Sphingosine-1-phosphate receptor subtype-specific positive and negative regulation of Rac and haematogenous metastasis of melanoma cells

Hironori YAMAGUCHI*, Joji KITAYAMA*, Noriko TAKUWA†, Kayo ARIKAWA†‡, Isao INOKI†, Kazuhiko TAKEHARA‡, Hirokazu NAGAWA* and Yoh TAKUWA†¹

*Department of Surgical Oncology, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan, †Department of Physiology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan, and ‡Department of Dermatology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan

We have recently reported that S1P (sphingosine-1-phosphate) differentially regulates cellular Rac activity and cell migration in either a positive or a negative direction via distinct G-protein-coupled receptor subtypes, i.e. S1P₁/Edg1 (endothelial differentiation gene) and S1P₂/Edg5 respectively, when each of the S1P receptor subtypes is expressed in CHO (Chinese-hamster ovary) cells. In B16F10 mouse melanoma cells, in which S1P₂, but not the other S1P receptor subtypes, is endogenously expressed, S1P inhibited cell migration with concomitant inhibition of Rac and stimulation of RhoA in dose-dependent manners. Overexpression of S1P₂ in the melanoma cells resulted in potentiation of S1P inhibition of both Rac and cell migration. In contrast, overexpression of S1P₁ led to stimulation of cell migration, particularly at the lower S1P concentrations. Treatment of B16F10

cells with S1P inhibited lung metastasis 3 weeks after injection into mouse tail veins. Intriguingly, overexpression of S1P₂ greatly potentiated the inhibition of metastasis by S1P, whereas that of S1P₁ resulted in aggravation of metastasis. Suppression of cellular Rac activity by adenovirus-transduced expression of N¹⁷Rac, but not N¹⁹RhoA, strongly inhibited cell migration *in vitro* and lung metastasis *in vivo*. These results provide the first evidence that G-protein-coupled receptors could participate in the regulation of metastasis, in which ligand-dependent, subtype-specific regulation of the cellular Rac activity is probably critically involved as a mechanism.

Key words: B16 melanoma cells, metastasis, Rac, sphingosine-1-phosphate, S1P/Edg receptor.

INTRODUCTION

S1P (Sphingosine-1-phosphate) is a pleiotropic lysophospholipid mediator that is present in plasma and is released in a large amount from activated platelets [1,2]. Investigations from several laboratories, including our own, have identified the Edg (endothelial differentiation gene) family of G-protein-coupled receptors for S1P, which include the ubiquitously expressed isotypes S1P₁/Edg1, S1P₂/Edg5/AGR16/H218 and S1P₃/Edg3 [1–6]. It has been known for a decade that S1P regulates cell motility in either a positive or a negative direction, which appeared to depend upon the cell type tested [2,3,7–9]. Thus S1P inhibits motility of certain types of murine and human tumour cells, including B16 melanoma cells and vascular smooth muscle cells, whereas it acts as a chemoattractant for other cell types, such as vascular endothelial cells. We recently identified S1P₂ as a chemorepellant receptor and the S1P–S1P₂ system as the first example of a receptor-mediated signalling that negatively regulates cellular Rac activity [10]. We and others have also found that S1P₁ and S1P₃ mediate activation of Rac and function as typical chemotactic receptors [10,11]. These findings strongly suggest that cell-type-dependent, bimodal regulation of cell motility by S1P reflects the S1P-receptor-mediated, subtype-specific biological activities. Differently from Rac, whose activity is regulated in the same direction as cell motility by the S1P signalling system, we and others found that the repulsive receptor S1P₂ [10], and the

attractive receptor S1P₃ [10,11], similarly mediated stimulation of cellular RhoA activity. S1P did not affect cellular Cdc42 activity through either of the S1P receptor subtypes. In addition, the expression of dominant negative mutants N¹⁷Rac or N¹⁷Cdc42, but not N¹⁹RhoA, inhibited cell migration [10]. These observations strongly suggest that cellular Rac and Cdc42 activities, but not necessarily RhoA activity, are required for cell motility, and that S1P receptor subtype-specific bimodal regulation of Rac activity is involved as a mechanism for the S1P-elicited distinct regulation of cell motility [3].

Metastasis is a temporo-spatially arranged multistep phenomenon that includes cell motility, extracellular-material degradation and proliferation. Previous studies [12–17] have implicated Rho family GTPases and their activator GEFs (guanine nucleotide-exchange factors) in transformation and invasive phenotypes. However, most of these studies were performed in *in vitro* cultured cell systems, and their precise roles in development of metastasis *in vivo* have remained elusive thus far.

Our observations on S1P-receptor-mediated, subtype-specific bimodal regulation of Rac and cell migration prompted us to examine the possible involvement of the S1P signalling system in the regulation of metastasis *in vivo*. By employing a well-characterized mouse tail vein injection model of haematogenous metastasis, we found that S1P receptors could positively and negatively regulate metastasis in receptor-subtype-specific, ligand-dependent manners. We also adopted adenovirus-mediated

Abbreviations used: DH-S1P, dihydrosphingosine-1-phosphate; DMEM, Dulbecco's modified Eagle's medium; Edg, endothelial differentiation gene; GEF, guanine nucleotide-exchange factor; i.p., intraperitoneal; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; S1P, sphingosine-1-phosphate.

¹ To whom correspondence should be addressed (e-mail ytakuwa@med.kanazawa-u.ac.jp).

transient expression of N¹⁷Rac to investigate whether indeed Rac activity in tumour cells is required for the development of metastasis.

EXPERIMENTAL

Materials

S1P and DH-S1P (dihydrosphingosine-1-phosphate) were obtained from Biomol (Plymouth Meeting, PA, U.S.A.). Sphingosine was purchased from Avanti (Birmingham, AL, U.S.A.). These lipids were dissolved in DMSO to 2×10^{-3} M, divided into aliquots and stored at -80°C . Final solvent concentrations did not exceed 0.1 % (v/v). Fatty-acid-free BSA was obtained from Sigma (St. Louis, MO, U.S.A.). Mouse monoclonal anti-Rac and anti-RhoA antibodies and a goat polyclonal anti-RhoC antibody were purchased from Upstate Biotechnology (Charlottesville, VA, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) respectively.

Cells, plasmids and adenoviruses

B16 (B16F10) cells, a gift from Dr T. Irimura (Graduate School of Pharmaceutical Sciences, University of Tokyo), were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) foetal calf serum (Equitech-Bio, Ingram, TX, U.S.A.) and antibiotics. The cDNAs of S1P₁ and S1P₂ and adenoviruses encoding dominant-negative mutants N¹⁷Rac, N¹⁹RhoA or LacZ were described previously [10,18–20]. B16 cells were infected with adenoviruses at an MOI (multiplicity of infection) of approx. 100, as described in [10]. This condition conferred expression of LacZ as a marker gene in nearly 100 % of infected cells 48–72 h after infection, when the expression of transduced gene products was maximal. Therefore adenovirus-infected cells were harvested 48 h after infection and were subjected to *in vivo* and *in vitro* assays throughout the present study. To obtain B16 cell clones that stably overexpressed either S1P₁ or S1P₂, a parent B16F10 clone that had been obtained by limiting dilution was co-transfected with each of the S1P-receptor-expression vectors, or the empty vector, together with an expression vector of the neomycin resistance gene (pKM3) using LIPOFECTAMINE™ (Invitrogen, Carlsbad, CA, U.S.A.), and were selected in the presence of 1 mg/ml G418. The receptor-subtype-overexpressing clones, B16-S1P₁-1 and -9, and B16-S1P₂-7 and -14 were identified by Northern blot analysis.

Northern blotting

Total RNA (10 µg), prepared as described previously [21] was analysed for expression of mRNAs of S1P receptors and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) by Northern blotting as described in [21]. The cDNA probes employed were described previously [10,18–20].

Lung metastasis assay

B16 cells were harvested by trypsinization, suspended at a density of 1×10^6 cells/ml in DMEM containing 0.1 % (v/v) fatty-acid-free BSA, and incubated with S1P, other lipids or 0.1 % (v/v) DMSO (vehicle control) for 5 min at room temperature (25 °C). The cells were washed and resuspended in Hanks' solution at a density of 1×10^6 cells/ml at 4 °C and a 100 µl cell suspension was injected into the lateral tail veins of 6-week-old male C57BL/6 mice. In some experiments, mice were injected daily with 10 µg of S1P or 0.1 % (v/v) DMSO in a volume of

100 µl through the i.p. (intraperitoneal) route for 3 weeks. On the first day of this set of experiments, S1P was i.p. injected 30 min before the tail-vein injection of B16 cells. In a different set of experiments, B16 cells were infected with adenoviruses, as described above, and were harvested after 48 h and subjected to a lung metastasis assay. At this time point, viability of adenovirus-infected cells was consistently > 95 %. Actin cytoskeletal changes in N¹⁹RhoA-expressing cells at the time of injection were evaluated by phalloidin staining, as described previously [18,20]. At 3 weeks after injection, mice were killed by an excess amount of pentobarbital, and the lungs were excised, rinsed in Dulbecco's PBS, and fixed overnight in Bouin's solution [22,23]. The metastatic colonies were easily identified by the naked eye as clearly demarcated black or white nodules in the background of normal lung tissue, which stained deep yellow. The numbers of metastatic nodules present over the entire surface of the five lobes of the lung were counted under a dissecting microscope at a magnification of $\times 10$ by an examiner to whom the identity of the sample was not notified. Nodule counting was performed three times per sample and the mean value was determined. All animals received humane care in compliance with our institutional guidelines.

Proliferation assays

To evaluate the ability of the cells used for lung metastasis assays to proliferate, a portion from each cell-treatment group was saved, and was seeded at 4×10^4 cells/well in 2 ml of growth medium in 6-well plates. The cells in each well were maintained in exactly the same way at subconfluent states with routine subcultures every 3 or 4 days throughout the metastasis assay period (21 days). On days 3, 7, 14 and 21 after plating, cells were trypsinized and viable cells were counted in a haemocytometer by Trypan-Blue exclusion. In a different set of experiments, cell proliferation in the presence or absence of S1P was evaluated by using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] cell proliferation assay (Promega) [24]. MTS was added to each of 96 wells 72 h after plating, and the cells were incubated for a further 4 h. The numbers of living cells were determined by measuring attenuation at 490 nm using a 96-well microplate reader (Bio-Rad Model 3550; Hercules, CA, U.S.A.).

Trans-well migration assay, and Rho, Rac and Cdc42 pull-down assays

Haptotactic motility of B16F10 cells was measured in a modified Boyden chamber as described previously [10,18] with a polycarbonate filter (8 µm diameter pores) (Neuroprobe, Cabin John, MA, U.S.A.), the lower side of which was coated with 10 µg/ml fibronectin (Sigma). The cells were suspended at 1×10^6 cells/ml in DMEM containing 0.1 % (v/v) fatty-acid-free BSA and 300 µl of the cell suspension was loaded into the upper well and allowed to migrate across the porous filter for 4 h at 37 °C in a tissue culture incubator, in the presence or absence of S1P in the lower well. In some experiments, adenovirus-infected cells were harvested 48 h after infection and were subjected to migration assays. At the end of the assay, the filter was disassembled from the chambers and the cells that remained attached to both sides of the filter were fixed with methanol. After staining of the cells by using a Diff-Quick staining kit (International Reagents Corp., Kobe, Japan), as described previously [10,18], the cells attached to the upper side of the filter were wiped away, and the number of cells that migrated to the lower side of the filter was determined by measuring attenuation

Figure 1 Northern analyses of naïve B16F10 cells and stable clones that overexpress either S1P₁ (clone B16-S1P₁-1) or S1P₂ (clone B16-S1P₂-7) as compared with vector-transfected cells (vector)

Total RNAs from mouse brain, spleen and rat aortic vascular smooth muscle cells (VSMC) as positive controls for S1P₃, S1P₄ and S1P₅ transcripts were also analysed. The other clones, B16-S1P₁-9 and B16-S1P₂-14, showed similar results.

at 595 nm using the Model 3550 96-well microplate reader. Determinations of cellular amounts of GTP-bound, active forms of Rac, Cdc42 and RhoA by pull-down-assay techniques were described in detail previously [10,18,27]. A portion (1/200 for Rac and RhoA, and 1/30 for Cdc42) of cell extracts was also analysed by Western blotting for the amounts of total Rac and RhoA. The bands of GTP-Rac and GTP-RhoA were densitometrically quantified and values were expressed as fold increases over the basal unstimulated values (= 1) [10].

Statistics

The data are presented as means $\pm$ S.E.M. The statistical significance of differences between the two groups was determined by Student's *t* test, whereas multiple comparisons were analysed by Duncan's test.

RESULTS

Northern blot analysis revealed that naïve B16F10 cells showed a moderate level of endogenous S1P₂ mRNA. The expression level of endogenous S1P₂ mRNA remained relatively constant in the cells that overexpressed either S1P₁ or S1P₂, or in vector-control cells (Figure 1). None of these cell types expressed detectable amount of the other S1P receptor subtypes (Figure 1).

We first examined and compared the effects of S1P on haptotactic cell migration *in vitro* (Figure 2). S1P, when present in the lower well of a Boyden chamber, inhibited the migration of vector-control B16 cells in a dose-dependent manner, with 10, 30 and

Figure 2 Differential effects of S1P on haptotactic cell migration of the S1P-receptor-subtype-overexpressing and vector-control cells toward immobilized fibronectin

Vector-control B16F10 cells and the B16F10 clones that overexpress S1P₁ (clone B16-S1P₁-1) or S1P₂ (clone B16-S1P₂-7) were allowed to migrate across the porous membrane in the presence or absence of the indicated concentrations of S1P in the lower wells for 4 h. Results are means $\pm$ S.E.M. (*n* = 3). *, *P* < 0.05; ***, *P* < 0.001, compared with the vehicle (DMSO) control. The other clones showed similar results.

56% inhibition at 10^{-9} , 10^{-8} and 10^{-7} M respectively, as compared with the basal value in the absence of S1P (Figure 2). Similar results were observed for naïve B16 cells. S1P₂-overexpressing cells (clone B16-S1P₂-7) were more sensitive to S1P; S1P at 10^{-9} M induced 65% inhibition of cell migration. In sharp contrast, S1P did not inhibit the migration of S1P₁-overexpressing cells (clone B16-S1P₁-1), except at the highest concentration examined, and it even stimulated migration at lower concentrations (10^{-9} M and 10^{-8} M). The enhancement of inhibition of cell migration by overexpression of S1P₂, and the attenuation by that of S1P₁, were also observed for the other B16 clones (B16-S1P₁-9 and B16-S1P₂-14) (results not shown).

We next studied the effects of S1P on cellular activity of Rac in these B16 cell types (Figure 3A). The expression level of total Rac protein or the basal level of GTP-bound active form of Rac (GTP-Rac) in the absence of S1P were not affected by overexpression of either S1P₁ or S1P₂. S1P induced dose-dependent reductions in the amount of GTP-Rac in vector-transfected B16F10 cells, with a 35% reduction at 10^{-8} M of S1P (Figure 3A). The suppression in the GTP-Rac level persisted for < 10 min (results not shown). Similar results were obtained for naïve B16F10 cells. S1P₂-overexpressing cells were more sensitive to S1P inhibition of cellular Rac activity; in this cell type, 10^{-9} M of S1P induced a significant 40% reduction in the level of GTP-Rac. In contrast, overexpression of S1P₁ rendered the cells resistant to S1P inhibition of Rac, and S1P at $\leq 10^{-8}$ M did not inhibit GTP-loading of Rac.

The expression level of total RhoA protein and the basal RhoA activity were comparable among the different B16 cell types (Figure 3B). S1P increased the amounts of GTP-RhoA in all of the three B16 cell types (Figure 3B). We also tried to determine the activity of RhoC [25]; however, its expression in B16 cells was below a detectable level. We measured the activity of Cdc42 in B16 cells and found that S1P at $\leq 10^{-7}$ M did not affect Cdc42 activity for at least 30 min in naïve B16F10 cells (Figure 3C) or the vector-control cells.

We then examined whether or not S1P could modulate pulmonary metastasis of B16F10 cells (Figure 4). We found that a

Figure 3 S1P regulates cellular Rac and RhoA activities in receptor-subtype-specific, ligand-dependent manners

B16 clones that overexpress either of the S1P receptor subtypes (B16-S1P₁-1 and B16-S1P₂-7) and vector-transfected B16 cells were stimulated with S1P at indicated concentrations or 0.1% (v/v) DMSO (vehicle control) for 1 min. Results are means $\pm$ S.E.M. ($n = 3$). Representative immunoblots are shown. (A) GTP-Rac in S1P-stimulated cells. (B) GTP-RhoA in S1P-stimulated cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$ compared with the vehicle (DMSO) control. (C) GTP-Cdc42 in S1P-stimulated parental B16 cells.

single and transient treatment of naïve B16F10 cells with S1P (10^{-7} M) for 5 min immediately before the tail vein injection resulted in a 40% decrease in the number of pulmonary metastatic nodules which were examined 3 weeks later (Figure 4A). Daily i.p. injection of S1P ($10 \mu\text{g/day}$) into mice, with the first S1P bolus given 30 min before injection of untreated B16 cells, also caused a similar extent of inhibition on pulmonary metastasis (Figure 4A). The combination of both treatments caused a larger decrease (by 60%) in metastasis than either managed alone. We also examined the effects of DH-S1P, which is a less potent agonist for S1P₂ [10,26], and sphingosine, which is not an agonist for the S1P receptors, on the melanoma cell metastasis (Figure 4B). The effect of DH-S1P was much limited compared with S1P, and sphingosine was essentially ineffective, consistent with the receptor-mediated actions of S1P. Shown in Figure 4(C) are the effects of pre-treatment with various concentrations of S1P on pulmonary metastasis of B16F10 vector-control cells and the cells that overexpress either S1P₁ or S1P₂. In vector-control cells, S1P at 10^{-7} M, but not at lower concentrations, caused a significant inhibition (56%) of the metastatic nodule formation, consistent with the results obtained in naïve B16 cells (Figures 4A and 4B). We found that S1P₂-overexpressing cells were approx. two orders of magnitude more sensitive to S1P inhibition of metastasis compared with the vector control; pre-treatment with S1P at 10^{-9} M resulted in a 53% inhibition of metastasis, with a maximal 81% inhibition at 10^{-7} M of S1P. Another S1P₂-overexpressing clone (B16-S1P₂-14) also showed augmented inhibition of metastasis by S1P; the numbers of metastatic nodules after S1P pre-treatment at 10^{-9} M and 10^{-7} M were $57 \pm 5\%$ and $24 \pm 4\%$ of the vehicle control respectively ($n = 8$). The results contrast sharply with those obtained with S1P₁-overexpressing cells, in which S1P at 10^{-9} M and 10^{-8} M stimulated pulmonary metastasis markedly (Figure 4C). We observed a similar stimulatory effect of S1P on metastasis in a different S1P₁-overexpressing clone (B16-S1P₁-9); the numbers of metastatic nodules after S1P treatment at 10^{-9} M were $206 \pm 11\%$ of the vehicle control ($n = 8$). Without

S1P pre-treatment, there was no difference in the numbers of metastatic nodules between S1P₁- or S1P₂-overexpressing B16 cells and vector-control cells. These results suggest that S1P₂ is the receptor subtype responsible for the S1P inhibition of metastasis, whereas S1P₁ mediates S1P stimulation, rather than inhibition, of metastasis. Blunting of the metastasis-stimulatory effect of S1P at high concentrations in S1P₁-expressing cells may reflect counteractions between exogenously expressed S1P₁ and endogenous S1P₂ as observed in *in vitro* systems [27].

We adopted adenovirus-transduced expression of a dominant-negative Rac, N¹⁷Rac, to evaluate the roles of endogenous Rac activity in tumour cells in developing metastasis. We infected naïve B16 cells with adenoviruses encoding either N¹⁷Rac, N¹⁹RhoA or β -galactosidase (LacZ). After 48 h, when the expression levels were maximal (Figure 5A), the cells were harvested and were subjected to the metastasis assay (Figure 5D), as well as migration (Figure 5B) and proliferation (Figure 5C) assays. We observed that the expression of N¹⁷Rac, but not N¹⁹RhoA, markedly (by 76%) inhibited trans-well migration of B16 cells (Figure 5B). The results indicate that Rac is required for migration of B16F10 cells, and that adenovirus-mediated expression of N¹⁷Rac effectively inhibits endogenous Rac activity. In addition, we found that the expression of N¹⁷Cdc42 also substantially inhibited cell migration *in vitro* as compared with the LacZ control (Figure 5B), although S1P did not affect cellular Cdc42 activity (Figure 3C). These observations suggest that the basal activity of Cdc42 is necessary for haptotactic cell motility of B16 cells.

As shown in Figure 5(D), the expression of N¹⁷Rac caused a 74% decrease in the number of metastatic nodules as compared with the LacZ control, providing compelling *in vivo* evidence that cellular Rac activity is required for metastasis. The expression of N¹⁹RhoA, which inhibited S1P-induced actin stress-fibre formation in B16 cells, caused a slight, although not significant, inhibition of metastasis formation.

To evaluate critically a possibility that the inhibition of metastasis by N¹⁷Rac expression had been the result of cell death,

Figure 4 S1P inhibits pulmonary metastasis of B16F10 cells

(A) Numbers of pulmonary metastatic nodules formed 3 weeks after injection of naïve B16F10 cells into tail veins. S1P was either added to the cells for 5 min just before injection (pre-treatment at a final concentration of 10^{-7} M), and/or administered to mice daily ($10 \mu\text{g/day}$) via the i.p. route. (B) Pre-treatment for 5 min with the weak S1P agonist DH-S1P (10^{-7} M), but not sphingosine (Sph), modestly inhibited lung metastasis. (C) S1P₂ overexpression augments S1P inhibition of metastasis, whereas that of S1P₁ aggravates it. Stable B16 clones, B16-S1P₁-1 and B16-S1P₂-7, and vector-transfected B16 cells were pre-treated with S1P at the indicated concentrations for 5 min before injection into mice. Additional clones showed similar receptor-subtype-specific, ligand-dependent responses (see text). Results are means $\pm$ S.E.M. (A) and (C), $n=8$; (B), $n=5$. *, $P < 0.05$; **, $P < 0.1$; ***, $P < 0.001$, compared with the vehicle (DMSO) control.

we plated a portion of the adenovirus-transduced cell populations employed for metastasis assays and examined their proliferation *in vitro* by counting numbers of the cells at 3, 7, 14 and 21 days after the tail vein injection. We found that the number of the cells relative to the LacZ control was decreased by 18% and 15% in the cells transduced with adenoviruses encoding N¹⁷Rac and N¹⁹RhoA respectively by day 3 (Figure 5C). However, there was no additional reduction in the cell number thereafter, consistent with the transient nature of the adenovirus-transduced expression system. The same extent (18%) of a modest inhibition of cell proliferation during the first 3 days for N¹⁷Rac-expressing cells was also observed when the MTS proliferation assay was adopted. The results strongly suggest that a mechanism other than inhibition of proliferation is involved as a major mechanism for inhibition of metastasis.

DISCUSSION

The present study demonstrates for the first time that S1P inhibited metastasis of B16F10 melanoma cells in S1P-receptor-isoform-

specific, ligand-dependent manners (Figures 4A–4C) in the tail vein injection model. We have also demonstrated in the present study that inhibition of endogenous Rac activity in mouse B16 melanoma cells by adenovirus-mediated transient expression of N¹⁷Rac potently inhibited metastasis (Figure 5D).

Several lines of evidence obtained in the present study indicate that S1P₂ is responsible for mediating S1P inhibition of metastasis. They include (i) naïve B16 cells detectably expressed mRNA of S1P₂, but not the other members of the S1P receptors (Figure 1); (ii) the overexpression of S1P₂ strongly potentiated S1P inhibition of metastasis, compared with vector-control cells, whereas that of S1P₁ resulted in aggravation of metastasis in response to S1P (Figure 4); (iii) consistent with the results on metastasis, the overexpression of S1P₂, but not S1P₁, strengthened the S1P inhibition of migration (Figure 2); (iv) DH-S1P, which is less potent as an agonist for S1P₂ than S1P by at least one order of magnitude [26], was less effective in inhibiting metastasis compared with S1P in naïve B16F10 cells, whereas sphingosine, which is not an agonist for S1P receptors, was totally ineffective (Figure 4B); (v) the overexpression of S1P₂, but

Figure 5 Adenovirus-mediated expression of N¹⁷Rac, but not N¹⁹RhoA, in naïve B16 cells potentially inhibits migration *in vitro* and pulmonary metastasis *in vivo*, but only modestly inhibits proliferation

Cells were infected with adenoviruses expressing either N¹⁷Rac, N¹⁹RhoA or LacZ for 48 h before being harvested, counted and subjected to Western analysis (A), migration (B) or proliferation (C) assays, or injected into mouse tail veins (D). (A) Expression of proteins of N¹⁷Rac and N¹⁹RhoA examined at the time of injection (2 days after infection). wt, wild-type. (B) Expression of N¹⁷Rac and N¹⁷Rac42, but not N¹⁹RhoA, strongly inhibits haptotactic migration of B16F10 cells as compared with the LacZ control. (C) Cell proliferation in the presence of foetal calf serum (10%). Adenovirus-transduced cells of the same population as used for metastasis assays were maintained at subconfluent states *in vitro* for 3 weeks in parallel to metastasis experiments, with routine subcultures of each population performed in exactly the same way. At the indicated time points, the numbers of the viable cells were counted and expressed as percentages of the LacZ control. (D) Numbers of pulmonary metastatic nodules examined 3 weeks after injection. Results in (B)–(D) are means $\pm$ S.E.M. (B) and (C), $n = 3$; (D), $n = 8$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with the LacZ control.

not S1P₁, potentiated S1P inhibition of cellular Rac activity (Figure 3A), which is essential for the processes of cell migration and metastasis (Figure 5D), like S1P inhibition of metastasis and cell motility.

Since Rac is essential for metastatic process (Figure 5D), it is reasonable to consider that S1P inhibition of metastasis is mediated at least in part through S1P₂-selective, receptor-mediated negative regulation of Rac activity. We found in the present study that pre-treatment with S1P for 5 min was sufficient for reducing pulmonary metastasis formation (Figures 4A–4C). With this regard, we observed that S1P-induced, S1P₂-mediated suppression of cellular Rac activity was sustained for > 10 min in B16 cells, as well as in other cell types [10,18]. These observations suggest that a transient change in the cellular Rac activity serves as a crucial signal for such long-term biological responses as metastasis formation in the tail vein injection model. A previous study demonstrated that injected B16 cells formed microemboli in pulmonary capillary beds within several minutes of intravenous injection, and that this process is critical for later

stages of metastasis to proceed [28]. It is suggested that very early events in the process of haematogenous metastasis, including microembolus formation, is dependent upon cellular Rac activity. We are now investigating this possibility.

The present results, however, do not exclude roles for Rac in the following stages of metastasis, in which tumour cells invade and proliferate. In support of this latter possibility, we observed that the expression of N¹⁷Rac in B16F10 cells markedly inhibited migration (Figure 5B) and invasion [29] through Matrigel, and also suppressed proliferation, although to a much limited extent (Figure 5C). In addition, we found that daily i.p. administration of S1P, in combination with S1P pre-treatment of the tumour cells before intravenous injection, caused an additional reduction in metastasis (Figure 4A), suggesting that late stages of metastasis development are also modulated by S1P. Thus Rac may be necessary for multiple processes of metastasis. To explore roles for Rac in more depth in the late stages of metastasis, different experimental designs would be required, such as inducible expression of N¹⁷Rac and delayed administration of S1P in future

studies. During the final stage of the preparation of the present paper, it was reported that stable overexpression of N¹⁷Rac in rat mammary adenocarcinoma cells resulted in reduced metastasis formation [30]. However, inhibitory effects of a receptor ligand on metastasis and cellular Rac activity were not addressed. It was also reported previously that stable overexpression of a constitutively active form of Rac in NIH3T3 fibroblasts resulted in stimulation of metastasis [31].

In the present study, we found that the expression of N¹⁹Rho slightly inhibited metastasis (Figure 5D), without inhibiting migration (Figure 5B). It is possible that a modest (15%) inhibition of proliferation by transient N¹⁹RhoA expression (Figure 5C) could be responsible, at least in part, for the observed slight inhibition of metastasis. However, we also found that the C3 toxin, which exerted a stronger inhibition of Rho than N¹⁹Rho expression, as estimated by its inhibitory effect on precipitated RhoA, failed to inhibit migration, but significantly inhibited metastasis. These latter observations indicate that, although migration may not require RhoA, metastasis requires RhoA activity in tumour cells *in vivo*, which is consistent with the suggested role for Rho in metastasis in previous reports [30,31]. With respect to this, it is of note that S1P, especially at its lower concentrations, stimulated metastasis of S1P₁-overexpressing B16 cells (Figure 4C). This might have resulted from RhoA stimulation without Rac inhibition in this cell type, which was most likely brought about by the counteraction of S1P₂-mediated Rac inhibitory signal by S1P₁-mediated Rac stimulatory signal. Precise roles of RhoA in each of the multistep processes of metastasis should be elucidated in future studies.

It is also the subject of future work to determine whether or not Cdc42 is required for metastasis. In the present study, we observed that S1P was without effect on cellular Cdc42 activity (Figure 3C). We also found that the expression of N¹⁷Cdc42 inhibited haptotactic cell motility (Figure 5B). These observations together may suggest that the basal activity of Cdc42 is required for migration of B16 cells. Alternatively, the expression of N¹⁷Cdc42 might have inhibited a GEF(s) for Rac, resulting in inhibition of Rac activation and thereby of migration, since several GEFs are known to act on both Rac and Cdc42 [32].

In the tail vein injection model, we failed to detect any change in the extent of metastasis of B16 cells without S1P pretreatment by overexpression of either S1P₁ or S1P₂. It has been reported that S1P is present in the circulation at a concentration of approx. 200 nM in forms mostly bound to plasma proteins, which give rise to 7% in man [33,34]. Since the present results were obtained in the presence of 0.1% (v/v) BSA in medium for the treatment of B16 cells, it is reasonable to consider that much higher concentrations of S1P would have been required if 7% plasma proteins were present in the medium. Although the free S1P concentration in the mouse plasma is not known at present, it is likely to be below the threshold level that would be required to affect metastasis of intravenously injected B16 cell clones in the present experimental design, in which plasma S1P must act on injected cells before they reach pulmonary microvasculature where critical initial events of metastasis take place. It is suggested that, in a living animal, the sites of platelet activation, which often associate with inflammation and immune responses, probably face higher concentrations of S1P than plasma, as platelets reportedly release a large amount of S1P upon activation [33]. It is a possibility that deserves future study that increased levels of S1P at the site of tumour–host interaction would affect motility of tumour cells through S1P-receptor-isotype-specific manners, resulting in positive or negative regulation of tumour cell invasion, extravasation and metastasis. In addition to platelets, a variety of cells and tissues are

potential sources of extracellular S1P, as sphingosine kinase, the S1P synthesizing enzyme, is widely expressed [35]. In addition, it is reported that cytokines such as TNF α (tumour necrosis factor α) are capable of stimulating sphingosine kinase activity [36]. On the other hand, in light of the fact that the S1P receptor subtypes such as S1P₁, S1P₂ and S1P₃ are ubiquitously expressed [1,3–5], it is not surprising to find S1P receptor mRNA expression in a variety of tumour cell types of different tissue origins. (H. Yamaguchi and Y. Takuwa, unpublished work) These composite observations raise an intriguing possibility of targeting tumour cell Rac activity through endogenous G-protein-coupled S1P receptors. Development of S1P₂-selective agonists and S1P₁-specific antagonists may enable tailor-made medical intervention against metastasis.

We thank Dr T. Irimura for providing us with B16F10 cells. Dr N. Sugimoto, Dr H. Okamoto and Ms C. Uchikawa are appreciated for technical help, and Ms N. Yamaguchi for secretarial assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, the Japan Society for the Promotion of Science Research for the Future Program and the Hoh-Ansha Foundation.

REFERENCES

- 1 Spiegel, S. and Milstien, S. (2000) Sphingosine-1-phosphate: signaling inside and out. *FEBS Lett.* **476**, 55–57
- 2 Igarashi, Y. (1998) Sphingosine-1-phosphate as an intercellular signaling molecule. *Ann. N. Y. Acad. Sci.* **845**, 19–31
- 3 Takuwa, Y. (2002) Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta* **1582**, 112–120
- 4 Hla, T., Lee, M. J., Ancellin, N., Paik, J. H. and Kluk, M. J. (2001) Lysophospholipids – receptor revelations. *Science* **294**, 1875–1878
- 5 Fukushima, N., Ishii, I., Contos, J. J. A., Weiner, J. A. and Chun, J. (2001) Lysophospholipid receptors. *Annu. Rev. Pharmacol. Toxicol.* **41**, 507–534
- 6 Tigyi, G. (2001) Physiological responses to lysophosphatidic acid and related glycerophospholipids. *Prostaglandins Other Lipid Mediators* **64**, 47–62
- 7 Sadahira, Y., Ruan, F., Hakomori, S. and Igarashi, Y. (1992) Sphingosine 1-phosphate, a specific endogenous signaling molecule controlling cell motility and tumor cell invasiveness. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9686–9690
- 8 Yamamura, S., Yatomi, Y., Ruan, F., Sweeney, E. A., Hakomori, S. and Igarashi, Y. (1997) Sphingosine 1-phosphate regulates melanoma cell motility through a receptor-coupled extracellular action and in a pertussis toxin-insensitive manner. *Biochemistry* **36**, 10751–10759
- 9 Wang, F., Van Brocklyn, J. R., Edsall, L., Nava, V. E. and Spiegel, S. (1999) Sphingosine-1-phosphate inhibits motility of human breast cancer cells independently of cell surface receptors. *Cancer Res.* **59**, 6185–6191
- 10 Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H. and Takuwa, Y. (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol. Cell. Biol.* **20**, 9247–9261
- 11 Paik, J. H., Chae, S. S., Lee, M. J., Thangada, S. and Hla, T. (2001) Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and Rho-dependent activation of α v β 3- and β 1-containing integrins. *J. Biol. Chem.* **276**, 11830–11837
- 12 Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J. and Parise, L. V. (1997) Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature (London)* **390**, 632–636
- 13 Shaw, L. M., Rabinovitz, I., Wang, H. H.-F., Toker, A. and Mercurio, A. M. (1997) Activation of phosphoinositide 3-OH kinase by the α 6 β 4 integrin promotes carcinoma invasion. *Cell* **91**, 949–960
- 14 Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* **279**, 509–514
- 15 Yoshioka, K., Matsumura, F., Akedo, H. and Itoh, K. (1998) Small GTP-binding protein Rho stimulates the actomyosin system, leading to invasion of tumor cells. *J. Biol. Chem.* **273**, 5146–5154
- 16 Banyard, J., Anand-Apte, B., Symons, M. and Zetter, B. R. (2000) Motility and invasion are differentially modulated by Rho family GTPases. *Oncogene* **19**, 580–591
- 17 Whitehead, I. P., Zohn, I. E. and Der, C. J. (2001) Rho GTPase-dependent transformation by G protein-coupled receptors. *Oncogene* **20**, 1547–1555

- 18 Ryu, Y., Takuwa, N., Sugimoto, N., Sakurada, S., Usui, S., Okamoto, H., Matsui, O. and Takuwa, Y. (2002) Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ. Res.* **90**, 325–332
- 19 Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H. and Takuwa, Y. (1998) EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a $G_{i/o}$ to multiple signaling pathways, including phospholipase C activation, Ca^{2+} mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J. Biol. Chem.* **273**, 27104–27110
- 20 Gonda, K., Okamoto, H., Takuwa, N., Yatomi, Y., Okazaki, H., Sakurai, T., Kimura, S., Sillard, R., Harii, K. and Takuwa, Y. (1999) The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. *Biochem. J.* **337**, 67–75
- 21 Takuwa, N. and Takuwa, Y. (1999) Cyclin D1 expression mediated by phosphatidylinositol 3-kinase through mTOR-p70^{S6K}-independent signaling in growth factor-stimulated NIH3T3 fibroblasts. *Mol. Cell. Biol.* **19**, 1346–1358
- 22 Poste, G., Doll, J., Hart, I. R. and Fidler, I. J. (1980) *In vitro* selection of mouse B16 melanoma variants with enhanced tissue-invasive properties. *Cancer Res.* **40**, 1636–1644
- 23 Honn, K. V. (1983) Inhibition of tumor cell metastasis by modulation of the vascular prostacyclin/thromboxane A_2 system. *Clin. Exp. Metastasis* **1**, 103–114
- 24 Cory, A. H., Owen, T. C., Barltrop, J. A. and Cory, J. G. (1991) Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Comm.* **3**, 207–212
- 25 Clark, E. A., Golub, T. R., Lander, E. S. and Hynes, R. O. (2000) Genomic analysis of metastasis reveals an essential role for RhoC. *Nature (London)* **406**, 532–535
- 26 Tamama, K., Kon, J., Sato, K., Tomura, H., Kuwabara, A., Kimura, T., Kanda, T., Ohta, H., Ui, M., Kobayashi, I. and Okajima, F. (2001) Extracellular mechanism through the Edg family of receptors might be responsible for sphingosine-1-phosphate-induced regulation of DNA synthesis and migration of rat aortic smooth-muscle cells. *Biochem. J.* **353**, 139–146
- 27 Sugimoto, N., Takuwa, N., Okamoto, H., Sakurada, S. and Takuwa, Y. (2003) Inhibitory and stimulatory regulation of Rac and cell motility by the $G_{12/13}$ Rho- and the G_i -pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Mol. Cell. Biol.* **23**, 1534–1545
- 28 Crissman, J. D., Hatfield, J., Schaldenbrand, M., Sloane, B. F. and Hon, K. V. (1985) Arrest and extravasation of B16 amelanotic melanoma in murine lungs. *Lab. Invest.* **53**, 470–478
- 29 Arikawa, K., Takuwa, N., Yamaguchi, H., Sugimoto, N., Kitayama, J., Nagawa, H., Takehara, K. and Takuwa, Y. (2003) Ligand-dependent inhibition of B16 melanoma cell migration and invasion via endogenous $S1P_2$ G protein-coupled receptor: requirement of inhibition of cellular Rac activity. *J. Biol. Chem.*, DOI 10.1074/jbc.M305024200
- 30 Bouzahzah, B., Albanese, C., Ahmed, F., Pixley, F., Lisanti, M. P., Segall, J. D., Condeelis, J., Joyce, D., Minden, A., Der, C. J. et al. (2001) Rho family GTPases regulate mammary epithelium cell growth and metastasis through distinguishable pathways. *Mol. Med.* **7**, 816–830
- 31 del Peso, L., Hernandez-Alcoceba, R., Embade, N., Carnero, A., Esteva, P., Paje, C. and Lacal, J. C. (1997) Rho proteins induce metastatic properties *in vivo*. *Oncogene* **15**, 3047–3057
- 32 Van Aelst, L. and D'Souza-Schorey, C. (1997) Rho GTPases and signaling networks. *Genes Dev.* **11**, 2295–2322
- 33 Yatomi, Y., Ozaki, Y., Ohmori, T. and Igarashi, Y. (2001) Sphingosine 1-phosphate, synthesis and release. *Prostaglandins Other Lipid Mediators* **64**, 107–122
- 34 Murata, N., Sato, K., Kon, J., Tomura, H., Yanagita, M., Kuwabara, A., Ui, M. and Okajima, F. (2000) Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. *Biochem. J.* **352**, 809–815
- 35 Olivera, A. and Spiegel, S. (2001) Sphingosine kinase: a mediator of vital cellular functions. *Prostaglandins Other Lipid Mediators* **64**, 123–134
- 36 Xia, P., Wang, L., Gamble, J. R. and Vadas, M. A. (1999) Activation of sphingosine kinase by tumor necrosis factor- α inhibits apoptosis in human endothelial cells. *J. Biol. Chem.* **274**, 34499–34505

Received 10 March 2003/27 May 2003; accepted 12 June 2003

Published as BJ Immediate Publication 12 June 2003, DOI 10.1042/BJ20030381