Experimental Cell Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Vascular endothelial growth factor mediates ceramide 1-phosphate-stimulated macrophage proliferation

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ARTICLE INFO

Keywords:
Cell proliferation
Ceramide 1-phosphate
Macrophages
Sphingolipids
VEGF

ABSTRACT

The bioactive sphingolipid ceramide 1-phosphate (C1P) regulates cell division in a variety of cell types including macrophages. However, the mechanisms involved in this action are not completely understood. In the present work we show that C1P stimulates the release of vascular endothelial growth factor (VEGF) in RAW264.7 macrophages, and that this growth factor is essential for stimulation of cell proliferation by C1P. The stimulation of VEGF release was dependent upon activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB-1 also known as Akt-1), and mitogen-activated protein kinase-kinase (MEK)/extracellularly regulated kinase-2 (ERK-2) pathways, as inhibition of these kinases with selective pharmacological inhibitors or with specific gene silencing siRNA, abrogated VEGF release. A key observation was that sequestration of VEGF with a neutralizing antibody, or treatment with VEGF siRNA abolished C1P-stimulated macrophage growth. Also, inhibition of the pathways involved in C1P-stimulated VEGF release inhibited the stimulation of macrophage growth by C1P. Moreover, blockade of VEGF receptor-2 (VEGFR-2), which is the primary receptor for VEGF, with the pharmacological inhibitor DMH4, or with specific VEGFR-2 siRNA, substantially inhibited C1P-stimulated cell growth. It can be concluded that stimulation of VEGF release is a key factor in the promotion of macrophage proliferation by C1P.

1. Introduction

Ceramide 1-phosphate (C1P) is a bioactive phosphosphingolipid with mitogenic properties. Initially, we showed that C1P stimulated DNA synthesis as determined by the incorporation of [^{3}H]thymidine into DNA, and that it potently enhanced cell division in rat fibroblasts [1,2], macrophages [3–5], and myoblasts [6]. Subsequently, we demonstrated that C1P played a key role in cell survival through inhibition of apoptosis [7–9]. The latter action implicated direct inhibition of the acidic form of sphingomyelinase (ASMase), a signal-activated enzyme that generates ceramides from sphingomyelin [8], or blockade of serine palmitoyl transferase (SPT) [7–9], which is a key regulatory enzyme of the *de novo* synthesis pathway of ceramides. Inhibition of either of these enzymes resulted in potent depletion of ceramides, which are pro-apoptotic for macrophages, thereby blocking apoptosis in these cells. C1P has also been involved in inflammatory responses [10–12], phagocytosis [13,14], neutrophil degranulation [15], and macrophage migration [16,17]. The latter action required the interaction of exogenous C1P with a putative Gi protein-coupled receptor, whilst intracellularly generated C1P was unable to induce macrophage migration [17–19]. C1P also stimulated migration in other cell types including hematopoietic and stem cells [20], primary human coronary artery endothelial cells [21], fibroblasts [22], or pancreatic cancer cells [23].

Although some of the signaling pathways involved in the mitogenic effects of C1P have been described (reviewed in [12]), the molecular mechanisms by which C1P stimulates cell proliferation are not completely understood. In the present work we show that C1P stimulates vascular endothelial growth factor (VEGF) secretion in macrophages through a mechanism involving activation of the PI3K/PKB (also

Abbreviations: BSA, bovine serum albumin; C1P, ceramide 1-phosphate; PKB, protein kinase B; ERK, extracellularly regulated kinases; FBS, fetal bovine serum; PTX, pertussis toxin; PI3K, phosphatidylinositol 3-kinase; DMH4, 6-[4-[2-(4-morpholinyl)ethoxy]phenyl]-3-phenylpyrazolo[1,5-*a*]pyrimidine; BHNB, 4-bromo-5-hydroxy-2-nitrobenzhydryl

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http://dx.doi.org/10.1016/j.yexcr.2017.10.027
Received 24 June 2017; Received in revised form 7 October 2017; Accepted 24 October 2017

known as Akt) and MEK/ERK1-2 pathways, and demonstrate that VEGF is a key mediator of C1P-induced macrophage proliferation.

2. Materials and methods

2.1. Materials

N-Hexadecanoyl-D-*erythro*-sphingosine-1-phosphate (C16:0-Ceramide 1-phosphate) (C1P) was supplied by Matreya. Culture medium, Dulbecco's Modified Eagle's Medium (DMEM) was from Lonza. LY 294002, PD 98059, pertussis toxin, Protease Inhibitor Cocktail, prostaglandin E_2 and 6-[4-[2-(4-morpholinyl)ethoxy]phenyl]-3-phenylpyrazolo[1,5-*a*]pyrimidine were from Sigma-Aldrich. 10-DEBC was from Tocris. Fetal bovine serum (FBS) was from Gibco. 4-bromo-5-hydroxy-2-nitrobenzhydryl (BHNB)-C1P was kindly donated by Dres. R.S. Lankalapalli and R. Bittman ♀ (City University of New York, NY, USA). Nitrocellulose membranes, protein markers, and BCA assay reagents were purchased from Bio-Rad. Oligofectamine was from Invitrogen Life Technologies. The mouse VEGF ELISA kit was from R & D Systems. Antibodies to phospho-VEGFR-2, VEGFR-2, PI3K, PKB and ERK were from Cell Signaling. Antibodies to GAPDH were purchased from Santa Cruz. PI3K, PKB1, PKB2, PKB3, ERK2 and VEGF siRNAs were from Ambion. All of the other chemicals and reagents were of the highest grade available.

2.2. Cell culture

RAW 264.7 macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA). RAW 264.7 cells were cultured in low glucose DMEM supplemented with 10% heat-inactivated FBS, 50 mg/l of gentamicin, and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. For most experiments, cells were seeded in 12-, 6- or 96-well dishes with 0.1–1 ml of culture medium, respectively, and used when ~40% confluent. The cells were passaged to a maximum of 15 passages. For experiments on VEGFR-2 expression, cells were seeded in 60 mm plates.

2.3. Delivery of C1P to cells in culture

An aqueous dispersion (in the form of liposomes) of C1P was added to cultured macrophages, as reported previously [5,7,8]. Stock solutions were prepared by sonicating C1P (1.66 mg) in sterile nanopure water (1 ml) on ice using a probe sonicator until a clear dispersion was obtained. The final concentration of C1P in the stock solution was approximately 2.6 mM. This procedure is considered preferable to using dispersions prepared by adding C1P in organic solvents because droplet formation is minimized and exposure of cells to organic solvents is avoided. We also delivered C1P to cells by using the photolabile caged C1P analog, BHNB-C1P [24], which was dissolved in ethanol at 1.62 mM. The final ethanol concentration was < 0.16%. The cells were exposed to 400–500 nm light in a transilluminator equipped with a 9 W lamp for 60 min at a distance of 1.5 cm at 37 °C, so as to release the C1P moiety into the cytosol.

2.4. Treatment of cells with siRNA

RAW 264.7 macrophages were seeded in 6-well dishes at 5×10^5 cells/well and incubated in DMEM containing 10% FBS. Four hours later, the medium was removed and the cells were washed twice with PBS. The cells were incubated for 24 h with 800 µl of Opti-MEM without antibiotics. Transfection of siRNA in the RAW 264.7 cells (20 pmol siRNA in 200 µl) was performed using an oligofectamine reagent according to the manufacturer's instructions (Invitrogen Life Technologies). Cells were then incubated for 5 h in culture medium containing 1 ml of Opti-MEM and 20% FBS. The next day, the cells were washed with PBS and the medium was replaced by fresh DMEM supplemented with 10% FBS. After 24 h of incubation, the medium was removed and the macrophages were washed twice with PBS. The cells were then preincubated with DMEM without FBS for 2 h, as indicated [25,26], and C1P or inhibitors were added as required.

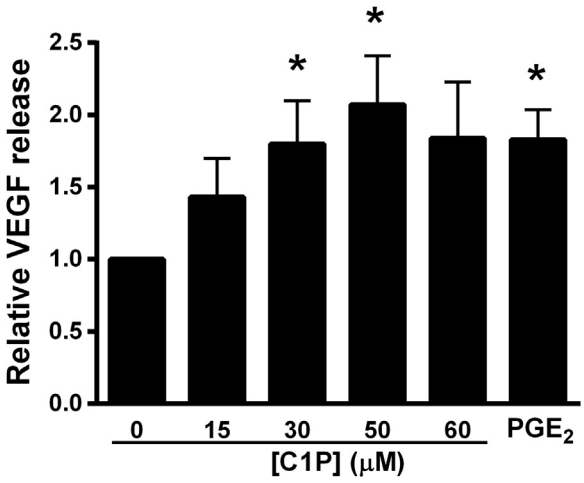

Fig. 1. C1P stimulates VEGF secretion in macrophages. Cells were stimulated with increasing concentrations of C1P or 100 ng/ml PGE_2 for 24 h, as indicated. VEGF concentration in supernatants was measured as described in Materials and Methods section. The results are expressed as the mean ± SEM of 4 independent experiments performed in duplicate (*$p < 0.05$).

2.5. VEGF determination in the culture medium

RAW 264.7 cells were seeded in 6-well plates (0.75×10^6 cells/ml) in DMEM supplemented with 10% FBS and incubated overnight to allow cell attachment. The next day, cells were washed and incubated in DMEM supplemented with 1% FBS. Agonists were added and 24 h later the cell medium was collected in microcentrifuge tubes. Samples were centrifuged at $10,000 \times g$ for 5 min and the supernatants were used for the VEGF measurement. VEGF concentration in the supernatants was measured using a "Mouse VEGF Quantikine" kit following manufacturer's instructions. This kit provides precoated ELISA plates, so that samples and standard solutions were directly added to 96-well plates and incubated for 2 h at room temperature. The wells were washed and a horseradish peroxidase-conjugated detection antibody was added. After 2 h of incubation, the wells were washed again and 3,3',5,5'-tetramethylbenzidine solution was added as a chromogenic substrate. Color development was stopped using the "Stop Solution" and the absorbance was measured as indicated above. VEGF secretion is given as pg/mg of total cell protein, or relative to the control value in each experiment.

2.6. Cell growth and viability assay

Macrophages were seeded at 25,000 cells/well in 96-well plates and incubated for 24 h in DMEM medium. The medium was then replaced by fresh DMEM without serum in the presence or absence of agonists and/or inhibitors as appropriate for 48 h. Cell viability was estimated by measuring the rate of reduction of the tetrazolium dye MTS as described [27].

2.7. Western blotting

Macrophages were harvested and lysed in ice-cold homogenization buffer as described [28]. Protein (20–40 µg) from each sample was loaded and separated by SDS-PAGE, using 12% separating gels. Proteins were transferred onto nitrocellulose paper and blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.01% NaN_3 and