Ceramide 1-phosphate induces macrophage chemoattractant protein-1 release: involvement in ceramide 1-phosphate-stimulated cell migration

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Arana L, Ordoñez M, Ouro A, Rivera I-G, Gangoiti P, Trueba M, Gomez-Muñoz A. Ceramide 1-phosphate induces macrophage chemoattractant protein-1 release: involvement in ceramide 1-phosphate-stimulated cell migration. Am J Physiol Endocrinol Metab 304:E1213–E1226, 2013. First published April 2, 2013; doi:10.1152/ajpendo.00480.2012.—The bioactive sphingolipid ceramide 1-phosphate (C1P) is implicated in inflammatory responses and was recently shown to promote cell migration. However, the mechanisms involved in these actions are poorly described. Using J774A.1 macrophages, we have now discovered a new biological activity of C1P: stimulation of monocyte chemoattractant protein-1 (MCP-1) release. This novel effect of C1P was pertussis toxin (PTX) sensitive, suggesting the intervention of G_{i} protein-coupled receptors. Treatment of the macrophages with C1P caused activation of the phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase kinase kinase (MEK)/extracellularly regulated kinases (ERK), and p38 pathways. Inhibition of these kinases using selective inhibitors or specific siRNA blocked the stimulation of MCP-1 release by C1P. C1P stimulated nuclear factor-κB activity, and blockade of this transcription factor also resulted in complete inhibition of MCP-1 release. Also, C1P stimulated MCP-1 release and cell migration in human THP-1 monocytes and 3T3-L1 preadipocytes. A key observation was that sequestration of MCP-1 with a neutralizing antibody or treatment with MCP-1 siRNA abolished C1P-stimulated MCP-1 release completely. Also, inhibition of the pathways involved in C1P-stimulated MCP-1 release completely blocked the stimulation of cell migration by C1P. It can be concluded that C1P promotes MCP-1 release in different cell types and that this chemokine is a major mediator of C1P-stimulated cell migration. The PI3K/Akt, MEK/ERK, and p38 pathways are important downstream effectors in this action.

ceramide 1-phosphate; monocyte chemoattractant protein-1 release; macrophage migration; sphingosine 1-phosphate

REGULATION OF CELL MIGRATION is a complex process involving hundreds of molecules. It is necessary for tissue homeostasis and is crucial for regulation of vital biological processes including embryogenesis, organogenesis, or regeneration (reviewed in Refs. 38, 48). Also, cell migration is fundamental to inflammatory responses (1, 49), but inadequate migratory signals may induce the migration of the wrong cell type to the wrong place, which may have severe effects in the organism. Some examples include autoimmune syndromes, angiogenesis, or the process of metastasis. Although cell migration is a subject of intense investigation, the mechanisms involved in controlling cell movements are incompletely understood. In this connection, growing evidence suggests that some sphingolipids are key metabolites for controlling chemotaxis (52). Our group recently reported that ceramide 1-phosphate (C1P), a sphingolipid metabolite that is present in serum or plasma (25), and which we found to regulate cell growth and survival (15, 17, 18, 20, 22), is a chemoattractant molecule for macrophages (23). This finding is particularly relevant when considering that macrophages play critical roles in chronic and acute inflammation and that these cells facilitate cancer cell migration (21, 46). In addition, Barth et al. (4) recently showed that ceramide kinase, the enzyme responsible for the phosphorylation of ceramide to C1P, regulates NADPH oxidase activity and eicosanoid biosynthesis in neuroblastoma cells, an action that is consistent with our recent finding that C1P increases the production of reactive oxygen species through activation of NADPH oxidase in macrophages (2). Besides these proinflammatory actions, increasing evidence suggests that C1P can also function as an anti-inflammatory agent under different experimental settings. Specifically, Hankins et al. (26) recently reported that C1P reduces lipopolysaccharide (LPS)-mediated nuclear factor-κB (NF-κB) activation and cytokine secretion, and Goldsmith et al. (16) reported that phosphoceramide analog-1 simultaneously suppresses TNFα and induces the production of anti-inflammatory interleukin-10 in activated macrophages.

The most important chemokine that regulates the migration and infiltration of monocytes/macrophages and works as a key factor in initiating the various inflammatory responses is monocyte chemoattractant protein-1 [MCP-1, also known as chemo- kine (C-C motif) ligand 2 (CCL2)] (11). MCP-1-induced migration of monocytes is mediated through interaction with its receptor CCR2, a G_{i} protein-coupled receptor (10). There are two subtypes of MCP-1 receptors, CCR2a and -b, with the CCR2b receptor isofrom being about fivefold more sensitive to induction of chemotaxis by MCP-1 than CCR2a (50). Furthermore, monocytes and activated NK cells express predominantly the CCR2b isofrom (10).

The present study was undertaken to examine whether C1P could stimulate the release of MCP-1 by macrophages and to assess whether this cytokine mediates the chemotactic effect of C1P in macrophages.

MATERIALS AND METHODS

Materials. N-hexadecanoyl-D-erythrosphingosine-1-phosphate (C16:0-ceramide 1-phosphate; C1P) was supplied by Matreya. Culture medium Dulbecco’s modified Eagle’s medium (DMEM) and RPMI (Roswell Park Memorial Institute) were from Lonza. Fibronectin, JE (MCP-1) from mouse, RS-102895, LY-294002, PD-98059, pertussis toxin (PTX), and SP-600125 were from Sigma-Aldrich. Fetal bovine serum (FBS) and opti-MEM were from GIBCO. Nitrocellulose mem-
branes, protein markers, and BCA assay reagents were purchased from Bio-Rad. C1P-1 neutralizing antibody (cell tested) was purchased from ebioscience. β-Actin, GAPDH, sphingosine 1-phosphate receptor (S1PR2; EDG-5), and S1PR4 (EDG-6) antibodies were from Santa Cruz Biotechnology. S1PR1, S1PR3, and S1PR5 antibodies were supplied by Abcam. Other antibodies were from Cell Signaling. Mouse CCL2 (MCP-1) ELISA Ready-SET-Go! was supplied by ebioscience. Oligofectamine reagent was from Molecular Probes (Invitrogen). SC-514 and 10-DEBC were from Tocris. Akt1 siRNA, Mapk1 (ERK2) siRNA, Pim3rl (PI3K) siRNA, Mapk8 (JNK1) siRNA, Mapk9 (JNK2) siRNA, Mapk10 (JNK3) siRNA, Mapk14 (p38α) siRNA, S1PR1 (slpr1) siRNA, S1PR3 (slpr3) siRNA and S1PR4 (slpr4) siRNA were from Applied Biosystems (Ambion) and Ckr-2 siRNA, negative siRNA, S1PR2 (EDG-5) siRNA, S1PR5 (EDG-8) siRNA, and MCP-1 siRNA were supplied by Santa Cruz Biotechnology. All of the other chemicals and reagents were of the highest grade available.

**Cell culture.** The J774A.1, THP-1, and 3T3-L1 cell lines used in this work were purchased from ATCC (Manassas, VA) and were cultured following the manufacturer’s indications. J774A.1 macrophages were grown in DMEM supplemented with 10% heat-inactivated FBS, 50 mg/l gentamicin, and 200 μM L-glutamine. Cells were incubated at constant temperature (37°C) in a humidified atmosphere containing 5% CO2. The THP-1 cells were grown in RPMI supplemented with 10% heat-inactivated FBS, 25 mg/l gentamicin, and 200 μM L-glutamine. Cells were incubated at constant temperature (37°C) in a humidified atmosphere containing 5% CO2. The 3T3-L1 cell line was grown in DMEM supplemented with 10% heat-inactivated FCS, 4.5 g/l D-glucose, 50 mg/l gentamicin, and 200 μM L-glutamine. Cells were incubated at constant temperature (37°C) in a humidified atmosphere containing 5% CO2. Bone marrow-derived macrophages (BMDM) were isolated from femurs of 6- to 8-wk-old female CD-1 mice as described (24). Cells were plated for 24 h in RPMI 1640 medium containing 10% FBS and 10% L cell conditioned medium as the source of macrophage colony-stimulating factor (MCSF) (27). The nonadherent cells were removed and cultured for 4–6 days in the same medium until ~80% confluence was reached.

**Delivery of CIP to cells in culture.** An aqueous dispersion (in the form of liposomes) of C1P was added to cultured macrophages as previously described (15, 19, 20). Specifically, stock solutions were prepared by sonicating C1P (5 mg) in sterile nanopure water (3 ml) on ice using a probe sonicator until a clear dispersion was obtained. The final concentration of C1P in the stock solution was ~2.62 mM. This procedure is considered preferable to dispersions prepared by adding C1P in organic solvents, because droplet formation is minimized and exposure of cells to alcohols or dodecane is avoided.

**Measurement of MCP-1 concentration.** J774A.1 macrophages were seeded in 24-well plates (2 × 10⁴ cells/well) and incubated in 0.525 ml of DMEM containing 10% FBS overnight. The next day, the cells were washed twice with PBS, and the medium was replaced by fresh DMEM containing 1% FBS. Macrophages were incubated for two additional hours, and agonists were then added as required. After incubation, the cell media were collected into microcentrifuge tubes, and cells were scrapped for counting. The 3T3-L1 preadipocytes were seeded in 24-well plates (10⁵ cells/well) and incubated in DMEM containing 10% FCS overnight. The next day, the cells were washed twice with PBS, and the medium was replaced by fresh DMEM in the absence of FCS. Cells were incubated for two additional hours, and agonists were then added as required. Then, the media were collected, and cells were trypsinized for counting. 3T3-L1 cells were seeded in 24-well plates (10⁵ cells/well) and incubated in 0.5 ml of RPMI. After 2 h, agonists were added as required. Then, cell media along with monocytes were collected into microcentrifuge tubes, and cells were counted. The harvested media were centrifuged at 10,000 g for 5 min at 4°C, and supernatants were diluted for determination of MCP-1 concentration. The amount of MCP-1 in the medium was determined using a Mouse CCL2 (MCP-1) ELISA Ready-SET-Go! kit according to the manufacturer’s instructions. Sample concentration values (pg/ml) obtained by the calibration curve were normalized by the number of cells counted in each well or by the amount of protein in each well.

**Determination of cell migration.** Cell migration was measured using a Boyden chamber-based cell migration assay, as described (23). Twenty-four-well chemotaxis chambers (Transwell, Corning Costar) precoated with 30 μg of fibronectin were used for the experiments. For J774A.1, 3T3-L1, and BMDM cell migration studies, 8 μm pore size was used, and for THP-1 cells the pore size was 5 μm. Cell suspensions (100 μl, 5 × 10⁴ cells for J774A.1; 100 μl, 5 × 10⁵ cells for 3T3-L1; 1 × 10⁶ cells for BMDM; and 100 μl, 10⁵ cells for THP-1) were then added to the upper wells of 24-well chemotaxis chambers. Agonists were added to the lower wells diluted in 300 μl of medium supplemented with 0.2% fatty acid-free bovine serum albumin (BSA) and 0.2% FBS treated with activated carbon. When used, inhibitors were added to the upper and lower wells and...
Fig. 2. The PI3K/Akt pathway is involved in C1P-stimulated MCP-1 release. Cells were seeded and treated as in Fig. 1. A: cells were preincubated with 1 μM LY-294002 (a selective PI3K inhibitor; filled bars) or with vehicle (open bars) for 30 min prior to stimulation with 20 μM C1P. Cells were then incubated further for 24 h, and MCP-1 concentration was measured by ELISA, as indicated in MATERIALS AND METHODS. MCP-1 values were normalized to total cell number, and results are expressed as means ± SE of 5 independent experiments performed in duplicate. B: cells were preincubated with 1 μM 10-DEBC (a selective Akt inhibitor; filled bars) or with vehicle (open bars) for 30 min and then treated with 20 μM C1P for 24 h. MCP-1 concentration was determined by ELISA as indicated in MATERIALS AND METHODS. Values were normalized to total cell number, and results are expressed as means ± SE of 5 independent experiments performed in duplicate. C: cells were seeded in 60-mm dishes (2 × 10^5 cells/dish) and treated with vehicle (open bars), negative siRNA (filled bars), or PI3K siRNA (hatched bars), as described in MATERIALS AND METHODS. Vehicle (control) or 20 μM C1P was then added for 24 h, as indicated. Cells were then scraped and treated as in A. MCP-1 concentration was determined by ELISA and normalized to total cell number. D: cells were incubated and treated as in C, except that siRNA Akt1 was used instead of PI3K siRNA. Data are expressed as means ± SE of 4 independent experiments performed in duplicate. E: PI3K siRNA inhibitory efficiency was confirmed by Western blotting using specific antibodies against PI3K. Equal loading of protein was monitored using a specific antibody to GAPDH. Similar results were obtained in each of 2 independent experiments. F: results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are means ± range of 2 independent experiments. G: Akt1 siRNA inhibitory efficiency was confirmed by Western blotting using specific antibodies against Akt1. Equal loading of protein was monitored using a specific antibody to GAPDH. Similar results were obtained in each of 2 independent experiments. H: results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are means ± range of 2 independent experiments. (n.s., not significant; *P < 0.05, **P < 0.01).
body at 1:4,000 dilution for 1 h. Thereafter, the proteins were visualized by enhanced chemiluminescence.

Statistical analyses. Results are expressed as means ± SE of three to six independent experiments performed in triplicate unless indicated otherwise. Confirmation for siRNA inhibition of each particular siRNA-targeted protein is expressed as the mean ± range of two independent experiments. Statistical analyses were performed using the two-tailed, paired Student’s t-test, where \( P < 0.05 \) was considered to be significant (GraphPad Prism software, San Diego, CA).

RESULTS

C1P stimulates MCP-1 release in J774A.1 macrophages.

Leukocyte chemotaxis toward sites of inflammation or tumorigenesis is primarily mediated by chemokine signaling (37), and we (23) have demonstrated that C1P stimulates macrophage migration. A major chemokine that regulates the migration and infiltration of macrophages is MCP-1 (37). Figure 1 shows that C1P significantly stimulated the release of MCP-1 by J774A.1 macrophages in a concentration-dependent (Fig. 1A) and time-dependent (Fig. 1B) manner. Optimal MCP-1 release was attained at 20 \( \mu \text{M} \) C1P after 24 h of incubation. A similar fold increase of MCP-1 release was still observed after 48 h of incubation with 20 \( \mu \text{M} \) C1P (Fig. 1B). Although this optimal concentration of C1P is relatively high compared with plasma levels (0.1 \( \mu \text{M} \) to 0.5 \( \mu \text{M} \)), it was shown that C1P concentrations vary according to the nutritional state of the organism and can be secreted by macrophages (5, 25) or by leaky damaged cells (29), so that local concentrations of C1P in vivo can be much higher than 0.5 \( \mu \text{M} \). In addition, it was reported that ATP at physiological concentrations (0.1 mM) can elevate intracellular MCP-1 production by macrophages, suggesting that C1P can be a potent stimulator of MCP-1 production under physiological conditions.

Western blotting. Cells were seeded in 60-mm diameter plates (2 \( \times 10^5 \) cells/well) in DMEM containing 10% FBS and incubated overnight. The next day, the cells were washed, and medium was replaced by 1.6 ml of opti-MEM containing 20% FBS was then added to the wells without removal of the transfection mixture. The cells were incubated further for 24 h, and the medium was then replaced by fresh DMEM containing 10% FBS. Cells were scrapped and counted and then used for experiments.

Treatment of cells with siRNA. siRNA transfection protocols were performed following the manufacturer’s instructions. Cells were seeded in 60-mm diameter plates (2 \( \times 10^5 \) cells/well) in DMEM containing 10% FBS. The medium was replaced by 1.6 ml of opti-MEM, and cells were then incubated for 24 h. siRNA (20 pmol/ml) in 0.4 ml of opti-MEM was added into each well. Cells were then incubated for 4–5 h, and 2 ml of opti-MEM containing 20% FBS was then added to the wells without removal of the transfection mixture. siRNA-targeted protein is expressed as the mean ± range of two independent experiments. Statistical analyses were performed using the two-tailed, paired Student’s t-test, where \( P < 0.05 \) was considered to be significant (GraphPad Prism software, San Diego, CA).

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Fig. 4. p38 MAPK is implicated in C1P-stimulated MCP-1 release. A: macrophages were seeded and treated as in Fig. 1. Cells were preincubated with vehicle (open bars) or with 1 μM SB 202190 (filled bars) for 30 min prior to stimulation with 20 μM C1P for 24 h, as indicated. MCP-1 was measured by ELISA and normalized to total cell number. Results are means ± SE of 3 independent experiments performed in duplicate. B: cells were seeded in 60-mm dishes (2 × 10⁵ cells/dish) and treated with vehicle (open bars), negative siRNA (filled bars), or p38α siRNA (hatched bars), as described in MATERIALS AND METHODS. Cells were then incubated with vehicle (Control) or with 20 μM C1P for 24 h, as indicated. MCP-1 concentration was normalized, and data are expressed as means ± SE of 5 independent experiments performed in duplicate. C: p38α siRNA inhibitory efficiency was confirmed by Western blotting using specific antibodies against p38α. Equal loading of protein was monitored using a specific antibody to GAPDH. Similar results were obtained in each of 2 independent experiments. D: results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are means ± range of 2 independent experiments (*P < 0.05).

Fig. 5. JNK is not implicated in C1P-stimulated MCP-1 release. J774A.1 cells were seeded in 60-mm dishes (2 × 10⁵ cells/dish), and siRNA treatment to silence JNK1 (A), JNK2 (B), and JNK3 (C) was performed as described in MATERIALS AND METHODS. Cells were seeded and treated with vehicle (Control) or with 20 μM C1P for 24 h, as indicated. MCP-1 concentration was normalized, and data are expressed as means ± SE of 5 independent experiments performed in duplicate. siRNA inhibitory efficiency was confirmed by Western blotting using specific antibodies against JNK1 (D), JNK2 (E), and JNK3 (F). Equal loading of protein was monitored using a specific antibody to GAPDH. Similar results were obtained in each of 2 independent experiments. Exposed films were scanned, and data obtained by densitometry are expressed as arbitrary units of intensity (JNK1 G, JNK2 H, and JNK3 I) and are means ± range of 2 independent experiments.
C1P up to ~4–5 nmol per million cells (32). So, if this amount of C1P were released into the extracellular milieu, local concentrations of ~5–10 μM, or even higher, would be easily achievable right after secretion. Also, in this work, C1P was added to the cells in the form of liposomes (sonicated in water), and so the actual concentration of C1P in touch with the cells was much lower than what was added to the culture medium. Therefore, the effective concentrations of C1P used in these experiments can be considered to be within or very close to physiological levels.

C1P-stimulated MCP-1 release is mediated by the PI3K/Akt (PKB), MEK/ERK1/2, and p38 pathways. We showed previously that C1P was able to stimulate PI3K/Akt (PKB) and MEK/ERK1/2 and that these pathways were involved in the mitogenic (15) and chemotactic (23) effects of C1P. Therefore, we tested to see whether these pathways were implicated in the release of MCP-1 by C1P. First, selective inhibitors of PI3K and Akt, as well as specific siRNA to silence the expression of the genes encoding these kinases, were used. Figure 2, A and B, shows that the PI3K inhibitor LY-294002 (1 μM) or 10-DEBC (1 μM), an inhibitor of Akt, completely inhibited C1P-stimulated MCP-1 release. Likewise, preincubation of these cells with specific siRNAs to silence PI3K or Akt1 completely blocked the stimulation of MCP-1 release by C1P (Fig. 2, C and D). Western blotting demonstrated potent knockdown of PI3K (Fig. 2, E and F), or Akt1 (Fig. 2, G and H) with 20 pmol/ml PI3K-targeted or Akt-targeted siRNA, respectively. To evaluate the possible involvement of MAP kinases in this process, the MEK inhibitor PD-98059 and specific siRNA against ERK were used. Both of these agents completely blocked C1P-stimulated MCP-1 release (Fig. 3, A and B). Western blotting demonstrated potent knockdown of ERK (Fig. 3, C and D) with 20 pmol/ml ERK-targeted siRNA. In addition, the p38 inhibitor SB 202190 and siRNA against p38α significantly decreased the stimulation of MCP-1 release by C1P (Fig. 4, A and B). Western blotting demonstrated potent knockdown of p38α (Fig. 4, C and D) with 20 pmol/ml p38α-targeted siRNA. However, specific siRNAs against the three different JNK isoforms (JNK1–3), although potently inhibited, did not significantly alter C1P-stimulated MCP-1 release, suggesting that this kinase is not involved in this process (Fig. 5). The effect of C1P on stimulation of ERK1/2, p38α, and Akt phosphorylation is shown in Fig. 6.

A well-known downstream target of Akt and ERK1/2 is NF-κB, a transcription factor that plays an essential role in the induction of inflammatory mediators. The activation of NF-κB by C1P in the J774A.1 macrophages was evaluated by determining its phosphorylation state after treatment with C1P. Figure 7, A and B, shows that C1P increases phosphorylation of NF-κB in a time-dependent manner, which is consistent with NF-κB activation. The implication of NF-κB in C1P-stimulated MCP-1 release was evaluated using SC-514, a selective inhibitor of this transcription factor. This inhibitor completely blocked the release of MCP-1 that was stimulated by C1P (Fig. 7C).

MCP-1 is essential for stimulation of cell migration by C1P. We reported recently (23) that C1P promotes RAW 264.7 leukemia monocytes cell migration and that the PI3K/Akt and MEK/ERK pathways were involved in this process. These observations together with the results shown above led us to hypothesize that MCP-1 might be the key mediator of C1P-stimulated macrophage migration. The ability of C1P to stimulate macrophage migration was studied by incubating the cells with increasing concentrations of MCP-1. Figure 8 shows that MCP-1 stimulates macrophage migration in a concentration- (Fig. 8A) and time-dependent (Fig. 8B) manner. Of interest, MCP-1 was as potent as C1P at stimulating cell migration (Fig. 8, A and C). To evaluate whether the release of MCP-1 was required for the stimulation of macrophage migration by C1P, two different experimental approaches were used. First, the cells were preincubated with a specific monoclonal antibody against MCP-1 prior to stimulation with C1P, and second, the macrophages were pretreated with specific MCP-1 siRNA. Both of these treatments completely blocked C1P-stimulated macrophage migration (Fig. 9), thereby demonstrating that MCP-1 release is absolutely required in this process. Western blotting demonstrated potent knockdown of MCP-1 (Fig. 9, C and D) with 20 pmol/ml MCP-1-targeted siRNA. Two key observations that further supported this hypothesis were that RS-102895, a selective inhibitor of the MCP-1 receptor CCR2b (Fig. 10A), and siRNA against this receptor (Fig. 10B), completely blocked both MCP-1- and C1P-stimulated macrophage migration (Fig. 10). Western blotting demonstrated potent knockdown of CCR2b (Fig. 10, C and D) with 20 pmol/ml CCR2b-targeted siRNA.
C1P-stimulated migration of J774A.1 macrophages involves activation of the PI3K/Akt, MEK/ERK1/2, and p38 pathways. The observation that MCP-1 release is essential for the stimulation of macrophage migration by C1P suggested that the pathways involved in C1P-stimulated MCP-1 release might also be implicated in cell migration. This was examined using selective inhibitors of these pathways (PD-98059 to inhibit MEK, 10-DEBC to inhibit Akt, and SB 202190 to inhibit p38), as well as specific siRNAs to silence the genes encoding these kinases. All of these inhibitory agents abrogated the induction of macrophage migration by C1P (Fig. 11, A and B), suggesting that these pathways are crucial in this process. We previously reported that NF-κB is a downstream target of Akt and ERK and that C1P stimulated the DNA binding activity of this transcription factor in macrophages (15). In agreement with the latter work, Fig. 11B shows that inhibition of NF-κB blocks C1P-stimulated macrophage migration.

In addition, we show in this work that C1P-stimulated MCP-1 release and macrophage migration are inhibited by PTX (Fig. 12), which is consistent with our previous studies suggesting the involvement of a G_i protein-coupled receptor in the stimulation of RAW 264.7 monocyctic leukemia cell migration by C1P (23).

Fig. 7. NF-κB is implicated in C1P-induced MCP-1 release. A: macrophages were seeded in 60-mm dishes (2.5 × 10^5 cells/dish) and treated as in Fig. 6. Cells were stimulated with 20 μM C1P for various times as indicated. Phosphorylation of the p65 subunit of NF-κB was detected by Western blotting using a specific antibody to phospho-p65. Equal loading of protein was monitored using a specific antibody to β-actin. Similar results were obtained in each of 3 independent experiments B: results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are means ± SE of 3 independent experiments.

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Fig. 8. Stimulation of cell migration by MCP-1 and C1P. Macrophage migration was measured using the Boyden chamber-based cell migration assay, as described in MATERIALS AND METHODS. Cells (5 × 10^4 cells/well) were plated in the upper wells of 24-well chambers coated with fibronectin and incubated for 1 h. A: cells were stimulated with increasing concentrations of MCP-1 or with 20 μM C1P as indicated and incubated further for 24 h. Results are expressed relative to control value and are means ± SE of 6 independent experiments performed in duplicate. B: cells were treated with 150 ng/ml MCP-1 for indicated time periods. Results are expressed relative to the value at 0 h and are means ± SE of 5 independent experiments performed in duplicate. C: cells were stimulated with increasing concentrations of C1P as indicated and incubated further for 24 h. Results are expressed relative to time at 0 h and are means ± SE of 3 independent experiments performed in duplicate (*P < 0.05, **P < 0.01).
Fig. 9. MCP-1 is essential for C1P-stimulated cell migration. A: macrophages were seeded and treated as in Fig. 8 and incubated with indicated concentrations of anti-MCP-1 antibody in the absence (squares) or presence (triangles) of 20 μM C1P. Results are means ± SE of 4 independent experiments performed in duplicate. B: cells were seeded in 60-mm dishes (2 × 10⁵ cells/dish) and treated with vehicle (open bars), negative siRNA (filled bars), or MCP-1 siRNA (hatched bars) as indicated in MATERIALS AND METHODS. Vehicle (Control) or 20 μM C1P was then added to the cells, and they were incubated further for 24 h. Results are expressed relative to control value and are means ± SE of 4 independent experiments performed in duplicate. C: MCP-1 siRNA inhibitory efficiency was confirmed by Western blotting using specific antibodies against MCP-1. Equal loading of protein was monitored using a specific antibody to GAPDH. Similar results were obtained in each of 2 independent experiments. D: results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are means ± range of 2 independent experiments (*P < 0.05, **P < 0.01).

Fig. 10. MCP-1 receptor (CCR2b) is involved in C1P-stimulated cell migration. A: macrophage migration was measured as indicated in Fig. 8. Cells were preincubated for 1 h with vehicle (open bars) or with 10 nM RS-102895 (a selective inhibitor of CCR2b; filled bars) and then treated with 20 μM C1P or 150 ng/ml MCP-1 for 24 h as indicated. Results are expressed relative to control value and are means ± SE of 4 independent experiments performed in duplicate. B: cells were seeded in 60-mm dishes (2 × 10⁵ cells/dish) and treated with vehicle (open bars), negative siRNA (filled bars), or CCR2b siRNA (hatched bars) as described in MATERIALS AND METHODS. Vehicle (Control) or 20 μM C1P was then added to the cells as indicated, and they were incubated further for 24 h. Results are expressed relative to control values and are means ± SE of 4 independent experiments performed in duplicate. C: CCR2b siRNA inhibitory efficiency was confirmed by Western blotting using specific antibodies against CCR2b. Equal loading of protein was monitored using a specific antibody to GAPDH. Similar results were obtained in each of 2 independent experiments. D: results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are means ± range of 2 independent experiments (*P < 0.05, **P < 0.01, #P < 0.05, cells treated with MCP-1 vs. cells treated with MCP-1 and RS-102895).
receptors are also coupled to Gi proteins and sensitive to is closely related to C1P. In fact, like the C1P receptor, S1P in independent of interaction with sphingosine 1-phosphate receptors.

Fig. 11. Involvement of PI3K/Akt, MEK/ERK1/2, p38, and NF-κB in C1P-stimulated cell migration. A: cells were seeded in 60-mm dishes (2 × 10^5 cells/dish) and treated with vehicle, negative siRNA, or specific siRNA to inhibit ERK, PI3K, Akt, or p38α, as indicated (see MATERIALS AND METHODS). Vehicle (Control) or 20 μM C1P was then added to the cells, and they were incubated further for 24 h. Results are expressed relative to control value and are means ± SE of 4 independent experiments performed in duplicate. B: macrophages were seeded and treated as in Fig. 8. Cells were then preincubated with 10 μM PD-98059, 1 μM 10-DEBC, 1 μM SB 202190, or 25 μM SC-514 for 1 h and then treated with or without 20 μM C1P, as indicated, and incubated for 24 h. Results are means ± SE of 4 independent experiments performed in duplicate (*P < 0.05).

**Stimulation of MCP-1 release and cell migration by C1P are independent of interaction with sphingosine 1-phosphate receptors.** Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that is closely related to C1P. In fact, like the C1P receptor, S1P receptors are also coupled to Gi proteins and sensitive to inhibition by PTX (13, 52). There are five S1P receptors, which are named S1PR1–5. Therefore, it could be speculated that C1P might be able to interact with any of these receptors to stimulate MCP-1 release and cell migration. To test this possibility, we treated the macrophages with specific siRNAs to silence the genes encoding for each S1PR. Figure 13 shows that pretreating cells with specific S1PR siRNAs did not significantly affect C1P-stimulated MCP-1 release (Fig. 13A) or cell migration (Fig. 13B), thereby demonstrating that these effects of C1P are independent of interaction with S1P receptors. Western blotting demonstrated potent knockdown of S1PR1 (Fig. 13, C and D), S1PR2 (Fig. 13, E and F), S1PR3 (Fig. 13, G and H), S1PR4 (Fig. 13, I and J), and S1PR5 (Fig. 13, K and L) with 20 pmol/ml of each S1PR-targeted siRNA.

**C1P stimulates MCP-1 release and cell migration in human THP-1 monocytes and 3T3-L1 preadipocytes.** In addition to stimulating MCP-1 release and cell migration in murine macrophages, we found that C1P also exerted these actions on other cell types, including human THP-1 monocytes and 3T3-L1 preadipocytes. Figure 14 shows that C1P promoted MCP-1 release (Fig. 14A) and cell migration (Fig. 14B) in THP-1 cells and that migration was inhibited when the cells were incubated in the presence of a specific antibody to MCP-1 (Fig. 14C), in analogy to the results obtained in J774A.1 macrophages (Figs. 1, 8, and 9). Likewise, C1P was able to stimulate MCP-1 release (Fig. 15A) and cell migration (Fig. 15B) in 3T3-L1 preadipocytes, and these effects were also abolished in the presence of a specific antibody to MCP-1 (Fig. 15C).

**DISCUSSION**

We reported recently that C1P stimulates cell migration in RAW 264.7 cells (23). Although this finding has increased our knowledge on how chemotaxis can be regulated in cells, the mechanisms or signaling pathways involved in this process are only beginning to be elucidated. In the present study, we...
present strong evidence for a novel action of C1P: stimulation of MCP-1 secretion. This chemokine plays an important role in the recruitment of mononuclear cells into sites of inflammation and has been associated with tumor metastasis (7, 41, 57). This proinflammatory action of C1P is consistent with previous work by Pettus and coworkers (43–45), who found that this phosphosphingolipid potently stimulates calcium-dependent cytosolic phospholipase A2 (cPLA2) activity and the subsequent generation of arachidonic acid and prostaglandins leading to inflammation. Although C1P can increase synthesis of prostaglandins, it is unlikely that they participate in the stimulation of MCP-1 production and stimulation of cell migration by C1P, as prostaglandins were reported to inhibit MCP-1 production (53). We have confirmed this latter observation in the J774A.1 macrophages, and have also observed that C1P-stimulated macrophage migration is inhibited by prostaglandin...
E2 (unpublished observations). MCP-1 has also been associated with stimulation of cell proliferation in both healthy and malignant cells (7, 55), and although it did not affect the growth of bladder cancer cells, it mediated migration and invasion of those cells (9) and facilitated metastasis to bone, lung, and prostate (6, 36, 47, 57).

There are several pathways involved in the regulation of secretion of chemokines, and we showed previously that C1P...
could activate some of these pathways (3, 14). In particular, we found that C1P was able to stimulate PI3K/Akt (PKB) and MEK/ERK1/2, and that these pathways were involved in the mitogenic (15) and chemotactic (23) effects of C1P. In the present study, we demonstrate that C1P-stimulated MCP-1 release is mediated by these pathways. In addition, we found that C1P promoted phosphorylation of the MAP kinase p38 and that blockade of this enzyme activity using siRNA or chemical inhibition completely abrogated C1P-stimulated MCP-1 release. Moreover, C1P-stimulated MCP-1 release was completely abolished by PTX, an action that is compatible with the existence of a specific G protein-coupled receptor for C1P (23). The implication of Akt, ERK1/2, and p38 in this C1P action is consistent with previous work by other groups showing that MCP-1 upregulated these kinases in different cell types, including rat aortic smooth muscle cells (55), proximal tubular cells (51), 3T3-L1 preadipocytes (56), vascular smooth muscle cells (12), and monocytes (33). Nonetheless, although MCP-1 release has been associated with activation of p38 in many instances (8, 33, 56; and our unpublished observations), other studies have indicated that this kinase is not involved in this process (31, 35), and therefore the role of p38 in stimulation of MCP-1 secretion remains controversial.

Of note, two related phosphosphingolipid mediators, sphingosine phosphorylcholine (SPC) and S1P, also induced secretion of proinflammatory MCP-1 in human umbilical vein endothelial cells (34) and vascular smooth muscle cells (54), and human mast cells (42), respectively. As for C1P, the stimulating effect of SPC on MCP-1 secretion was also dependent on activation of a specific, albeit still not well-defined, receptor (40). However, unlike C1P, the stimulation of MCP-1 release by S1P was not dependent on receptor activation (42). In addition, we demonstrate here that C1P-stimulated MCP-1 release and cell migration are independent of interaction of C1P with S1P receptors.

A key observation in this work was that incubation of the macrophages with an MCP-1-neutralizing antibody or with MCP-1 siRNA or the MCP-1 receptor antagonist RS-102895 or CCR2b-targeted siRNA abrogated C1P-stimulated cell migration, thereby demonstrating that MCP-1 is the principal mediator of C1P-stimulated macrophage migration. These findings are consistent with recent work by Mitsutake et al. (39), who recently demonstrated that MCP-1-induced cell migration was significantly reduced in BMDM from mice lacking ceramide kinase, the enzyme responsible for C1P generation. We also observed that C1P was able to stimulate migration of BMDM, but this effect was only marginal compared with the extent of activation seen in J774A.1 macrophages, in agreement with Mitsutake et al. (data not shown).

Although the molecular identity of the target receptor whose activation by C1P leads to MCP-1 release and subsequent migration of the macrophages is not yet clear, we show here that a PTX-sensitive G protein-coupled receptor is likely to be involved in mediating these effects of C1P. In fact, PTX blocked C1P-stimulated macrophage migration, which is consistent with the inhibition of C1P-stimulated MCP-1 release by this toxin and with our previous work on RAW 264.7 cells (23). These observations suggest that MCP-1 release depends on the interaction of C1P with its putative receptor to trigger macrophage migration.

In terms of the signaling events downstream of C1P stimulation in macrophages, our previous work showed that PI3K/Akt, MEK/ERK1/2, and JNK are involved in the mitogenic or antiapoptotic effects of C1P, and these pathways are also known to act downstream of the C1P receptor (23). In the present work, we found that PI3K/Akt, MEK/ERK, and their downstream target NF-κB are involved in the stimulation of cell migration by C1P. However, inhibition of JNK did not alter macrophage migration. Of interest, and unlike PI3K/Akt and MEK/ERK, JNK was also not involved in upregulation of MCP-1 expression in endometrial stroma cells (35).

Finally, it should be emphasized that the effects of C1P on MCP-1 release and cell migration are not restricted to murine macrophages, as C1P also promoted these actions in human THP-1 monocytes and 3T3-L1 preadipocytes. Moreover, recent work by Ratajczak’s group demonstrates that C1P also stimulates migration of hematopoietic stem progenitor cells (30), multipotent stromal cells, and endothelial progenitor cells (29), as well as migration of bone marrow-derived stem cells in patients suffering from acute myocardial infarction (28).

In conclusion, we have demonstrated here that C1P promotes MCP-1 release and cell migration in macrophages. This stimulatory effect of C1P is accomplished through activation of the PI3K/Akt, MEK/ERK1/2, and p38 pathways, but not JNK, via PTX-sensitive G proteins, leading to NF-κB activation. C1P also stimulated MCP-1 release and migration in human THP-1 monocytes and 3T3-L1 preadipocytes. Because MCP-1 is proinflammatory and may be involved in tumorigenesis and tumor metastasis, C1P and its putative plasma membrane receptor may have potential as therapeutic targets to combat inflammation and cancer.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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