Inhibition of Monocyte Chemotaxis to C-C Chemokines by Antisense Oligonucleotide for Cytosolic Phospholipase A<sub>2</sub>*

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Monocyte chemotactic protein (MCP)-1, a member of the C-C (or β) branch of the chemokine superfamily, at chemotactic concentrations, induced a rapid release of [³H]arachidonic acid but not of [¹⁴C]oleic acid from pre-labeled human monocytes. This effect was associated with an increase in the intensity of the immunoreactive band corresponding to the phosphorylated form of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>). To address the role of cPLA<sub>2</sub> in the induction of monocyte chemotaxis, cells were treated with a specific antisense oligonucleotide. Monocytes cultured in the presence of 10 μM antisense oligonucleotide for 48 h showed a marked decrease (57 ± 5%, n = 4) of cPLA<sub>2</sub> expression, as evaluated by Western blot analysis and a nearly complete inhibition (81.8 ± 4.2%, n = 3) of [³H]arachidonic acid release in MCP-1-stimulated cells. Monocyte chemotaxis in response to MCP-1 also was inhibited in a concentration-dependent manner by cPLA<sub>2</sub> antisense oligonucleotide (IC<sub>50</sub> = 1.9 ± 1.1 μM; n = 3), with complete inhibition observed between 3 and 10 μM. No inhibition of chemotactic response was observed in monocytes treated with a control oligonucleotide. Monocyte migration in response to MCP-3, RANTES (regulated on activation normal T cells expressed and secreted), and MIP-1α/LD78 was also inhibited (>70%) in antisense oligonucleotide-treated cells. On the contrary, the chemotactic response elicited by formyl-methionyl-leucyl-phenylalanine and C5a, two classical chemotactic agonists, was minimally affected (<20%) by antisense oligonucleotide treatment. These data show that cPLA<sub>2</sub> plays a major role in [³H]arachidonic acid release by MCP-1 in human monocytes and provide direct evidence for the involvement of cPLA<sub>2</sub> in C-C chemokine-induced monocyte chemotaxis.

The recruitment of leukocytes from the blood compartment to the site of inflammation represents one of the characteristic elements of the inflammatory process (1). Locally produced chemotactic agonists are believed to play a crucial role in the “multistep paradigm” of leukocyte accumulation in tissues (2, 3).

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The abbreviations used are: fMLP, formyl-methionyl-leucyl-phenylalanine; MCP, monocyte chemotactic protein; MIP-1α/LD78, macrophage inflammatory protein-1α; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; cPLA<sub>2</sub>, cytosolic PLA<sub>2</sub>; PBS, phosphate-buffered saline; FCS, fetal calf serum; RANTES, regulated on activation normal T cells expressed and secreted.
ity for arachidonic acid and that translocates to the membrane fraction by a Ca$^{2+}$ (nonomparp-dependent) mechanism upon receptor stimulation (25-27); and a Ca$^{2+}$-independent ATP-regulated cytosolic PL$\alpha_2$ that does not show a preference for the fatty acid at the sn-2 position (28). Because of the lack of specific inhibitors, the relative contribution of these enzymes in arachidonic acid metabolism in human monocytes is still uncertain.

In this paper we report that MCP-1-stimulated monocytes selectively released $[^3H]$arachidonic acid; no detectable release of $[^14C]$oleic acid was observed. This effect paralleled the phosphorylation of cPLA$2$ evaluated by Western blot analysis. In addition, by the use of a specific antisense oligonucleotide, we show that cPLA$2$ plays a crucial role in the chemotactic response of human monocytes to C-C chemokines.

**EXPERIMENTAL PROCEDURES**

**Chemotacticants—**Human recombinant MCP-1 and RANTES were from PeproTech Inc. (Rocky Hill, NJ). Human recombinant MCP-3 and Ip-10 were kindly donated by Dr. L. Marshall (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) and Dr. J. D. Clark (Genetics Institute Inc., Cambridge, MA), respectively. Human recombinant C5a was a generous gift from Dr. H. S. Showell (Pfizer Central Res., Groton, CT). Recombinant products were endotoxin free as assessed by Limulus Amebocyte Lysate assay (Bio Whittaker, Walkersville, MD). IMLP and platelet-activating factor were from Sigma.

**Human Monocytes Purification—**Human monocytes were obtained from buffy coats of normal blood donors through the courtesy of Centro Transfusionale Ospedale Sacco (Milan, Italy) and Centro Transfusionale Ospedale Caduti Bollate (Bollate, Italy) as described previously (18). To reduce platelet contamination, monocytes were isolated according to the procedure described by Pawlowski et al. (29) with minor modifications (18). Briefly, anticoagulated whole blood was diluted 1:4 with cold phosphate-buffered isotonic saline without Ca$^{2+}$ and Mg$^{2+}$ (PBS; Life Technologies, Grand Island, NY) and centrifuged at 800 $g$ for 5 min to allow sedimentation of platelets. The supernatant and platelet rich plasma were then removed and monocytes were isolated by density centrifugation on Ficoll (Becton Dickinson, Heidelberg, Germany) for 40 min at 4°C at a density of 1.077 to 1.080 g/mL. Mononuclear cells were resuspended in PBS containing 0.3 mM sodium orthovanadate, 200 $M$ NaF) and disrupted by sonication. Sonicated cells were centrifuged at 800 $g$ for 10 min, and the supernatants were further centrifuged at 150,000 $g$ at 4°C for 1 h. Supernatants and pellets obtained with this procedure were used as cytosol and membrane fractions, respectively (33).

**Release of Labeled Fatty Acids—**Monocytes ($10^9$/ml) were labeled with $[^3H]$arachidonic acid (200 Ci/mmole) and/or $[^14C]$oleic acid (60 Ci/mmole) (Amersham) during the last 18 h of oligonucleotide treatment. Incubation did not affect cell viability ($>95\%$, by trypan blue dye exclusion) nor the ability of monocytes to respond to MCP-1. At the end of the incubation, cells were washed twice and resuspended in RPMI 1640 medium supplemented with 0.2% fatty acid free bovine serum albumin (Sigma). Monocytes ($10^6$/ml) were prewarmed at 37°C for 5 min and then stimulated. The reaction was terminated by the addition of 2 ml of chloroform/methanol/ formic acid (1:2:0, v/v/v) followed by agitation. Cell extracts were transferred to centrifuge tubes, and 1 ml of water and 2 ml of chloroform were added. Chromatographic separation of lipids was performed by evaporating the organic phase under a stream of nitrogen, redissolving the residue in chloroform, and loading the extract on silica gel G plates (Merck). Fatty acids were separated by thin layer chromatography using hexane/ethyl ether/formic acid (15:10:1, v/v/v) as a solvent system. The positions of standards were detected by exposure to iodine vapors. The amounts of fatty acids were determined by a specific instrumental assay exactly as described previously (18, 30). 27-cholesterol was determined by a specific instrumental assay exactly as described previously (31).

**Enzyme-linked Immunosorbent Assay for MCP-1—**Monocyte migration was evaluated using a microchamber technique (34) as described previously (18, 30). A $\mu$l of chemotacticant diluted in RPMI 1640 medium with 1% FCS were seeded in the lower compartment of the chemotaxis chamber (Nucleopore Corp., Pleasanton, CA), and 50 $\mu$l of cell suspension ($1.5 \times 10^6$/ml) were seeded in the upper compartment. The two compartments were separated by a 5- $\mu$m pore size PVP membrane (Nucleopore). Chambers were incubated at 37°C in air with 5% CO$_2$, for 90 min. At the end of the incubation, filters were removed, fixed, and stained with Diff-Quik (Baxter s.p.a., Rome, Italy). Migrated monocytes in five high power oil immersion fields were counted.

**RESULTS**

**Effect of MCP-1 on the Release of $[^3H]$Arachidonic Acid and $[^14C]$Oleic Acid from Labeled Human Monocytes—**Initial studies were designed to examine the selectivity of MCP-1-induced PL$\alpha_2$ activity for arachidonic acid. Monocytes cultured in nonadherent conditions in the presence of both $[^3H]$arachidonic acid and $[^14C]$oleic acid for 18 h showed a comparable uptake of the two labels (47.4 $\pm$ 2.5 and 46.9 $\pm$ 1.9, respectively; n = 4) with more than 90% of the labels incorporated in the phospholipid pools (data not shown). Distribution of the labels in phospholipids was: 60 $\pm$ 3.6 and 72 $\pm$ 2.6% phosphatidylincholine, 11
Maximal chemotactic activity of MCP-1 is observed at 50–100 ng/ml (6–12 nM; 30) and these concentrations were used throughout this study. As shown in Fig. 1, 100 ng/ml MCP-1 induced a rapid accumulation of [3H]arachidonic acid that peaked between 3 and 10 min and, at 3 min, corresponded to 196 ± 18% (n = 4) of control group activity (Fig. 1). MCP-1 activation appeared to be specific for arachidonic acid because no detectable release of [14C]oleic acid was observed up to 30 min of stimulation. In the same experimental conditions, 10−7 M fMLP for 3 min resulted in the release of 577 ± 128% and 163 ± 11% of control cells for [3H]arachidonic acid and [14C]oleic acid, respectively (n = 4).

Effect of MCP-1 on cPLA2 Phosphorylation—Agonist-triggered cPLA2 activation, including that mediated by seven transmembrane domain receptors, is associated with increased phosphorylation of the protein on serine residues resulting in a stable 3–4-fold increase of cPLA2 catalytic activity (36–39). cPLA2 phosphorylation correlates with the appearance of a more slowly migrating electrophoretic form of the protein. In agreement with previous reports (40, 41), Western blot analysis of resting human monocytes shows that in these cells cPLA2 migrates as a doublet (Fig. 2). Three min of stimulation with 100 ng/ml MCP-1 caused a decrease of the intensity of the faster migrating band and an increase in the intensity of the slower migrating species. Similar results were obtained with fMLP (Fig. 2) and with MCP-3 (data not shown). A short (1 min) preincubation of monocytes with 100 nM platelet-activating factor, before MCP-1 stimulation, resulted in the complete loss of the faster migrating band (Fig. 2). Three min of stimulation with 100 ng/ml MCP-1 caused a decrease of the intensity of the faster migrating band and an increase in the intensity of the slower migrating species. Similar results were obtained with fMLP (Fig. 2) and with MCP-3 (data not shown). A short (1 min) preincubation of monocytes with 100 nM platelet-activating factor, before MCP-1 stimulation, resulted in the complete loss of the faster migrating band (Fig. 2). Three min of stimulation with 100 ng/ml MCP-1 caused a decrease of the intensity of the faster migrating band and an increase in the intensity of the slower migrating species. Similar results were obtained with fMLP (Fig. 2) and with MCP-3 (data not shown). A short (1 min) preincubation of monocytes with 100 nM platelet-activating factor, before MCP-1 stimulation, resulted in the complete loss of the faster migrating band (Fig. 2).
that a shift in the ratio of cPLA_2_ immunoreactive bands was present in both cytosolic and membrane fractions. The effect was time-dependent, detectable 1 min after stimulation, reaching statistical significance in both cytosol and membrane between 3 and 5 min and declining to basal levels thereafter (data not shown).

Inhibition of cPLA_2_ Expression by Antisense Oligonucleotide—Because of the lack of specific inhibitors, it is difficult to correlate cell functions to activation of different PLA_2_ forms. Antisense technology provides a unique approach to this problem and was successfully used in monocytic cells (42, 43) also to inhibit PLA_2_ isoforms (23, 43). Table I reports that monocytes treated with 10 \( \mu \text{M} \) cPLA_2_ antisense oligonucleotide for 48 h showed a marked decrease of the immunoreactive bands detected by quantitative Western blot, when compared with cultured untreated cells or with monocytes exposed to a similar concentration of control oligonucleotide in the same experimental conditions. Table I also shows that [\( ^{3} \text{H} \) arachidonic acid release was almost completely blocked in antisense oligonucleotide-treated monocytes challenged with 100 ng/ml MCP-1. Inhibition was not due to a toxic effect of the treatment because cell viability was higher than 90% (data not shown), and inhibition was not the result of homologous desensitization by MCP-1 released in the culture medium, because at the end of the incubation MCP-1 levels in untreated, antisense, and control oligonucleotide-treated cultures were similar (0.37 ± 0.05, 0.44 ± 0.15, 0.44 ± 0.15, and 0.39 ± 0.12 ng/ml, respectively; \( n = 3, p > 0.05 \) of oligonucleotide versus untreated groups).

Effect of cPLA_2_ Antisense Oligonucleotide on Chemotaxis—The effect of cPLA_2_ antisense oligonucleotide treatment on monocyte chemotactic response to MCP-1 was investigated. fMLP was used as reference chemoattractant. Fig. 4 shows that antisense oligonucleotide treatment (0.3–3 \( \mu \text{M} \) for 48 h) did not significantly alter the spontaneous migration of monocytes when compared with cells treated with control oligonucleotide or to untreated cultured monocytes. On the contrary, the number of cells migrated across polycarbonate filters in response to 50 ng/ml of MCP-1 was inhibited in a concentration-dependent manner (IC_{50} = 1.9 ± 1.1 \( \mu \text{M} \); \( n = 3 \), with complete inhibition observed between 3 and 10 \( \mu \text{M} \) antisense oligonucleotide (Fig. 4, upper panel, and data not shown). Control oligonucleotide did not affect basal or activated cell migration. Inhibition of MCP-1 response could partially be overcome by the use of higher concentrations of the agonist (Fig. 5A). In parallel experiments, the same monocyte population migrated normally in response to both optimal (10^{-8} \( \mu \text{M} \)) and suboptimal (10^{-9})

10^{-10} \( \mu \text{M} \) chemotactic concentrations of fMLP, indicating that inhibition was not the result of toxicity (Figs. 4, lower panel, and 5B).

Effect of cPLA_2_ Antisense Oligonucleotide on Chemotaxis to C-C Chemokines and C5a—Because of the discrepancy of effect of cPLA_2_ antisense oligonucleotide treatment on monocyte chemotaxis to MCP-1 and fMLP, other C-C chemokines and a second classical chemoattractant were tested in the chemotaxis assay. All the agonists were used at their optimal chemotactic concentrations. In the same experimental condi-
cPLA2 Antisense Oligonucleotide Inhibits Monocyte Chemotaxis

The release of arachidonic acid and the production of eicosanoids is an early event in the activation of phagocytic cells by several inflammatory agonists including chemotactic factors (14, 44, 45). PLA2 activation represents the most direct and the main mechanism of arachidonic acid release from the sn-2 position of membrane phospholipids. Thus, activation of PLA2 is the rate-limiting step in arachidonic acid mobilization (21, 22, 46).

In the present study we report that chemotactic concentrations of MCP-1, a prototypic C-C chemokine, induced [3H]arachidonic acid release and phosphorylation of cPLA2 in a time-dependent manner (Figs. 1–3). Similar results (not shown) were obtained with MCP-3, another member of the C-C chemokine family that shows 72% homology to (8) and shares binding sites (19, 47) with MCP-1, in human monocytes. The kinetics of cPLA2 phosphorylation after MCP-1 and MCP-3 stimulation were fast and correlated with arachidonic acid release from labeled monocytes. Both release and phosphorylation were already detectable 1 min after stimulation, peaked between 3 and 10 min, and returned to baseline within the following 10 min (Refs. 18 and 19 and Figs. 1 and 2). cPLA2 is a 85-kDa protein that preferentially hydrolyzes phospholipids containing arachidonic acid at the 2 position and that was recently purified and cloned from the cytosol of myelomonocytic cell lines (25–27, 48–50). Ca2+ is not required for cPLA2 catalytic activity (51, 52), but nanomolar concentrations of Ca2+ are needed for interfacial association with the lipid bilayer (25, 26). In ionophore-permeabilized human monocytes it was shown that maximal arachidonic acid release by MCP-1 was observed in the presence of 300–700 nM free Ca2+ concentration (18). These concentrations are compatible with MCP-1-activated intracellular Ca2+ levels in monocytes (30, 53) and with the calcium concentrations required for cPLA2 membrane association (25, 26). In the experimental conditions used, MCP-1 did not release oleic acid from labeled monocytes, suggesting that the activated phospholipase(s) is specific for arachidonic acid-labeled phospholipid pools (Fig. 1). Finally, cPLA2 antisense oligonucleotide-treated monocytes released only a minute fraction (20% of control oligonucleotide-treated cells) of [3H]arachidonic acid when challenged with MCP-1 (Table I). Taken together, these data indicate that cPLA2 plays a major role in the mobilization of arachidonic acid in MCP-1-stimulated monocytes.

Monocytes treated with a specific antisense oligonucleotide were used to address the role of cPLA2 in the induction of monocyte chemotaxis by C-C chemokines. In 19 of 21 experiments performed with different monocyte cultures, a nearly 2 M. Locati and S. Sozzani, unpublished results.

**Fig. 5.** MCP-1 and fMLP dose-responses for monocyte chemotaxis. Human monocytes, obtained as detailed under "Experimental Procedures," were cultured in the absence or in the presence of 10 μM antisense or control oligonucleotides for 48 h. Cells were then washed, resuspended (1.5 × 106/ml) in RPMI 1640 medium in the presence of 1% FCS, and tested for their ability to migrate across a polycarbonate filter in response to different concentrations of MCP-1 or fMLP. One experiment performed in triplicate, representative of three similar experiments, is shown. The results are expressed as the number of migrated monocytes in five high power oil immersion microscopic fields.

**Fig. 6.** Effect of cPLA2 antisense oligonucleotide on monocyte chemotaxis to C-C chemokines and C5a. Human monocytes, obtained as detailed under "Experimental Procedures," were cultured in the absence or in the presence of 10 μM antisense or control oligonucleotides for 48 h. Cells were then washed, resuspended (1.5 × 106/ml) in RPMI 1640 medium in the presence of 1% FCS, and tested for their ability to migrate across a polycarbonate filter in response to an optimal concentration of chemokines, C5a or fMLP. The results are expressed as the percentage of inhibition of chemotactic response of cells treated with the antisense oligonucleotide at the net of basal migration (49 ± 10; n = 24). Chemotactic response of cells treated with control oligonucleotide at the net of basal migration (49 ± 9) to each agonist was assumed as 100% (93 ± 17, n = 18, 50 ng/ml MCP-1; 93 ± 23, n = 5, 50 ng/ml MCP-3; 82 ± 25, n = 3, 50 ng/ml MIP-1α/LD78; 70 ± 8, n = 3, 100 ng/ml RANTES; 122 ± 24, n = 7, 50 ng/ml C5a; 135 ± 24, n = 21, 10−6 M fMLP). The results are the average numbers (± S.E.) of multiple experiments (see above) performed with different monocyte cultures each one in triplicate. The results obtained with chemokines were statistically different (p < 0.01, by paired Student’s t test) from the control group (see “Results”).
complete inhibition of cell migration was observed (>85%) in response to an optimal concentration of MCP-1 (Figs. 4 and 5). Inhibition by the antisense oligonucleotide was concentration-dependent and -specific, because it was not observed in cells treated with a control oligonucleotide (Figs. 4 and 5) or with a c-myb-specific antisense oligonucleotide (data not shown). Inhibition of chemotactic response was not caused by toxicity of the treatment because: (i) cell viability was always higher than 90% by trypan blue dye exclusion, and treated monocytes were similar to untreated cells in terms of morphology (data not shown) and spontaneous migration (Fig. 4); (ii) cells exposed to control oligonucleotides showed a normal migration to MCP-1 (Fig. 4); and (iii) cPLA2 antisense oligonucleotide-treated cells migrated normally to fMLP (Figs. 4, 5 and 6). Finally, inhibition was not caused by homologous receptor desensitization (8) because comparable levels of MCP-1 were present in the supernatants of untreated and control or antisense oligonucleotide-treated cells.

A more extensive analysis showed that monocyte chemotaxis to all the C-C chemokines tested was strongly (>70%) inhibited by the antisense oligonucleotide treatment, whereas monocyte migration to fMLP or to C5a was only minimally (<20%) affected (Fig. 6). Thus, according to their requirement for cPLA2, it is possible to divide the chemotactic agonists tested in two groups, a first one, highly sensitive to the action of the antisense oligonucleotide that includes all the C-C chemokines investigated, and a second one that was poorly sensitive to this treatment and that comprises classical chemotactic factors. At the moment, the reason for this difference is unknown. It is possible that fMLP and C5a but not chemokine receptors might have access to the surviving cPLA2 molecules. fMLP and C5a receptors could also induce the required levels of free arachidonic acid through the activation of other types of PLA2 that are not efficiently coupled to chemokine receptors. Alternatively, fMLP and C5a receptors could bypass cPLA2 inhibition through the stronger activation of signaling pathways alternative to arachidonic acid mobilization. A similar hypothesis can be formulated to explain the ability of supraoptimal concentrations of MCP-1 to overcome oligonucleotide inhibition. The optimal chemotactic concentration (50 ng/ml MCP-1) is similar to or less than the Ka value of MCP-1 receptors (47, 54). A higher degree of receptor occupancy could activate residual cPLA2 or trigger alternative signaling pathways.

A direct role for arachidonic acid and its metabolites in cell movement was recently suggested in different cell types. Both 5-lipoxygenase and cyclooxygenase products were found to regulate epidermal growth factor-induced actin remodeling in A431 cells (55) and neutrophil migration in vivo (56). cPLA2-mediated arachidonic acid release was found to be required for basic fibroblast growth factor-stimulated migration of endothelial cells (57). A direct role for arachidonic acid in monocyte and macrophage adherence, expression of adhesion molecules, and chemotaxis was suggested (58–60). Recently, three chemotactic factors for phagocytic cells: macrophage colony-stimulating factor (40, 41), transforming growth factor-β (61), and fMLP (62) were shown to activate cPLA2 in human monocytes, elicited guinea pig macrophages, and human neutrophils, respectively.

In a previous study we found a strict correlation between C-C chemokine-induced arachidonic acid release and monocyte migration (18). In the present study, we show that cPLA2 appears to be the main effector enzyme for chemokine-elicited arachidonic acid release in human monocytes. In addition, by the use of specific antisense oligonucleotides, we provide evidence that arachidonic acid by itself or through its metabolites is strictly implicated in the induction of monocyte migration to C-C chemokines.
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