Platelet-derived growth factor (PDGF)-induced chemotaxis does not require the G protein-coupled receptor $S1P_1$ in murine embryonic fibroblasts and vascular smooth muscle cells

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Abstract Sphingosine 1-phosphate (S1P), a bioactive lipid mediator, signals via G protein-coupled receptors (GPCR). The prototypical S1P receptor, $S1P_1$ (also known as EDG-1), a G_i-linked receptor, is critical for vascular maturation during development. Recent work suggested that platelet-derived growth factor (PDGF)-induced cell migration required the $S1P_1$ receptor, representing a novel mechanism for cross-talk between receptor tyrosine kinases and GPCRs. Since both S1P and PDGF are implicated in vascular smooth muscle cell (VSMC) pathobiology and development, we investigated this issue in rat VSMC and in embryonic fibroblasts derived from $S1P_1$ null mice. Our data suggest that the $S1P_1$ receptor is critical for S1P-induced, G_i-dependent migration but not for PDGF-BB-induced, receptor tyrosine kinase-dependent chemotaxis in VSMC. In addition, lack of $S1P_1$ receptor in mouse embryonic fibroblasts did not significantly affect PDGF-induced cell migration. These data question the generality of the concept that $S1P_1$ GPCR is a critical downstream component of PDGF-induced chemotaxis.

Key words: Sphingosine 1-phosphate; Vascular smooth muscle cells; Platelet-derived growth factor; EDG-1; S1P1 receptor; G-protein-coupled receptor; Receptor tyrosine kinase; Migration; Chemotaxis; Vascular disease; Vascular development

1. Introduction

The migration of vascular smooth muscle cells (VSMC) is an important aspect of blood vessel development [1–4] and cardiovascular disease [5–8]. Many polypeptide growth factors regulate VSMC migration [9,10], especially platelet-derived growth factor (PDGF) [5–7,11].

In addition, the platelet-derived bioactive lipid sphingosine 1-phosphate (S1P) has been demonstrated to regulate VSMC migration. Originally, S1P was shown to inhibit the migration of human VSMC [12]. However, further investigation has revealed that S1P can induce the migration of VSMC if they express the $S1P_1$ receptor, a G protein-coupled receptor (GPCR) originally known as endothelial differentiation gene (EDG)-1 [13,14]. In addition, antagonistic signaling between $S1P_1$ and $S1P_2$ receptors was shown to be critical for S1P-induced VSMC migration, as these different receptors regulate migration pathways in an opposite manner [15,16].

Recently, cross-talk between the S1P and PDGF signaling pathways has been proposed [14,17,18]. Hobson et al. [18] proposed a model that included a critical requirement for the $S1P_1$ GPCR in PDGF-induced cell migration. In this model, PDGF-BB, a high-affinity ligand for the receptor tyrosine kinase PDGF-β receptor, induced the intracellular activation of sphingosine kinase enzyme, followed by the activation of $S1P_1$ receptor in an autocrine manner. This event was shown to be critical for activation of chemotaxis signaling pathways, such as the stimulation of the small GTPase Rac. The authors argued that this might be a physiologically important mechanism, as both PDGF-β and $S1P_1$ receptor gene deletion studies resulted in vascular maturation defects in mice. In a second study, Alderton et al. [17] proposed that $S1P_1$ is critical for PDGF-induced mitogen-activated protein (MAP) kinase activation in airway smooth muscle cells. Overexpression of PDGF-β receptor and $S1P_1$ receptor in human embryonic kidney (HEK) 293 cells resulted in co-precipitation of the two receptors, suggesting that $S1P_1$ is an important component of PDGF-induced MAP kinase activation. These proposals are novel in that they suggest cross-communication between receptor tyrosine kinases and GPCRs [17]. However, some aspects of the model, such as the pertussis toxin sensitivity of PDGF-induced migration, have recently been questioned [14].

In an attempt to improve our understanding of the potential involvement of $S1P_1$ in PDGF-induced migration, we investigated the cultured VSMC cells from rat aorta, a model system widely used in studies of VSMC pathobiology [19]. Contrary to the previous studies, we show that $S1P_1$ expression is not critical for PDGF-BB-induced VSMC migration.

2. Materials and methods

2.1. Materials

S1P (Biomol) was prepared as previously described [13]. Recombinant human PDGF-BB (R&D Systems) was resuspended in 4 mM

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Abbreviations: S1P, sphingosine 1-phosphate; PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cell; GPCR, G protein-coupled receptor; MAP kinase, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; RT-PCR, reverse transcriptase-polymerase chain reaction

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HCl with 0.1% fatty acid-free bovine serum albumin (BSA). Pertussis toxin (Calbiochem) was resuspended in phosphate buffer.

2.2. Cell culture

The following cell lines were used in this study: (a) rat adult medial VSMC [13,19]. These cells express $S1P_2$ and $S1P_3$ receptors and undetectable $S1P_1$ receptor [13]. In addition, $S1P_1$ −/− mouse embryo fibroblasts were derived as described below. These cells express $S1P_2$ and $S1P_3$ receptors as described before [3]. In some experiments, cells were infected with adenoviral constructs expression $S1P_1$ receptor as described previously [25]. To determine the effect of increasing the receptor expression level, cells were infected with increasing numbers of viral particles, as determined by multiplicity of infection (MOI) units. An MOI is defined as one viable particle of adenovirus per host cell.

Rat adult medial VSMC were maintained and infected with adenovirus as described previously [13]. Heterozygous $S1P_1$ knockout mice were generously provided by R. Proia (NIH) [3]. They were crossed to obtain embryos of different genotypes. Mouse embryonic fibroblasts were prepared from 12.5 day embryos (of all three genotypes, namely, +/+, +/− and −/−) by removing the head and liver and homogenizing the remaining embryo through a needle and syringe. Although +/+ and +/− embryos were healthy at the time, occasional bleeding was seen in −/− embryos. However, at all times only embryos that were alive (defined as those with a beating heart) were used for the isolation of cells. Genotyping was done by extracting DNA from yolk sacs. Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum with antibiotics and were genotyped as described previously [3].

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNA-Stat 60 reagent (Tel Test). In some experiments, poly-A^+ RNA was prepared using the oligo-dT cellulose chromatography as described previously [24].

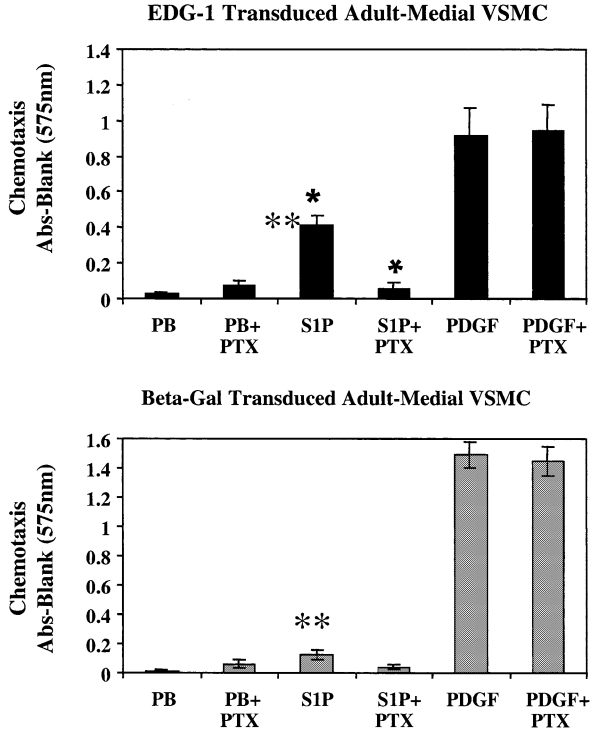

Fig. 1. Rat adult medial VSMC were grown until 75–100% confluence and were infected with either $S1P_1$ or β-galactosidase adenovirus. Two days after infection, cell migration assay was conducted as described. In some conditions, cells were pretreated with pertussis toxin (PTX, 200 ng/ml) and migration toward S1P (5 nM) or PDGF (40 ng/ml) was tested. Cell migration was quantified by measuring absorbance at 575 nm. PB = PBS/BSA control. Data represent mean ± S.D. Statistical significance was calculated using the unpaired two-tailed *t*-test (**P* = 0.004 and ***P* = 0.015).

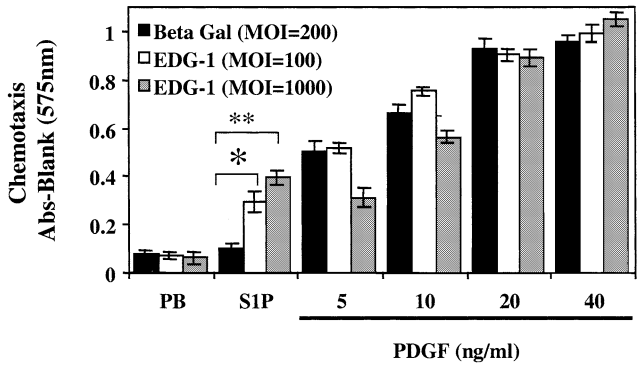

Fig. 2. Rat adult medial VSMC were infected with β-galactosidase or $S1P_1$ adenovirus (MOI indicated in figure legend) and migration toward 5 nM S1P or various doses of PDGF was tested as described. Increasing number of viral particles (as determined by increasing MOI) is used to increase the level of $S1P_1$ receptor. PB = PBS/BSA control. Data represent mean ± S.D. Statistical significance was calculated using the unpaired two-tailed *t*-test (**P* = 0.032 and ***P* = 0.001). NS = not significant.

RNA was reversed-transcribed and PCR was performed to determine the expression of $S1P_1$, $S1P_2$/EDG-5 and $S1P_3$/EDG-3 using the primers and conditions described [20]. Amplified products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

2.4. Migration assays

Rat adult medial VSMC were used in migration assay as described previously [13]. Mouse embryonic fibroblasts were serum-starved overnight in DMEM with 0.5% charcoal-stripped serum. The next day, cells were trypsinized, resuspended in DMEM with 0.5% fatty acid-free BSA and loaded into the upper chambers. The lower chambers were loaded with DMEM, 0.5% fatty acid-free BSA alone or supplemented with PDGF (10 ng/ml). Fibroblasts were allowed to migrate for 6 h and then were quantitated as described elsewhere [21].

3. Results and discussion

We compared the migration of rat VSMC which express different levels of $S1P_1$ receptor. As previously described, rat adult medial VSMC abundantly express $S1P_3$/EDG-3 and $S1P_2$/EDG-5 mRNA but do not express detectable $S1P_1$ mRNA [13,15,22]. Expression of $S1P_1$ in rat adult medial VSMC has been shown to enhance proliferation and migration in response to S1P [13], however, in this system, the role of $S1P_1$ in PDGF-induced migration was not tested. Furthermore, a recent publication [18] suggests that $S1P_1$ may play a role in regulating PDGF-induced migration in HEK 293 cells, mouse embryonic fibroblasts and human VSMC. Therefore, we tested whether expression of $S1P_1$ in rat adult medial VSMC would alter the migratory responses to PDGF.

Expression of $S1P_1$ in adult medial VSMC by adenoviral infection clearly enhanced migration toward S1P in a pertussis toxin-sensitive manner (Fig. 1). However, expression of $S1P_1$ did not enhance PDGF-induced migration and PDGF-induced migration was not pertussis toxin-sensitive. These findings suggest that the $S1P_1/G_i$ signaling pathway may not be required for PDGF-induced migration.

In order to rule out the possibility that different levels of $S1P_1$ expression may influence PDGF receptor signaling, two different MOIs for the $S1P_1$ adenovirus were used in combination with different doses of PDGF. As seen in Fig. 2, the $S1P_1$ adenovirus infection clearly enhanced S1P-induced migration at both doses. However, PDGF-induced migration

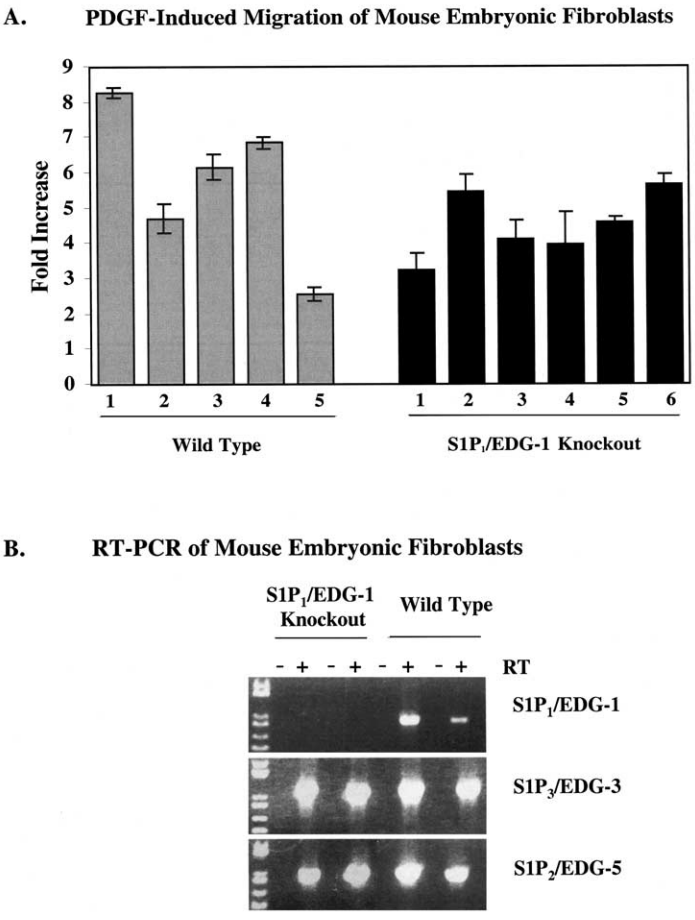

Fig. 3. A: PDGF-induced migration of wild-type embryonic fibroblasts isolated from five different embryos and $S1P_1$ knockout (−/−) embryonic fibroblasts isolated from six different embryos are shown. The data represent several independent experiments with all conditions performed in triplicate. Data represent mean ± S.D. Statistical significance was calculated using the unpaired two-tailed *t*-test, and the *P* values did not indicate significant differences ($P = 0.244$). B: The expression of $S1P_1$, $S1P_3$/EDG-3 and $S1P_2$/EDG-5 mRNAs was determined by RT-PCR using poly-A^+ RNA isolated from either wild-type or $S1P_1$ knockout fibroblasts. Shown here are the results for fibroblasts isolated from two different embryos of each genotype. Note that, as expected, wild-type fibroblasts express mRNA for $S1P_1$, $S1P_3$/EDG-3 and $S1P_2$/EDG-5 while $S1P_1$ knockout fibroblasts express mRNA only for $S1P_3$/EDG-3 and $S1P_2$/EDG-5. − = minus reverse transcriptase; + = plus reverse transcriptase.

was not enhanced even when multiple doses of PDGF (5–40 ng/ml) were used. Taken together, these results demonstrate that the expression of $S1P_1$ receptor does not enhance PDGF-induced migration in rat VSMC and that PDGF-induced migration is not pertussis toxin-sensitive. Since $S1P_1$ expression is not detectable in adult medial VSMC, these data question the generality of the concept that PDGF-induced chemotaxis requires signal transduction from the $S1P_1$ GPCR [18].

To further test the requirement for $S1P_1$ in PDGF-induced chemotaxis, we utilized several independent isolates of mouse embryonic fibroblasts (MEF) from wild-type and well as $S1P_1$ −/− mice [3]. The endogenous $S1P_1$ gene was disrupted by homologous recombination in these cells; therefore these cells completely lack the $S1P_1$ receptor [3]. We found that PDGF clearly induced the migration of mouse embryonic fibroblasts isolated from both wild-type and $S1P_1$ knockout mouse embryos (Fig. 3). Interestingly, there appeared to be some variation in migratory responses of fibroblasts with the same genotype isolated from different embryos. Nevertheless, the average PDGF-induced migration of all wild-type fibroblast clones (5.72 ± 0.97-fold increase above control) did not differ significantly ($P = 0.244$, unpaired *t*-test) from the average PDGF-induced migration of the $S1P_1$ knockout fibroblasts (4.50 ± 0.38-fold increase above control). As expected, $S1P_1$ was expressed in wild-type embryonic fibroblasts but not in fibroblasts isolated from $S1P_1$ knockout mice (Fig. 3). These data are in agreement with our findings from adult medial VSMC that $S1P_1$ receptor is not required for PDGF-induced chemotaxis.

In conclusion, expression of $S1P_1$ receptor in rat VSMC enhanced S1P-induced migration in a pertussis toxin-sensitive manner, consistent with signaling via the $S1P_1/G_i$ pathway. However, expression of $S1P_1$ in rat VSMC did not enhance PDGF-induced migration and such migration was not pertussis toxin-sensitive. These data are not consistent with the model proposed by Hobson et al. [18], where $S1P_1$ was proposed to be necessary for PDGF-induced migration. There may be several reasons for the apparent discrepancies between our results and those of Hobson et al. In the model proposed by these authors, PDGF induced the activity of sphingosine kinase enzyme, which produced the ligand S1P. In addition, the authors further suggested that S1P is somehow exported out of the cell and activated $S1P_1$ receptor, resulting in the activation of the chemotaxis program. Many of the conclu-

sions of that study were based on the use of *N*,*N*′-dimethylsphingosine, an inhibitor of sphingosine kinase that also inhibits other signaling proteins [18,23]. In addition, the authors reported data on only one MEF cell line which may not be truly representative. Analysis of several independent isolates in our study did not reveal significant differences in PDGF sensitivity of $S1P_1$ −/− cell lines from the wild-type counterparts. It is also possible that sphingosine kinase activation in the rat adult medial VSMC and MEF may be significantly different from those employed by Hobson et al. [18]. Despite these caveats, our data question the generality of the concept that $S1P_1$ is an essential downstream mediator of PDGF-induced chemotaxis.

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