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Signaling mechanisms for positive and negative regulation of cell motility by sphingosine-1-phosphate receptors

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1. Introduction

Sphingosine-1-phosphate (S1P) is a lysophospholipid growth factor circulating in blood, which induces a wide variety of biological responses in diverse cell types. S1P is present at 10^{-7} M order of concentration in the blood largely in a form bound to plasma proteins including albumin and lipoproteins, and released by activated platelets and other cell types [1]. Identification of cell surface receptors for S1P provided strong support for the notion that many, if not all, of the diversity of biological activities of S1P are mediated through either of these receptors [2-5]. Among 5 S1P receptor subtypes, S1P₁/Edg (for endothelial differentiation gene)-1, S1P₂/Edg5, S1P₃/Edg3, are widely expressed in various tissues [2-5].

Cell migration is a critical component of cellular responses, which is involved in such diverse physiological and pathological processes as embryonic morphogenesis,

angiogenesis, wound healing, atherogenesis, inflammation and tumor cell dissemination [6]. Cell migration is regulated in both positive and negative directions by a variety of extracellular cues called chemoattractants and chemorepellants, respectively. A number of biologically active substances, including chemokines, cytokines and growth factors, have been demonstrated to act as chemoattractants to induce chemotaxis, i.e. cell migration directed toward a higher concentration of a chemoattractant. Certain factors including some members of the semaphorine and the ephrine families were shown to directly repel neuronal and vascular cells as chemorepellants. However, our knowledge about chemorepellants or migration-inhibitory substances is generally much limited at present, compared to chemoattractants [6].

More than 10 years ago, Igarashi and his colleagues demonstrated for the first time that S1P exerted inhibitory effects on migration and invasion of certain tumor cells including melanoma cells and osteosarcoma cells [7]. They also showed that S1P inhibited migration of vascular smooth muscle cells (VSMCs) [8] and neutrophils [9] that were directed toward chemoattractants. More recently, it was shown that S1P by itself was capable of stimulating, rather than inhibiting, chemotaxis of vascular

endothelial cells [10] and mouse embryonic fibroblasts [11]. Thus, S1P appeared to be a unique, bimodal regulator of cell migration.

In this chapter we describe molecular mechanisms underlying this bimodal regulation by S1P, i.e. S1P receptor subtype-specific, positive and negative regulation of cell motility and the Rho family GTPases, and S1P regulation of invasion and metastasis of malignant melanoma cells via endogenous S1P receptor through the control of Rho GTPases.

2. Receptor subtype-specific, bimodal regulation of cell motility and the small GTPase Rac by S1P

After the identification of the S1P-specific Edg family receptors, S1P was found to stimulate chemotaxis in vascular endothelial cells via endogenously expressed S1P₁ and S1P₃ [10, 11]. However, it remained unknown how S1P induced inhibition of cell migration in several other cell types, although an extracellular mode of this inhibitory S1P action was suggested [12]. We showed the activity of each of the S1P receptors on cell migration in Boyden chamber assay by employing Chinese hamster ovary (CHO)

cells that stably overexpressed each of the widely expressed subtypes, S1P₁, S1P₂ and S1P₃. It is known that CHO cells overexpressing exogenous chemoattractant receptors can vigorously respond to a concentration gradient of a respective chemoattractant with stimulation of directed cell migration in a trans-well migration assay. S1P stimulated directed cell migration, i.e. chemotaxis, of either S1P₁- or S1P₃-expressing CHO cells [13, 14]. These two cell types were quite sensitive to S1P with the threshold concentrations as low as 0.1-1 nM of S1P. It was also reported that S1P stimulated chemotaxis in HEK293 cells overexpressing S1P₁ [15]. In sharp contrast, S1P did not stimulate trans-well migration of S1P₂-expressing cells and vector-transfected control cells [13]. However, the addition of a potent chemoattractant insulin-like growth factor (IGF-I) unveiled a strong inhibitory effect of S1P on cell migration via S1P₂; IGF-I stimulated chemotaxis in three S1P receptor subtype-expressing cells and vector control cells to comparable degrees. When S1P was present together with IGF-I in the lower well of Boyden chamber, IGF-I-directed chemotaxis was completely abolished only in S1P₂-expressing CHO cells. The S1P₂-mediated inhibitory effect of S1P on cell migration was potent with the threshold concentration of as low as 1 nM, and was

dependent on an S1P concentration gradient. Thus, S1P acts as a chemorepellent.

Chemoattractants receptors activate complex, multiple signaling cascades including protein tyrosine kinases, lipid kinases and the low molecular weight GTPases [6]. These signaling events lead in concert to regulate actin organization and myosin motor function, which constitute essential processes for cell migration. Among the low molecular weight G proteins, the Rho family GTPases comprise three major members, Rho, Rac and Cdc42, and exert distinct regulatory actions on actin cytoskeletons, thus playing an important role in cell motility [16 17]; Rho mediates formation of stress fibers and focal adhesions, while Rac and Cdc42 direct peripheral actin assembly that results in formation of lamellipodia and filopodia, respectively, especially at the leading edge of a migrating cell. For example, expression of a dominant negative form of Rac, N¹⁷-Rac, in a variety of cell types inhibits directed cell migration toward diverse chemoattractants, including PDGF, LPA, epidermal growth factor (EGF), insulin and colony stimulating factor-1 [16-18], indicating that Rac is required for chemotaxis toward these attractants. The expression of dominant negative forms of the Rho family GTPases, N¹⁷-Rac and N¹⁷-Cdc42, but not N¹⁹-RhoA, inhibited chemotaxis toward S1P

in S1P₁- and S1P₃-expressing CHO cell types and IGF-I in all the CHO cell types, indicating that IGF-I- and S1P-directed chemotaxis was dependent on Rac and Cdc42 [13]. S1P as well as IGF-I increased an amount in a GTP-bound, active form of Rac in S1P₁- and S1P₃-expressing CHO cells and all the CHO cell types, respectively. In S1P₂-expressing cells, S1P by itself did not change Rac activity. However, S1P dramatically abolished IGF-I-induced Rac activation and lamellipodia formation with a dose-response relationship similar to that for S1P inhibition of IGF-I-directed chemotaxis [13]. Either S1P or IGF-I did not affect GTP-loading of Cdc42 in any of CHO cell types. Rho regulation by S1P and IGF-I was a little complicated; stimulation of the attractant receptor S1P₁ did not change RhoA activity, but both the repellent receptor S1P₂ and the attractant S1P₃ stimulated RhoA. IGF-I did not affect RhoA or Cdc42 activity [13]. Thus, all these data in CHO cells was consistent with the notion that stimulation of Rac activity, but not of Cdc42 or RhoA, above the resting level was necessary for stimulation of chemotaxis in this cell type. The three Edg family S1P receptors transmit overlapping but distinct sets of signals to the Rho family GTPases [13, 14, 19, 20], thus allowing receptor subtype-specific, distinct regulatory activities on

cell migration (Fig. 1).

Chemoattractants that act via G protein-coupled receptors are generally shown to mediate chemotaxis and Rac activation via the heterotrimeric G_i protein. However, signaling mechanisms of chemorepellant receptors were largely unknown. The C-terminal mini-peptide of the heterotrimeric G protein α -subunits ($G\alpha$ -CTs) serve as inhibitors specific for respective G proteins [21]. The expression of either $G_{12}\alpha$ -CT or $G_{13}\alpha$ -CT, but not $G_s\alpha$ -CT or $G_q\alpha$ -CT or pertussis toxin, abrogated $S1P_2$ -mediated inhibition of Rac and cell migration. Moreover, either $S1P_2$ - $G_{12\alpha}$ or $S1P_2$ - $G_{13\alpha}$ fusion receptor [22], but not $S1P_2$ - $G_{q\alpha}$ fusion receptor, mediated S1P inhibition of Rac and cell motility [14]. Our data indicated that the $G_{12/13}$ family protein coupled $S1P_2$ to inhibition of Rac, cell migration and lamellipodia in CHO cells [14]. Furthermore we found that downstream of $G_{12/13}$, Rho mediated $S1P_2$ inhibition of Rac and cell migration through a mechanism not involving Rho kinase as a Rho effector [14]. Very recently, Sanchez et al. [23] showed that $S1P_2$ mediated inhibition of cell migration through stimulating the 3'-phosphatase of phosphoinositides, PTEN, in a Rho kinase-dependent manner. This may suggest the possibility that more than a single

mechanism could contribute to S1P₂ mediated inhibition of cell migration and that the mechanism behind S1P₂-mediated cell migration inhibition may be different among cell types.

S1P₃ mediated Rac activation and chemotaxis despite stimulating Rho [13]. We found in pertussis toxin-pretreated CHO cells expressing S1P₃ that S1P induced inhibition of Rac, cell migration and lamellipodia just as in CHO cells expressing S1P₂ [14]. Thus, inactivation of G_i by pertussis toxin treatment converted S1P₃ into the S1P₂-like repellent receptor. These results indicate that integration of counteracting signals from the G_i- and the G_{12/13}-Rho pathways directs either positive or negative regulation of Rac and thus cell migration, upon activation of a single S1P receptor isoform.

3. Stimulation and inhibition of melanoma cell invasion and metastasis by

S1P receptors

Igarashi and his colleagues [7] first reported that S1P inhibits trans-well migration as well as invasion across the Matrigel layer of B16 mouse melanoma cells. They showed that S1P immobilized on glass beads exhibited similar inhibitory action on migration of

B16 melanoma cells [12]. They also demonstrated the presence of a single class of specific, binding site for a radio-labelled S1P on B16 melanoma cells. These observations suggested that S1P inhibited melanoma cell migration through an extracellular action by specific binding to a cell surface receptor. On the other hand, other studies suggested S1P-induced migration inhibition through its intracellular action [24]. Our observation that the heterologous expression of S1P₂ mediated cell migration inhibition in CHO cells prompted us to investigate the possibility that S1P₂ receptor might mediate migration inhibition of B16 melanoma cells in response to S1P. We found that B16 melanoma cells detectably expressed S1P₂, but not other S1P receptor subtypes [25]. The S1P₂-selective antagonist JTE013, which specifically blocks S1P binding to S1P₂ and prevents Ca²⁺ mobilization and ERK activation mediated by S1P₂, completely abolished the inhibitory effect of S1P on B16 cell migration. Thus, B16 melanoma cell is the example of inhibitory regulation of cell migration by endogenously expressed S1P₂.

In B16 cells, S1P inhibited and stimulated Rac and RhoA, respectively, as in S1P₂-overexpressing CHO cells, and that both of these responses were totally abrogated

by JTE013. S1P did not affect Cdc42 activity. Consistent with the observation in S1P₂-expressing CHO cells [13], suppression of endogenous Rac activity by adenovirus-mediated expression of N¹⁷Rac resulted in inhibition of migration of B16 cells [25]. On the other hand, the inactivation of cellular Rho by C3 toxin reversed S1P inhibition of migration in B16 cells. In addition, random migration activity of B16 cell in the absence of S1P was also stimulated, rather than inhibited, after C3 toxin pretreatment, suggesting that the basal Rho activity is inhibitory for cell migration. This latter observation is consistent with a previous report in Rat1 fibroblasts [18]. Similar to CHO cells, the Rho kinase inhibitors Y27632 or HA1077, at the concentrations that effectively inhibited stress fiber formation, did not reverse S1P inhibition of migration. The results indicate that a RhoA effector other than Rho kinase, and cell responses different from formation of stress fibers or focal adhesions, are responsible for mediating negative regulation of cell motility and invasion by S1P.

S1P₂ also mediated inhibition of B 16 cell invasion across the Matrigel matrix, which is Rac-dependent [25]. With regard to the latter point, it is of note that Rac1 is reported to mediate matrix metalloproteinase (MMP)-2 activation in fibrosarcoma cells [26].

Requirement of Rac activity for both cell motility and invasion through extracellular matrix has been reported in Rat1 fibroblasts and T lymphoma cells as well [18]. Wang et al. [24] showing that S1P inhibited migration of MDA-MB-231 breast carcinoma cells which express S1P₁ and S1P₃ but not S1P₂, and postulated that this S1P-inhibitory action of tumor cell migration was due to an intracellular action of S1P. It is possible that the role for Rac in regulation of migration or invasion might be distinct among cells of epithelial and non-epithelial origin through mechanisms involving the regulation of cell adhesion molecules. Invasion of B 16 cells was inhibited by N¹⁷Rac, like cell migration. On the other hand, unlike cell migration, invasion in the absence of S1P was inhibited by C3 toxin, suggesting that invasion is dependent upon both Rac and Rho. Inhibition of invasion by the expression of N¹⁷RhoA, as well as V¹⁴RhoA, was reported previously in fibroblasts [18]. Despite this, the inhibitory effect of S1P on B16 cell invasion was partially reversed by C3 toxin pretreatment, underscoring the role for Rho in S1P inhibition of invasion. These composite observations indicate that S1P₂-mediated Rho stimulation and Rac inhibition are both involved as mechanisms for S1P-induced inhibition of B16 cell migration and invasion. However, Rho stimulation

by itself does not appear to be sufficient for migration inhibition in the absence of Rac inhibition. This notion is based on the observation obtained in S1P₃-overexpressing B16 cells (see below), in which the addition of S1P together with JTE013 stimulated RhoA activity via S1P₃, but stimulated, rather than inhibited, Rac activity and migration. Rho may be involved in migration and invasion inhibition through negatively regulating Rac, as in migration inhibition in CHO cells [14].

Metastasis is a temporo-spacially architected multistep phenomenon that takes place in the context of host environment. Rho family GTPases and their extracellular regulators were implicated in transformation and invasive phenotypes [18], however, their roles in metastasis *in vivo* were largely unknown. B16 cells that are injected into mouse tail veins form multiple metastatic nodules in the lung two or three weeks later. We found that daily intraperitoneal injection of S1P reduced the number of metastatic nodules [27]. More interestingly, transient treatment of B16 cells with S1P just before the tail vein injection also effectively reduced the number of metastatic nodules, suggesting that S1P might inhibit very early steps of pulmonary metastasis including attachment to the pulmonary capillary endothelium. Adenovirus-mediated transient

expression of N¹⁷Rac markedly inhibited pulmonary metastasis, suggesting that Rac inhibition might underly S1P inhibition of metastasis. The stable overexpression of S1P₂ augmented S1P-induced cell migration inhibition compared to vector control cells, whereas the overexpression of either S1P₁ or S1P₃ greatly attenuated S1P inhibition of migration or stimulated migration. In S1P₂-overexpressing cells S1P-induced metastasis inhibition was augmented. In contrast, that of either S1P₁ or S1P₃ resulted in aggravation of metastasis in an S1P-dependent manner. These observations suggest that Rac is required at a very early stage of hematogenous metastasis before invasion or proliferation and that G-protein-coupled receptors could participate in regulation of metastasis in receptor subtype-specific ligand-dependent manners.

4. Differential regulation of Rac and cell migration by endogenous Edg receptors in vascular smooth muscle and endothelial cells

As mentioned above, S1P displays unique bimodal activities on two vascular cell types, endothelial cells [10, 15] and VSMCs [28]. S1P inhibits VSMC migration, whereas it stimulates endothelial cell migration. Both VSMCs and endothelial cells in culture

express multiple S1P receptor subtypes. VSMCs express abundant levels of S1P₂ and S1P₃, and endothelial cells express S1P₁ and S1P₃ [28, 29]. The expression of S1P₁ in VSMCs and S1P₂ in endothelial cells is very faint. The observations, together with the fact that S1P₁ and S1P₃ are chemotactic whereas S1P₂ is inhibitory, suggest that the vascular cell type-specific, distinct expression pattern of the S1P receptor subtypes can account for differential cell motility responses of the two vascular cell types. The S1P receptors exerted bimodal regulation on Rac activity in vascular cells; S1P inhibited PDGF-induced Rac activation in VSMCs although S1P alone did not affect Rac very much, whereas S1P by itself robustly stimulated Rac activity in endothelial cells. The overexpression of S1P₁ in VSMCs rendered the cells chemotactic toward S1P with elevation of Rac, whereas that of S1P₂ augmented S1P inhibition of PDGF-directed chemotaxis. The overexpression of S1P₁ in endothelial cells also made the cells repulsive in response to S1P. These observations are consistent with the notion that an integration of the S1P receptor subtype-selective, positive and negative signals on cellular Rac activity is a critical determinant for eventual direction of regulation on cell motility by S1P.

5. Future directions

The S1P receptors, especially S1P₁, S1P₂ and S1P₃, are expressed widely in a variety of tissues during the fetal life as well as the adult life. Both attractive and repulsive signals guide migrating cells in tissue remodeling and angiogenesis as well as organogenesis and morphogenesis. It is likely that S1P₂, a unique negative regulator of Rac, might be involved in these pathological and physiological processes in concert with S1P₁ and S1P₃, which are positive regulators of Rac, and presumably also other S1P receptors. It would be particularly interesting to see whether these S1P receptors play any role in pathological processes such as inflammation, tumor cell progression and atherosclerosis. Elucidation of pathological roles for the S1P signaling system is expected to lead to development of novel therapeutic strategies including the use of specific antagonists and agonists for S1P receptor subtypes and inhibitors of synthesis and degradation of S1P, and therefore should deserve intensive investigation.

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Figure legend

Figure 1. Bimodal regulation of the Rho family GTPases and cell migration by the attractant receptors S1P₁ and S1P₃, and the repellent receptor S1P₂. S1P₁ and S1P₃ mediate Rac activation and chemotaxis via Gi. S1P₂ mediates Rac inhibition and migration inhibition (chemorepulsion), which is mediated by G12/13 and Rho. S1P₃ also stimulates Rho and generates an inhibitory signal for Rac, which is uncovered by inactivation of Gi with pertussis toxin.

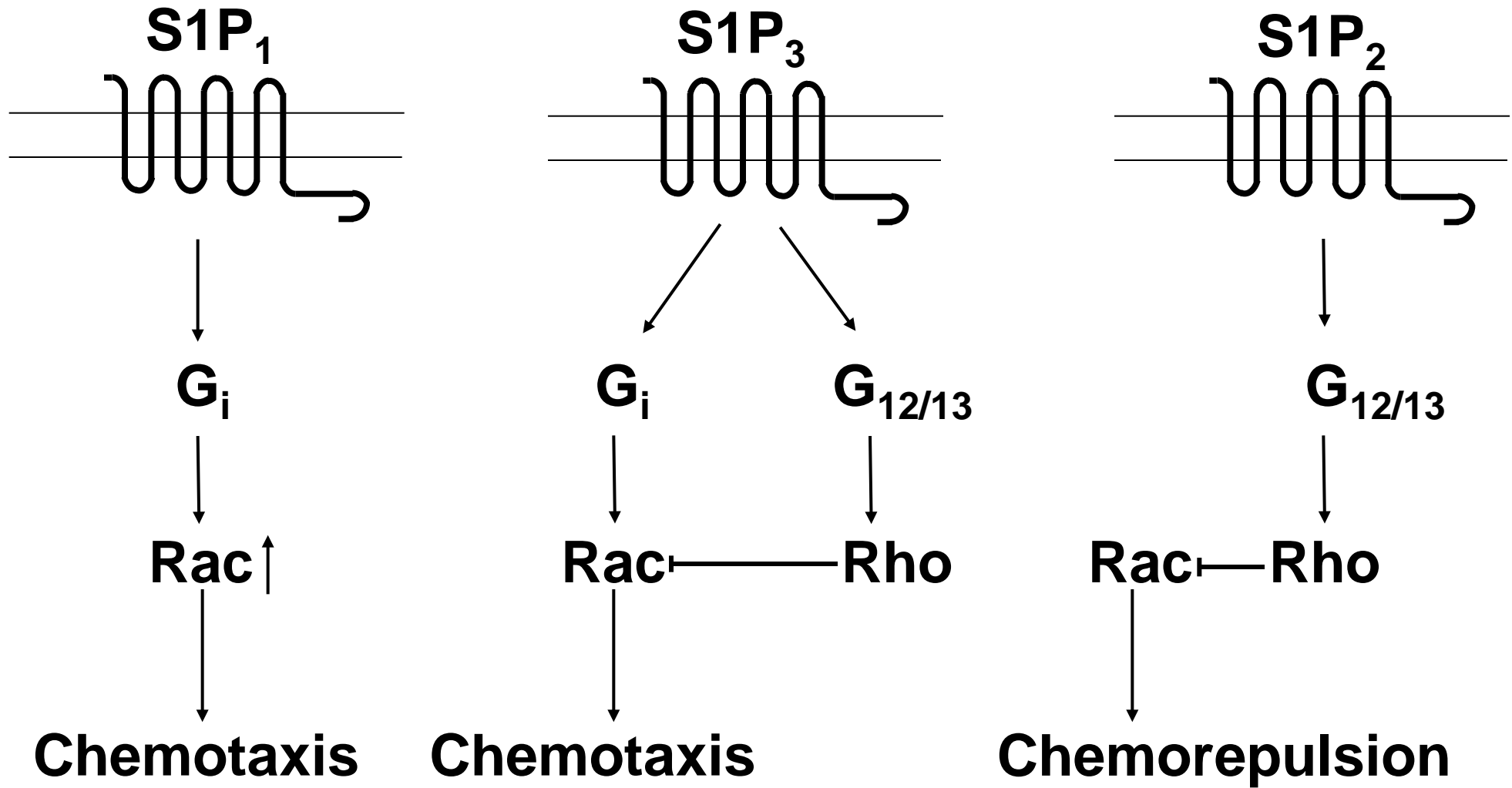


Figure 1