalian 15-lipoxygenases are capable of directly oxygenating phospholipids (22) and biomembranes (3). This oxygenation of membrane lipids is proposed to initiate the degradation of intracellular organelles during red-cell maturation (23, 24). Hence monocyte 15-lipoxygenase may be recruited to degrade intracellular organelles during differentiation or to digest extracellular particles during phagocytosis. Interestingly, IL-4 has been shown to induce monocytes to differentiate into multinucleated giant cells, a process that requires fusion and remodeling of cellular membranes (25). Furthermore, IL-4 has been shown to induce another lipid-metabolizing enzyme, the cytotoxic-T-cell lipase, which is capable of enhancing the cytotoxicity of this subpopulation of murine T cells (26).

The oxidative modification of LDL has been proposed as a key event in the development of early atherosclerotic lesions (27). 15-Lipoxygenase could contribute to the oxidative modification of LDL through the production of intracellular hydroperoxy lipids, which could then initiate the extracellular oxidation of LDL. Our results suggest that the first step in this process would be the specific enzymatic oxygenation of free fatty acids or esterified membrane lipids by activated monocyte/macrophages. In this scenario, radical intermediates produced during this reaction could be transferred outside the cell to LDL, where they would initiate a nonenzymatic oxidation of LDL.

Whether IL-4 or IFN- γ regulates 15-lipoxygenase expression in atherosclerosis is not known. However, activated T cells, a major cell source for IL-4, have been localized to atherosclerotic lesions (28). In addition, IFN- γ has been

FIG. 4. Metabolism of endogenous lipids in IL-4-treated monocytes. Chromatograms are shown for extracts of human monocyte suspensions (6×10^6 cells; viability 96%) radiolabeled with [1- 14 C]arachidonic and [1- 14 C]linoleic acid and cultured in the presence of IL-4. (A) Reverse-phase HPLC of hydrolyzed and nonhydrolyzed lipid extracts of monocytes suspension. (B) Straight-phase HPLC of hydroxy fatty acids of the hydrolyzed lipid extracts after reverse-phase HPLC purification. The compounds eluted between 32 min and 42 min in A were collected and analyzed by straight-phase HPLC. Retention times of standards are indicated. (Inset) Chiral-phase HPLC. The 15-HETE fraction of B was mixed with a molar excess of racemic 15-HETE. The mixture was methylated, repurified by straight-phase HPLC, and analyzed on a chiral-phase column.

shown to inhibit the size and extent of atherosclerotic lesions in hypercholesterolemic rabbits (29). The results presented here imply that IFN- γ may inhibit foam-cell formation, in part, by decreasing the formation of oxygenated lipids by 15-lipoxygenase. While the role of IL-4 and IFN- γ in determining 15-lipoxygenase expression in atherosclerosis remains to be established, the hypothesis can now be tested *in vivo* and such experiments will potentially delineate new therapeutic interventions.

The specific, potent induction of 15-lipoxygenase by IL-4 offers insight into the lipoxygenase reaction in inflammatory disorders. IL-4 is a highly pleiotropic cytokine produced by the T_{H2} subset of T helper lymphocytes as well as mast cells (30). The T_{H2} cytokine response, characterized by high levels of IL-4 and low levels of IFN- γ , has been increasingly associated with allergic or immediate hypersensitivity responses. IL-4 is responsible for the class switching that results in increases in serum IgE (31), and IL-4 is known to increase expression of class II major histocompatibility molecules as well as Fc receptors for IgE (32, 33). Transgenic mice that overexpress IL-4 develop complex inflammatory lesions characteristic of human allergic disorders (34). Because IL-4 receptors are expressed along the airway epithelium in human lung in a distribution identical to that of 15-lipoxygenase (35), we suggest that the IL-4 regulation of 15-lipoxygenase may be a general mechanism and that the epithelial 15-lipoxygenase may serve a proinflammatory function in human airway. Further study of the interrelationship of IL-4, IFN- γ , and the lipoxygenase reaction is expected to increase our understanding of the immune/inflammatory response in a variety of disorders, including bronchial asthma and atherosclerosis.

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