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Tyrosine Sulphation of Sphingosine 1-Phosphate 1 ($S1P_1$) is Required for S1P-mediated Cell Migration in Primary Cultures of Human Umbilical Vein Endothelial Cells

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Sphingosine 1-phosphate (S1P), a lysophospholipid mediator, regulates diverse functions of many types of cells by binding to specific G protein-coupled receptors termed $S1P_1$–$S1P_5$. In T-cells, tyrosine sulphation of $S1P_1$ is required for high-affinity binding of S1P and fully functional signalling. In this study, we showed that tyrosine sulphation of $S1P_1$ is necessary for S1P-induced Src phosphorylation and migration in human umbilical vein endothelial cells (HUVECs). Both substitution of phenylalanine (F) for tyrosine (Y) in $S1P_1$ and inhibition of tyrosine sulphation blocked c-Src phosphorylation and migration in HUVECs. In addition, overexpression of mutant (F19, 22F) $S1P_1$, lacking tyrosine sulphation sites, suppressed native $S1P_1$ effects on migration, actin rearrangement and lamellipodia formation. Therefore, tyrosine sulphation of $S1P_1$ is required for its optimal transduction of signals from S1P in HUVECs.

Key words: endothelial cells, migration, sphingosine 1-phosphate, Src, tyrosine sulphation.

Sphingosine 1-phosphate (S1P), a multifunctional bioactive lipid mediator, plays an important role in regulating vascular homeostasis (*1*). By binding to its specific receptor, S1P regulates many cellular responses, including cell proliferation, migration and differentiation in endothelial cells (*2*, *3*). To the present, five S1P receptors have been identified, namely $S1P_1$–$S1P_5$, which are members of the seven transmembrane G-protein-coupled receptors (GPCRs) (*4*, *5*). Through interacting with $S1P_1$ and $S1P_3$, the most abundant S1P receptors expressed in human umbilical vein endothelial cells (HUVECs) (*6*), S1P up-regulates intracellular adhesion molecule (ICAM)-1 messenger (m)RNA, total protein and cell surface expression in HUVECs (*7*), suggesting its role in regulating inflammatory processes.

Tyrosine sulphation, a post-translational modification process, occurs almost exclusively on secreted and transmembrane spanning proteins (*8*). It was shown that through tyrosylprotein sulphotransferase, sulphate is transferred from the universal sulphate donor, 3′-phosphoadenosine-5′-phosphosulphate, to the hydroxyl group of tyrosine residues to form tyrosine *O*-sulphate esters (arylsulphate) and 3′,5′-ADP (*8*, *9*). Tyrosine sulphation events were found to be involved in various biological processes including homeostasis regulation and leucocyte trafficking (*8*). However, the biological function of tyrosine sulphation in endothelial cells remains unknown.

Previous studies showed that $S1P_1$ regulates cell survival, proliferation and migration in endothelial cells (*2*, *3*). Moreover, two tyrosine–aspartic acid sequences in $S1P_1$ were identified as targets for tyrosine *O*-sulphation (*10*). Using sodium chlorate, a tyrosine sulphation inhibitor, and overexpression of the mutant (Y19, 22F) $S1P_1$, Fieger *et al*. (*10*) revealed that tyrosine sulphation of $S1P_1$ is required for high-affinity binding with S1P and $S1P_1$-mediated cell proliferation and migration in mouse CD4 T-cells. However, little is known about the biological function of tyrosine sulphation of $S1P_1$ in endothelial cells. In this study, by suppressing the tyrosine sulphation process in $S1P_1$ using sodium chlorate and transfection with mutant (Y19, 22F) $S1P_1$ in HUVECs, we present evidence that tyrosine sulphation of $S1P_1$ is required for S1P-induced Src phosphorylation, cell migration and actin remodelling. These results suggest that tyrosine sulphation of $S1P_1$ modulates S1P-mediated cellular responses, thus facilitating endothelial cell migration.

MATERIALS AND METHODS

Reagents—S1P and fatty acid-free bovine serum albumin (FAF-BSA) were purchased from Sigma (St Louis, MO, USA). Mouse anti-ICAM-1 (clone 6.5B5), rabbit anti-c-Src (clone SRC 2), goat anti-actin (clone I-19) and rabbit anti-c-myc (clone A-14) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture—HUVECs were isolated from fresh umbilical cords as described earlier (*7*) and cultured in M199 medium supplemented with 10% fetal bovine serum

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(FBS) and 25% EGM (Cell Applications, San Diego, CA, USA). Cells were maintained under standard cell culture condition at 37°C in humidified air with 5% CO_2.

Flow Cytometry—After treatment with 10 mM sodium chlorate and 5 μM S1P, HUVECs were collected in 0.05% trypsin. Suspensions of 10^6 HUVECs in phosphate buffered saline (PBS) (200 μl) with 0.1% FAF-BSA were supplemented with 1 μl of FITC-conjugated human ICAM-1 and incubated for 30 min at 4°C. Antibody binding of HUVECs was determined by CyFlow® SL (Partec, Munster, Germany) and analysed by WinMDI version 2.8 software.

Whole-cell Extractions and Western Blot Analysis—Whole-cell lysates were isolated from HUVECs as described earlier (7). The protein concentration was measured by Bio-Rad reagents using BSA as the standard reference. Twenty micrograms of whole-cell lysates was separated by 12% sodium dodecylsulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and processed for immunoblotting.

Wound-healing Assay—Serum-starved HUVECs at 80% confluency were treated with or without 10 mM sodium chlorate for 4 h, followed by making a scratch with a 200-μl pipette tip. Cells were washed twice with PBS and then incubated with 5 μM S1P for 18 h. Cell migration was then assessed by microscopy.

Cell-migration Assay—Transwell migration of HUVECs towards S1P treatment was analysed using a modified Boyden chamber (Neuro Probe, Cabin John, MD, USA) as described earlier (7). Migratory cells on the lower membrane surface were fixed with 3.7% formalin for 30 min and stained with 2% crystal violet. Migration results were quantified by Image Gauge V4.0 software.

Plasmid Construction and Transfection—Plasmids encoding the full-length human wild-type (WT) and a human mutant in which tyrosine 19 and 22 both have been replaced with phenylalanine (Y19, 22F) were kindly provided by Dr Edward J. Goetzl (University of California, San Francisco, CA, USA). These plasmids were then transfected into HUVECs using the Amaxa Nucleofector™ biosystems (Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, 5×10^5 cells were mixed with 2 μg of plasmid DNA in 100 μl of HUVEC Nucleofector™ solution. The mixtures were electroporated with the HUVEC-specific A-34 program. After electroporation, transfected cells were cultured in complete medium for 24 h before experiments.

RNA Extraction and Real-time Polymerase Chain Reaction—Total cellular RNA was extracted from HUVECs using the TRIzol reagent, and a Superscript kit was used for the reverse transcription (RT) synthesis of complementary (c)DNA. Real-time polymerase chain reactions (PCRs) were conducted in an iCycler iQ Real-Time detection system (Bio-Rad, Hercules, CA, USA) using SYBR Green I (PerkinElmer Life Sciences, Boston, MA, USA). The PCR was performed using the following primers: 5′-AAGGTGAAGGTCGGAGTC-3′ and 5′-TGTAGTTGAGGTCAATGAAGG-3′ (for GAPDH), and 5′-CCACCACCTACAAGCTCACTCC-3′ and 5′-AATGGC GATGGCGAGGAGAC-3′ (for $S1P_1$) and 5′-TATGGCAAC GACTCCTTCTC-3′ and 5′-TCTCCTGGCTCTGGTTCC-3′ (for ICAM-1).

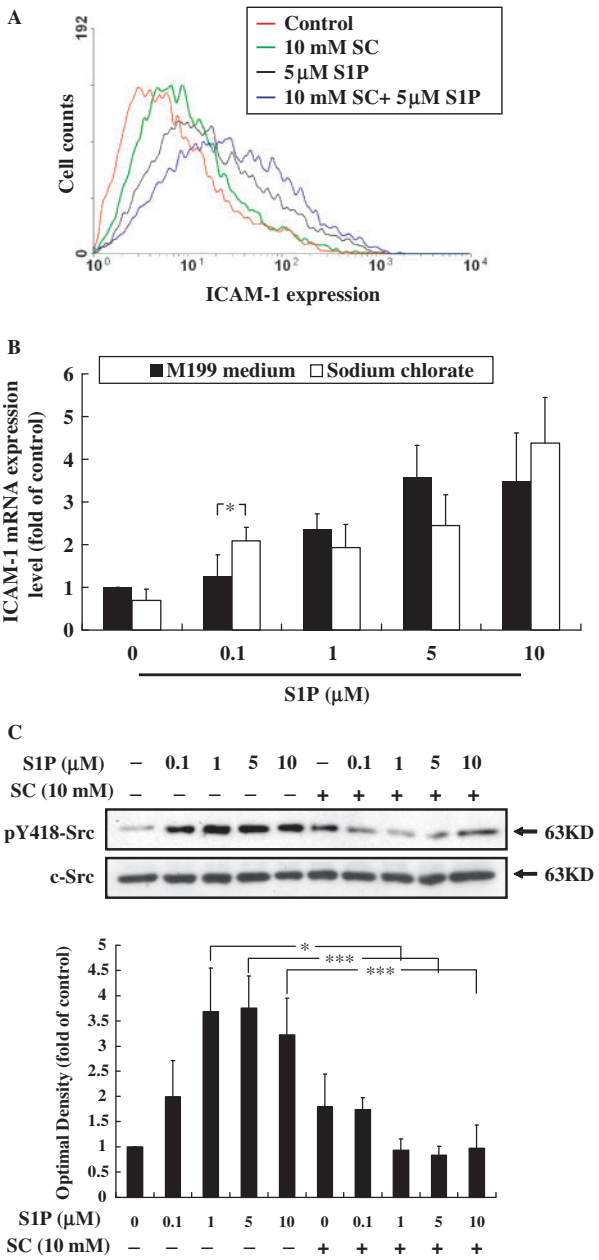

Fig 1. **Tyrosine sulphation activity is involved in S1P-mediated Src phosphorylation in HUVECs.** (A) HUVECs were serum-starved for 12–16 h, and pre-incubated for 4 h with 10 mM sodium chlorate, followed by co-treatment with vehicle or 5 μM S1P for another 8 h. Cells were then dissociated by trypsinization, incubated with an FITC-labelled anti-ICAM-1 antibody for 30 min, followed by Cyflow analysis. (B) Serum-starved HUVECs were pre-incubated for 4 h with 10 mM sodium chlorate, followed by co-treatment with vehicle, 0.1, 1, 5 and 10 μM S1P for another 4 h. mRNA were then extracted and ICAM-1 expression were analysed by quantitative real-time PCR. (C) Serum-starved HUVECs were pre-incubated for 4 h with 10 mM sodium chlorate, followed by co-treatment with vehicle, 0.1, 1, 5 and 10 μM S1P for another 3 min. Total cell lysates were isolated, and phospho-Src protein expression levels were monitored by western blotting using an anti-phospho-Src antibody. Immunoblots were reprobed with anti-c-Src to ensure equal loading amounts. Data are representative of three experiments and are expressed as the mean ± SE ($^*P < 0.05$, $^{***}P < 0.001$). SC, sodium chlorate.

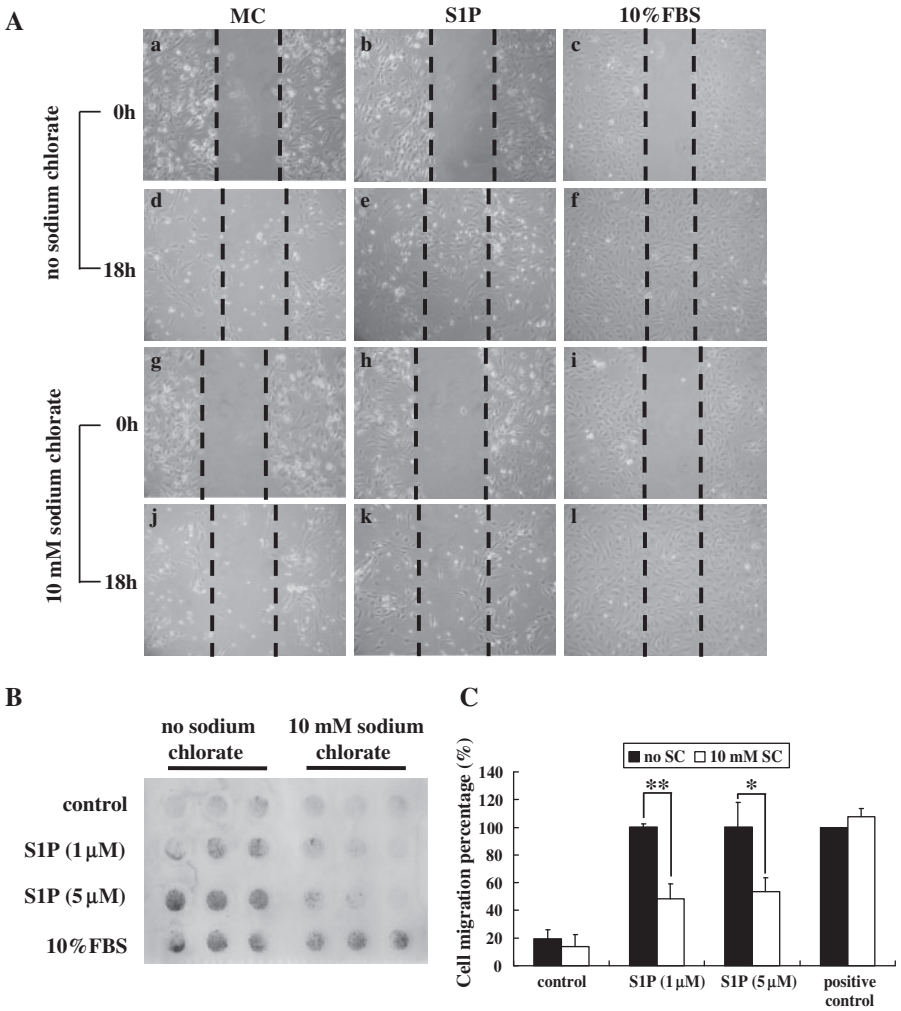

Fig. 2. **Tyrosine sulphation of S1P$_1$ is responsible for S1P-mediated cell migration in HUVECs.** (A) Serum-starved HUVECs were pre-incubated for 4 h with (g–l) or without (a–f) sodium chlorate, followed by the co-treatment with vehicle (a, d, g and j), 5 μM S1P (b, e, h and k) or 10% FBS-containing medium (c, f, i and l) for another 18 h, and cell migration was assayed by a wound healing analysis. (B) Serum-starved HUVECs were pre-incubated for 4 h with or without sodium chlorate, followed by co-treatment with vehicle, 1 or 5 μM S1P, or 10% FBS-containing medium for another 4 h, and cell migration was then assayed by a modified Boyden chamber analysis. (C) The histogram represents quantification results of migrating HUVECs ($^*P < 0.05$, $^{**}P < 0.01$). All data are relative multiples of expression compared with sodium chlorate-untreated cells.

Statistical Analysis—Significant differences between the groups were analysed using analysis of variance (ANOVA). Each experiment was repeated at least three times. The results are shown as the mean ± SE. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Tyrosine Sulphation is Essential for S1P-mediated Src Phosphorylation of HUVECs—Our previous study reported that through binding to S1P$_1$, S1P up-regulates the expression of ICAM-1 in HUVECs (*7*). In addition, S1P also phosphorylates Src, which causes its re-organization to the cell boundary in human patient-derived endothelial progenitor cells (*11*, *12*). Moreover, tyrosine sulphation of S1P$_1$ was recently revealed to be responsible for cell proliferation and migration in T-cells (*10*). To examine whether tyrosine sulphation activity is involved in S1P-mediated physiological behaviour in endothelial cells, a tyrosine sulphation inhibitor, sodium chlorate, was applied to HUVECs. As shown in Fig. 1, while inhibition of tyrosine sulphation failed to suppress S1P-induced ICAM-1 expression (Fig. 1A and B), it significantly blocked S1P-induced Src phosphorylation (Fig. 1C) and the distribution of phospho-Src in HUVECs (Supplementary Fig. 1). These results indicate that tyrosine sulphation activity is required for S1P-induced Src phosphorylation but not ICAM-1 induction in HUVECs.

S1P-induced Cell Migration is Dependent on Tyrosine Sulphation of S1P$_1$ in HUVECs—To determine if tyrosine sulphation activity is responsible for S1P-mediated cell migration, the migration abilities were assessed in HUVECs following treatment with sodium chlorate. As shown in Fig. 2, inhibition of tyrosine sulphation significantly blocked S1P-induced cell migration as

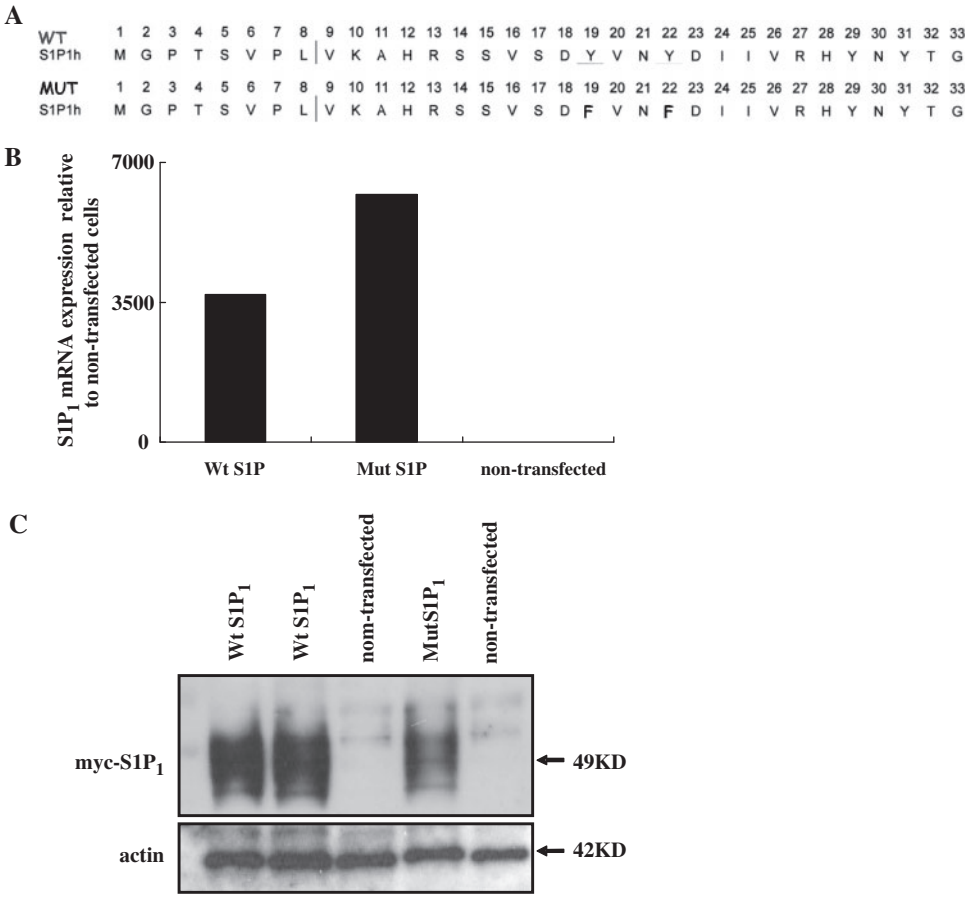

Fig. 3. **WT and mutant (Y19, 22F) of $S1P_1$ is over-expressed in HUVECs.** HUVECs were transfected with two different pcDNA3 expression constructs, one of which contains the sequence encoding the full-length human WT $S1P_1$ and the other a sequence encoding a human mutant $S1P_1$ in which tyrosine residues 19 and 22 were both replaced with phenylalanine (Y19, 22F). Each construct also contained sequences encoding a c-myc tag at the carboxyl terminal of $S1P_1$ (A). mRNA expression levels of human WT and mutant $S1P_1$ in HUVECs were determined by real-time PCR (B), and the protein expression levels of those constructs were assayed by Western blotting using an anti-c-myc antibody (C).

demonstrated by the wound-healing assay (Fig. 2A) and the modified Boyden's chamber assay (Fig. 2B and C). It was shown that $S1P_1$ contains two tyrosine residues (Y19 and Y22) at its N-terminal which are subject to sulphation (*10*). To further examine whether tyrosine sulphation of $S1P_1$ is responsible for S1P-induced cell migration in HUVECs, c-myc-tagged WT and mutant (Y19, 22F) $S1P_1$ forms were transfected to HUVECs. As demonstrated by real-time PCR (Fig. 3B) and western blotting (Fig. 3C), the c-myc-tagged WT and mutant $S1P_1$ forms were overexpressed in HUVECs. Using these constructs, we showed that mutant $S1P_1$ failed to respond to S1P-mediated cell migration in HUVEC cells, as demonstrated by the wound-healing assay (Fig. 4A) and modified Boyden's chamber assay (Fig. 4B and C).

To determine whether actin cytoskeletal remodelling is also regulated by S1P-mediated cell migration in HUVECs, rhodamine-conjugated phalloidin, which specifically binds to F-actin, was used. As shown in Supplementary Fig. 2A, re-arrangement of the actin cytoskeleton and formation of lamellipodia were both stimulated by S1P treatment in HUVECs. To further examine whether re-arrangement of the actin cytoskeleton and formation of lamellipodia are regulated by tyrosine sulphation of $S1P_1$ in HUVECs, the c-myc-tagged WT and mutant (Y19, 22F) $S1P_1$ forms were transfected into HUVECs. Results showed that neither S1P-mediated actin remodelling nor lamellipodia were observed in mutant (Y19, 22F) $S1P_1$-expressing HUVECs (Supplementary Fig. 2B, h and l). Taken together, these results indicate that sulphation of Y19 and Y22 on $S1P_1$ is essential for S1P-induced cell migration in HUVECs.

DISCUSSION

Tyrosine sulphation was reported to play important roles in blood coagulation, leucocyte adhesion and trafficking, hormonal regulation, retroviral and parasitic infections, and smooth muscle cell proliferation and migration (*13*, *14*). Our current results also demonstrated that S1P-induced migration was significantly suppressed by the inhibition of tyrosine sulphation in HUVECs, which is consistent with previous studies that tyrosine sulphation is important for the migration abilities of other cell types analysed (*10*, *15*, *16*).

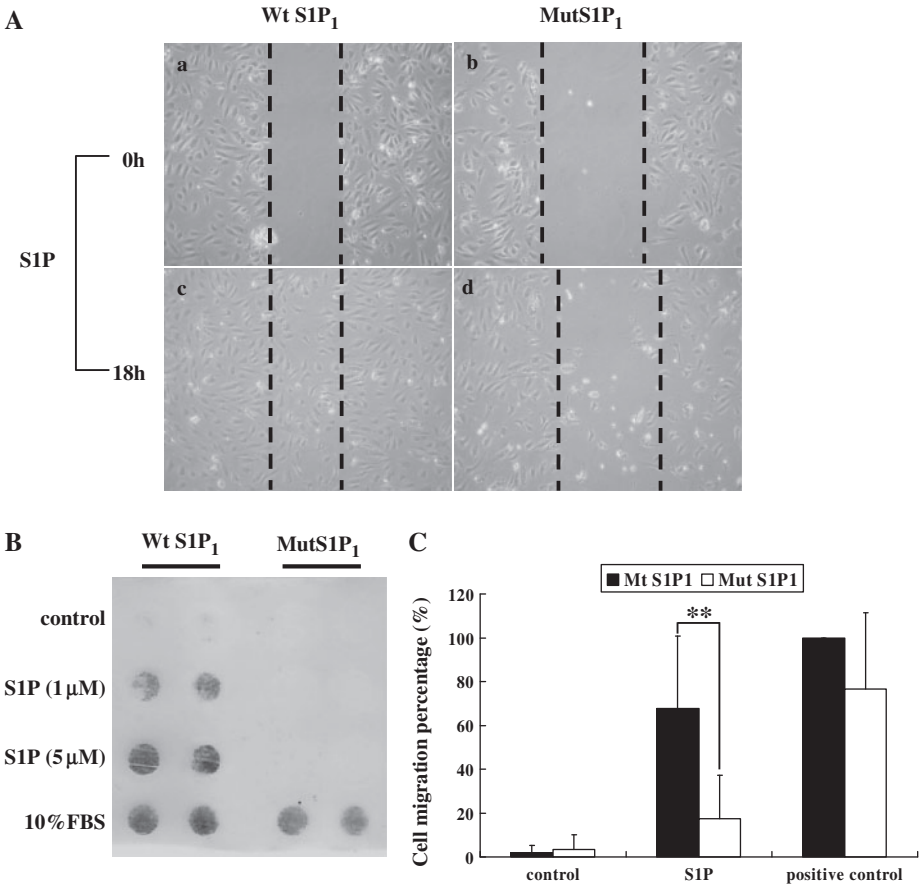

Fig. 4. **Tyrosine sulphation of S1P$_1$ is responsible for S1P-induced cell migration in HUVECs.** (A) HUVECs were transfected with WT (a and c) or mutant (b and d) S1P$_1$, and subjected to serum starvation for 12–16 h, followed by treatment with 5 μM S1P for another 18 h. Cell migration was then assayed by a wound-healing analysis. (B) HUVECs were transfected with WT or mutant S1P$_1$, subjected to serum starvation for 12–16 h, followed by treatment with 1 or 5 μM S1P, or 10% FBS-conta medium for 4 h. Cell migration was then assayed by a modified Boyden chamber analysis. (C) The histogram represents quantification results of migrating HUVECs ($^{**}P < 0.01$). All data are relative multiples of expression compared with mutant (Y19, 22F) S1P$_1$-transfected cells.

Acute inflammation is a rapid response to infectious microbes or injured tissues that involves local recruitment and activation of neutrophils, which results from endothelial cell activation (*17*). Meanwhile, activated platelets release lysophospholipids that regulate endothelial cell-induced ICAM-1, vascular cell adhesion molecule-1, E-selectin and S-selectin expressions, thus facilitating monocyte adhesion and chemotaxis of endothelial cells (*18*). In addition, inflammation also plays an important role in the progression of tumour formation and atherosclerosis (*19*). S1P up-regulates ICAM-1 expression (*7*) and Src phosphorylation (*11*, *12*) through activating its specific receptors, corresponding to its role in regulating the inflammatory process (*2*, *20*). In the present study, we further demonstrated that tyrosine sulphation is responsible for S1P-induced Src phosphorylation and cell migration in HUVECs. The inhibition of tyrosine sulphation failed to suppress 10% FBS-mediated cell migration in HUVECs (Fig. 2), suggesting that some growth factors in serum, such as fibroblast growth factor and vascular endothelial growth factor, induce HUVEC migration (*21*), and their receptors are not affected by tyrosine sulphation. In addition, by applying sodium chlorate, we showed that while S1P-induced ICAM-1 expression is independent of tyrosine sulphation (Fig. 1A and B), this sulphation modification is obviously involved in S1P-induced Src phosphorylation (Fig. 1C) and distribution (Supplementary Fig. 1). These results suggested that S1P-induced ICAM-1 expression and c-Src phosphorylation are mediated through different signalling pathways. However, it is interesting to note that S1P-induced ICAM-1 expression is mediated by S1P$_1$ but not S1P$_3$ (*7*), which lacks a tyrosine site for sulphation modification (*10*). Furthermore, both S1P$_1$ and S1P$_3$ are required for the S1P-induced Src phosphorylation (*7*, *11*). Taken together, these results further indicate that tyrosine sulphation is not necessary for all S1P-mediated cellular responses in HUVECs.

S1P$_1$ contains two tyrosine residues (19 and 22) flanked by aspartic acid, which can be sulphation-modified in T-cells (*10*). Through transfection with the mutant (Y19, 22F) S1P$_1$, we showed that S1P-induced cell migration is dependent on tyrosine sulphation at Y19 and Y22 residues of S1P$_1$ in HUVECs (Fig. 4).

These results correspond well to previous studies on T-cells (*10*). In addition, 10% FBS-induced cell migration was not affected by either transfection with WT or mutant (Y19, 22F) $S1P_1$ (Fig. 4B), suggesting that overexpression of either WT or mutant $S1P_1$ did not affect the normal migration behaviour of HUVECs. These results indicate that Y19 and Y22 on $S1P_1$ play essential roles in S1P-mediated cell migration in HUVECs.

In summary, we demonstrated that S1P-induced Src phosphorylation, Src distribution and cell migration are mediated through tyrosine sulphation in HUVECs. In addition, tyrosine sulphation sites within $S1P_1$ are responsible for S1P-mediated cell migration in HUVECs. Our findings suggest that tyrosine sulphation of $S1P_1$ might be an essential target for S1P-mediated cell migration, which provides valuable information for developing new therapeutics against the inflammatory process and atherosclerosis formation.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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