THE EFFECTS OF MARKEDLY RAISED INTRACELLULAR SPHINGOSINE KINASE-1 ACTIVITY IN ENDOTHELIAL CELLS

VIDYA LIMAYE¹,²,³*, MATHEW A. VADAS⁴, STUART M. PITSON²,⁵ and JENNIFER R. GAMBLE⁴

¹Royal Adelaide Hospital, Department of Rheumatology, North Tce, Royal Adelaide Hospital, Adelaide, SA 5000, Australia ²Division of Human Immunology, Institute of Medical and Veterinary Science, Frome Road, Adelaide, SA 5000, Australia, ³Department of Medicine, University of Adelaide, Frome Rd, Adelaide, SA, Australia, ⁴Centenary Institute, University of Sydney, NSW, Australia, ⁵School of Molecular and Biomedical Science, University of Adelaide, Australia

Abstract: The enzyme sphingosine kinase-1 (SK1) promotes the formation of sphingosine-1-phosphate (S1P), which is an important survival factor for endothelial cells (EC). Modest increases in intracellular SK1 activity in the EC are known to confer a survival advantage upon the cells. Here, we investigated the effects of more dramatic increases in intracellular SK1 in the EC. We found that these cells show reduced cell survival under conditions of stress, enhanced caspase-3 activity, cell cycle inhibition, and cell-cell junction disruption. We propose that alterations in the phosphorylation state of the enzyme may explain the differential effects on the phenotype with modest versus high levels of enforced expression of SK1. Our results suggest that SK1 activity is subject to control in the EC, and that this control may be lost in conditions involving vascular regression.

Key words: Sphingosine kinase-1, Endothelial cells, Cell survival

* Author for correspondence; e-mail: vidya.limaye@health.sa.gov.au, tel.: 61-8-82225190, fax: 61-8-82225895

Abbreviations used: EC – endothelial cell; EV – empty vector; HUVEC – human umbilical vein endothelial cell; PECAM-1 – platelet-endothelial cell adhesion molecule-1; pfu – plaque-forming unit; SK1 – sphingosine kinase-1 (SK1); S1P – sphingosine 1-phosphate; TNF – tumour necrosis factor
INTRODUCTION

There is much interest in the factors governing endothelial cell (EC) survival. Along with growth factors and cell-cell and cell-matrix interactions, the lipid mediator sphingosine-1-phosphate (S1P), which forms via the phosphorylation of membrane-associated sphingosine by sphingosine kinase-1 (SK1), is an important survival factor for the EC. S1P antagonizes the pro-apoptotic effects of ceramide, an intermediate in the sphingomyelin degradation pathway, and promotes cell survival [1]. S1P acts both as a ligand for the S1P family of G-protein-coupled receptors and as an intracellular messenger [2-5]. We previously showed that modest (5-fold) increases in SK activity in the EC enhance cell survival and render the cells resistant to the normal stressful stimuli of loss of serum or loss of attachment to the extracellular matrix [6]. These phenotypic changes were accompanied by the induction and de-phosphorylation of the cell junctional molecule called platelet endothelial cell adhesion molecule-1 (PECAM-1), which itself is known to have anti-apoptotic effects [7] and was shown to be obligatory in the activation of the major cell survival pathway, the PI-3K/Akt pathway [6].

In this study, we sought to determine the effects of more dramatic increases in intracellular SK activity on the EC. Given the oncogenic role of SK1 [8], and the finding that in NIH 3T3 fibroblast cells, large increases in SK1 activity confer a transformed phenotype [8] upon the cells, and our previous demonstration that modest increases in SK1 activity enhance cell survival, we hypothesized that profound increases in SK1 activity in the EC would further promote cell survival and confer upon the cells an oncogenic phenotype. Instead, we found that large increases in SK activity in the EC result in cell death.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described [9]. The cells were plated in non-tissue culture, non-adhesive 96-well microtiter trays coated with 1% bovine serum albumin at 8 x 10^3 cells/well in serum-free medium.

Cell survival was assayed as follows: EC were plated into gelatin-coated 96-well microtiter trays at 3 x 10^3 cells/well in serum-free medium. MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium; Promega) was used to measure cell viability. The optical density at 490 nm was measured on days 0 and 2.

Cell permeability was determined as follows: endothelial cells were seeded into fibronectin-coated 3.0-μm transwells with a polycarbonate membrane (Corning Inc, NY, USA) at 10 x 10^3 cells per well in 150 μl HUVE medium supplemented with ECGS and heparin. 600 μl of HUVE medium was added to the bottom of the transwell, and the cells were incubated at 37ºC for 24 h. The medium from
the top and bottom of the transwell was then replaced with 2% HUVE medium with growth factors and heparin, and the cells incubated for a further 4 h. Stimulation with thrombin (Sigma, St Louis, MO) at a concentration of 0.2 units/ml (in serum-free HUVE medium) was done immediately prior to the assay, whereas sphingosine 1 phosphate (1 μM) was added 20 min earlier. FITC-dextran (500 μg/ml diluted in serum-free HUVE medium) was added to each transwell, and then 20 μl of medium was collected from the bottom of each transwell at predetermined time points (commencing at time 0). This was dispensed into a 96-well flat bottom tray containing 60 μl serum-free medium per well. The fluorescence was then quantified with a well plate reader using excitation and emission wavelengths of 485 nm and 530 nm, respectively.

**Adenovirus production and generation of HUVECs overexpressing SK1**

The AdEasy system (Qbiogene, Carlsbad, CA) was used to produce a recombinant adenovirus carrying human SK1 (SK1; or an empty vector, EV, or G82D) according to the Qbiogene Version 1.4 AdEasy Vector system manual [10]. The 293 cells were cultured in 25-cm² flasks in complete Dulbecco modified Eagle medium (CSL Biosciences, Parkville, Australia) containing 10% fetal calf serum (FCS). The virus was amplified in 293 cells and purified on a cesium chloride gradient with centrifugation. The viral titer was determined using the tissue culture infectious dose50 method. Transfection of the HUVECs was achieved by infection with adenoviral preparations of SK or EV using equivalent plaque-forming units/cell, which yielded a similar level of green fluorescent protein expression. The cells were used for functional assays 1 to 3 days post-infection. Over-expression of SK1 was confirmed by Western blot and an SK activity assay. Cells with low levels of over-expression of SK1 using 1 pfu/cell of the adenovirus carrying SK1 or EV (respectively, EC<sub(SK)</sub> and EC<sub(EV)</sub>) were generated, as were cells with high levels of over-expression of the enzyme (EC<sub1<sub>(SK)</sub>50, EC<sub1<sub>(EV)</sub>50, or EC<sub1<sub>(G82D)</sub>50) using 50 pfu/cell.

**Western blotting**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on cell lysates using 12% acrylamide gels. The immunocomplexes were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). The commercially available antibodies used were: M2 mouse anti-FLAG antibody (Sigma); rabbit polyclonal anti-phospho-Akt and rabbit polyclonal anti-Akt (Cell Signaling Technology, Beverly, MA); antiphosphotyrosine (Cell Signaling Technology); and mouse monoclonal anti-cyclin D1 or anti-cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit anti-PECAM-1 antibody was an affinity-purified antibody as previously described [11]. Mouse monoclonal antibodies directed against β1 integrin (61-2C4) and VE-cadherin (55-7H1) were raised at the Hanson Centre for Cancer Research as previously described [11], and anti-phosphoSK-1 antibody was generated as previously
described [12]. A goat anti-PECAM antibody (Santa Cruz Biotechnology) was used for the Western blots as indicated.

Enzyme assays
SK1 activity in whole-cell lysates made from ultracentrifugated preparations was determined as previously described [13] using D-erythro sphingosine and -32P ATP as substrates. Caspase-3 activity was measured in the cell lysates using a Calbiochem kit (Calbiochem-Novabiochem, Darmstadt, Germany) according to the manufacturer’s instructions.

Statistical analysis
The analyses, stratified over replicate experiments, were performed by ANOVA-style regression using Statistica Version 6.1 (Statsoft, Tulsa, OK).

RESULTS

High intracellular SK1 activity inhibits cell accumulation due to enhanced caspase-3 activity and cell cycling inhibition

We previously showed that infection of HUVEC with 1 or 50 pfu/cell respectively results in approximately 5-fold or 100-fold increases in SK1 activity above that of the control cells [6], and here determined the effect of varying levels of SK1 on cell accumulation. As observed previously, low level (3- to 5-fold) over-expression of SK1 (EC\textsuperscript{SK}) enhanced cell accumulation compared to the effect with empty vector control cells (EC\textsuperscript{EV}; Fig. 1A). By contrast, high level (50-fold) over-expression of SK1 (EC\textsuperscript{SK50}) yielded a relative deficit in cell number (Fig. 1A).

EC\textsuperscript{SK50} showed a reduction in cell numbers over three days (Fig. 1B). The inhibition of cell accumulation was mediated specifically by SK1 activity, since over-expression of a catalytically inactive mutant of SK1 (G82D) did not alter cell accumulation (Fig. 1B).

EC\textsuperscript{SK50} showed enhanced basal caspase-3 activity compared with that for EC\textsuperscript{EV50} (Fig. 1C, D), consistent with the phenotype of reduced cell numbers. These cells also displayed a significant reductions in the expression of both cyclin D1 and cyclin E compared with EC\textsuperscript{EV50} (Fig. 1E). Together, this suggested the inhibition of cell accumulation by EC\textsuperscript{SK50} resulting from both enhanced apoptosis and decreased cell division.

High levels of SK1 reduce cell survival in serum-free conditions and in suspension

We next assessed the effect of high level SK1 over-expression on cell survival following stressful stimuli. Following serum deprivation for 24 h, there were significantly less viable EC\textsuperscript{SK50} compared with EC\textsuperscript{EV50} (p < 0.05) (Fig. 2A). EC\textsuperscript{G82D50} did not alter cell survival compared with EC\textsuperscript{EV50}, confirming that the effects observed are mediated specifically by the catalytic activity of SK1.
Fig. 1. The effect of SK on cell accumulation, caspase-3 activation and cyclin D1 and E expression. A – the relative cell accumulation in a medium containing 2% FCS for HUVEC infected with varying pfu/cell of an adenovirus carrying SK or EV. The results are normalized to the optical density on day 0, and are adjusted to EV = 1. The figure shows the composite analysis of six observations from two separate experiments (0.1 pfu/cell), 31 observations derived from 7 separate experiments (1 pfu/cell) and 15 observations from 5 separate experiments (50 pfu/cell), using Statistica Version 6.1 (Statsoft, Inc). B – cell numbers as reflected by the optical density, in EC\textsubscript{SK50}, EC\textsubscript{EV50} and EC\textsubscript{EV50+G82D}. The figure shows the results of five observations from a single experiment which is representative of at least two separate experiments. The bars represent SEM. *p < 0.001: SK compared with EV on day 3. C – the effect of EC\textsubscript{SK50} (dark columns) compared with EC\textsubscript{EV50} (light columns) on the caspase-3 activity under basal conditions. D – the composite analysis of four separate endothelial cell lines (from Fig. 1C), normalized to the control using Statistica Version 6.1 (Statsoft, Inc) thus indicating the mean fold increase in caspase-3 activity in EC\textsubscript{SK50} compared with EC\textsubscript{EV50}. *p < 0.001 compared with EC\textsubscript{EV50}. The bars represent 95% confidence intervals. E – cyclin D1 and cyclin E expression in EC\textsubscript{SK50} and EC\textsubscript{EV50}, under basal culture conditions. The mean fold change of cyclin D1 compared with EC\textsubscript{EV50} was 0.75 ± 0.03, n = 3, p < 0.05, and for cyclin E was 0.78 ± 0.01, n = 3, p < 0.05. A loading control of VE cadherin is indicated.
We then determined whether EC\textsuperscript{SK50} are less resistant to the additional stress of loss of attachment to the extracellular matrix. EC\textsuperscript{SK50} showed significantly reduced survival in suspension compared with EC\textsuperscript{EV50} (Fig. 2B). Over-expression of high levels of SK caused a consistent increase in caspase-3 activity in two endothelial cell lines subjected to serum deprivation for 24 h, as shown in Fig. 2C and D. There was a trend towards an exaggerated caspase-3 activation in response to serum deprivation, but due to the small sample size (n = 2), this did not reach statistical significance (p = 0.1; Fig. 2D).

Fig. 2. The effect of SK on cell survival under stress. A and B show cell numbers of EC\textsuperscript{EV50}, EC\textsuperscript{SK50} and EC\textsuperscript{G82D50} maintained (A) in SF conditions and (B) in suspension, as reflected by the optical density. *p < 0.05: EC\textsuperscript{SK50} compared with EC\textsuperscript{EV50} on day 1. The figure shows the results from a single experiment done in quadruplicate, which is representative of four separate experiments. The bars represent SEM. C – The response of two of endothelial lines to serum deprivation. D –The composite analysis of C, normalized to the control using Statistica Version 6.1 (Statsoft, Inc.) thus indicating the mean fold increase in caspase-3 activity in cells over-expressing SK compared with EV. *p < 0.001 compared with EV. The bars represent 95% confidence intervals.
The effects of over-expression of high levels of SK on cell junctions
In light of the recognized role of EC junctions and cell-cell contact in maintaining EC viability, permeability to FITC-dextran was measured to assess the structural integrity of EC monolayers in response to SK1 over-expression. Low-level SK1 over-expression is accompanied by a reduction in basal permeability to FITC-dextran (Fig. 3A) consistent with our previously published observation that small increases in SK1 activity increase proliferation and survival [6]. By contrast, EC<sup>SK<sub>50</sub></sup> showed increased basal permeability to FITC-dextran (Fig. 3B), indicating a disruption of cell-cell junctions. This is consistent with the observed reduction in EC viability. Whereas EC<sup>EV<sub>50</sub></sup> maintained the normal response to thrombin stimulation with increased permeability (Fig. 3C), EC<sup>SK<sub>50</sub></sup> showed a loss of this response (Fig. 3D). This is in contrast to EC<sup>SK</sup>, which, despite showing a reduced basal permeability, did not differ in their response to thrombin stimulation compared with EC<sup>EV</sup> (Fig. 3E).

The known role of PECAM-1 in the regulation of cell junctions and protection from apoptosis prompted a determination of the total PECAM-1 levels in EC<sup>SK<sub>50</sub></sup>. They showed a substantial reduction in total PECAM-1 expression (Fig. 4A, B). The effect was PECAM-1-specific, as there was no change in the expression of the other junctional protein, VE cadherin, in four separate EC lines.

Cells over-expressing high levels of SK failed to engage the PI-3Kinase pathway
The PI-3K/Akt pathway is a major cell survival pathway, and given the enhanced cell death in EC<sup>SK<sub>50</sub></sup>, we sought to determine whether these cells were able to activate the PI-3K/Akt pathway. EC<sup>SK<sub>50</sub></sup> showed a reduction in Akt phosphorylation under basal conditions and when stressed with serum deprivation (Fig. 4C). Moreover, they showed a further down-regulation in the phosphorylation of Akt with serum deprivation, as did EC<sup>EV<sub>50</sub></sup> (Fig. 4C), consistent with the enhanced cell death and reduction in cell survival seen in these cells.

The phosphorylation of SK under basal conditions was evident with the over-expression of high levels of SK
Phosphorylation of SK at Ser225 results in the activation of the enzyme [12]. We determined whether 5-fold or 100-fold increases in enforced SK activity result in differential effects on the phosphorylation state of SK. EC<sup>SK<sub>50</sub></sup> showed basal phosphorylation of the enzyme indicating activation (Fig. 4D). By contrast, phosphorylation was not detected in cell lysates of EC<sup>SK</sup>, even after stimulation with TNF (Fig. 4D).
Fig. 3. The effect of SK on cell permeability. The figure shows the permeability (normalized to time = 0) of EC^{SK} and EC^{EV} (A, E), and EC^{SK50} and EC^{EV50} (B, C, D) to FITC-dextran, across different time points under basal conditions (A, B) or in response to thrombin stimulation (0.2 units/ml) (C, D, E). C and D respectively show the response of EC^{EV50} and EC^{SK50} to thrombin stimulation. *p < 0.001: SK compared with EV under basal conditions across all time points (A, B), or untreated EV (C) compared with the thrombin-treated vector across all time points. The figure shows the composite analysis of 7 observations from 3 separate experiments using Statistica Version 6.1 (Statsoft, Inc). The bars represent 95% confidence intervals.
Fig. 4. The effect of SK on junctional proteins and the phosphorylation of Akt. A – PECAM-1 and VE cadherin expression by Western blot in EC<sub(SK50)</sub> and EC<sub(EV50)</sub>. B – the composite analysis of two separate experiments for PECAM-1 expression in EC<sub(SK50)</sub> normalized to EC<sub(EV50)</sub> using Statistica Version 6.1 (Statsoft, Inc). The bars represent 95% confidence intervals. *p < 0.05: EC<sub(SK50)</sub> compared with EC<sub(EV50)</sub>. C – a Western blot of phosphorylated Akt (phospho-Akt) and total Akt in EC<sub(SK50)</sub> and EC<sub(EV50)</sub>, under basal conditions (culture in HUVE medium, HM) and after incubation for 6 h in serum-free (SF) medium. D – by Western blot, the phosphorylation of SK in HUVEC infected with 1 pfu/cell or 50 pfu/cell of adenoviral supernatant carrying SK or EV. Cells infected with 1 pfu/cell were analysed in the presence (+) or absence (-) of stimulation with TNFα (0.5 ng/ml) for 10 min. ‘R’ indicates recombinant SK which is constitutively active and used as a positive control. The membrane was immunoblotted with rabbit anti-phospho SK peptide antibody, and then re-blotted with anti-flag antibody. The loading control of a non-specific background band detected by the anti-FLAG antibody is included.

DISCUSSION

Markedly raised SK1 intracellular levels and activity in the EC result in profound alterations in the EC phenotype, distinct from those we previously described with more modest increases in enzyme activity. Whereas 5-fold increases in SK1 activity result in enhanced cell survival, mediated by a PECAM-1-dependent activation of the PI-3K/Akt pathway, 100-fold increases in intracellular SK1 activity render the EC susceptible to apoptosis with a corresponding loss of cell numbers, down-regulation of PECAM-1, and an inability to engage the PI-3K/Akt pathway in response to stress. This, together with the reduction in G1-phase cyclins, results in a phenotype of inhibited cell growth and cell survival. This cellular ‘toxicity’, resulting from high levels of
SK1 in HUVEC, is distinct to HEK293 cells, NIH3T3 cells and tumour cell lines which do not show such toxicity with high levels of SK1 [14]. This indicates that cellular levels of SK are subject to control in the EC. Of note, the physiological relevance of a 100-fold increase in SK1 activity is questionable, and although the levels and activity of SK1 can vary in endothelial cells under physiological and pathological conditions, 100-fold increases in enzyme activity have not yet been reported. The divergence in the effects of high and low expression of SK is consistent with the biphasic dose-response curve of many active metabolites when given through a range from zero to conditions of over-abundance. This may be likened to the clinical biphasic dose-response curve of alcohol, a commonly encountered substance that exhibits a biphasic dose-response in terms of stimulation and sedation.

Whereas cells over-expressing low levels of SK1 showed a PECAM-1 dependent activation of the PI-3K/Akt pathway under conditions of stress, cells over-expressing high levels of SK showed reduced PECAM-1 expression, and were unable to engage the PI-3K/Akt pathway. It is tempting to speculate that the failure to engage the PI-3K/Akt pathway is due to a reduction in PECAM-1 signalling resulting from high levels of SK1 over-expression. The differential effects on PECAM-1 expression are also consistent with opposing effects on the structural integrity of EC monolayers, with the disruption of cell-cell junctions seen in EC^{SK50} (Fig. 3B), and the tightening of cell-cell contacts in EC^{SK} (Fig. 3A). Indeed, PECAM-1 is involved in anti-apoptosis [7], and it is intriguing that this protein is differentially regulated by variable levels of SK1. A critical difference between high and low level over-expression of SK1 is likely to be the degree to which the enzyme is phosphorylated under basal conditions. Basal phosphorylation (activation) of SK1 was evident when it was over-expressed at high but not low levels in the EC (Fig. 4D). Although a small degree of phosphorylation of SK1 in EC^{SK} is not excluded, the failure to detect phosphorylation even in immunoprecipitated SK (data not shown) makes it unlikely that this occurs to any significant degree. The 5-fold elevation in SK1 activity achieved with enforced expression of low levels of SK1, although not substantially dissimilar to the level of basal intrinsic activity, still produced significant functional changes in the EC. By contrast, transfection with the non-phosphorylatable hSK1 mutant (containing the substitution Ser225→Ala), while enhancing SK1 catalytic activity, did not result in functional alterations when over-expressed in HEK293 cells [12]. Under basal conditions in HEK293 cells, SK is not phosphorylated [12], and it is likely that the EC are comparable in this regard, with an absence of phosphorylation of SK in the unstimulated state. As for other protein kinases that are activated by phosphorylation, it is conceivable that phosphorylation of SK1 alters its ability to interact with downstream targets, and that activation of SK1 above its basal catalytic level is obligatory for its role in signal transduction cascades [15]. Furthermore, as phosphorylation of SK1 results in its translocation from the cytosol to the plasma membrane [12], it is
possible that the over-expression of high levels of SK alters its subcellular location, thereby changing the profile of its intracellular interactions. In summary, profound increases in intracellular SK1 activity in the EC result in cellular toxicity. Phosphorylation of the enzyme under basal conditions when it is subject to obligatory expression at high levels is postulated to be critical in accounting for the phenotypic differences to the state resulting from modest increases in SK activity. Demonstrating DNA fragmentation (via TUNEL assays) would clarify whether the reduction in cell numbers is due to apoptosis or necrosis, but the final outcome of cellular toxicity and reduction in cell numbers is clearly demonstrated. Measuring inflammatory mediators such as TNF in cells with both modest and markedly raised SK1 activities may give clues to the potential effect of these mediators on cell death and survival. Our findings of distinct effects on the cellular fate due to dramatic versus modestly raised SK1 activity should prompt an investigation into the role of sphingolipid mediators such as ceramide, S1P and S1P receptors in such processes. It would be of interest to measure cellular S1P levels in cells with varying levels of increased intracellular SK1 activity. Our results indicate that the cellular levels of SK are subject to control in the EC with the possibility that such control is lost in diseases of enhanced cell death. It is possible that the divergent effects observed with modest versus profound increases in SK activity in the EC correspond to distinct pathological phenomena, with the former resulting in enhanced angiogenesis and the latter associated with vascular regression. This suggests that therapeutic manipulation of intracellular SK1 activity may be a novel means of modulating the vascular network. Indeed our recent study has described the effects on endothelial cell function of more modest increases in intracellular SK1 activity, and has suggested a role for manipulating levels of SK1 in endothelial cells in diseases of aberrant inflammation and angiogenesis [16].

Acknowledgements. This study was supported by a Programme Grant from the National Health and Medical Research Council of Australia, a National Health and Medical Research Council (NHMRC) scholarship for Vidya Limaye, and a NHMRC R. Douglas Wright Biomedical Research Fellowship to Stuart Piston. Jennifer Gamble is a Medical Foundation Fellow, Faculty of Medicine, University of Sydney. We are grateful to Michelle Parsons for the adenoviral preparation, Paul Moretti for the preparation of the SK constructs and Sue Lester for her expert statistical assistance. We are indebted to Jenny Drew and Anna Sapa for the preparation of HUVEC and cell culture, and to the staff of the Maternity Wards of the Women’s and Children’s Hospital and Burnside War Memorial Hospital for the collection of the umbilical cords.

REFERENCES

1. Kwon, Y.G., Min, J.K., Kim, K.M., Lee, D.J., Billiar, T.R. and Kim, Y.M. Sphingosine 1-phosphate protects human umbilical vein endothelial cells...
from serum-deprived apoptosis by nitric oxide production. J. Biol. Chem. 276 (2001) 10627-10637.

2. Wang, F., Van Brocklyn, J.R., Hobson, J.P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S. and Spiegel, S. Sphingosine 1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis. J. Biol. Chem. 274 (1999) 35343-35350.

3. Lee, M.J., Van Brocklyn, J.R., Thangada, S., Liu, C.H., Hand, A.R., Menzeleev, R., Spiegel, S. and Hla, T. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. Science 279 (1998) 1552-1555.

4. Ghosh, T.K., Bian, J. and Gill, D.L. Intracellular calcium release mediated by sphingosine derivatives generated in cells. Science 248 (1990) 1653-1656.

5. Desai, N.N., Zhang, H., Olivera, A., Mattie, M.E. and Spiegel, S. Sphingosine-1-phosphate, a metabolite of sphingosine, increases phosphatidic acid levels by phospholipase D activation. J. Biol. Chem. 267 (1992) 23122-23128.

6. Limaye, V., Li, X., Hahn, C., Xia, P., Berndt, M.C., Vadas, M.A. and Gamble, J.R. Sphingosine kinase-1 enhances endothelial cell survival through a PECAM-1-dependent activation of PI-3K/Akt and regulation of BeI-2 family members. Blood. 105 (2005) 3169-3177.

7. Evans, P.C., Taylor, E.R. and Kilshaw, P.J. Signaling through CD31 protects endothelial cells from apoptosis. Transplantation 71 (2001) 457-460.

8. Xia, P., Gamble, J.R., Wang, L., Pitson, S.M., Moretti, P.A., Wattenberg, B.W., D’Andrea, R.J. and Vadas, M.A. An oncogenic role of sphingosine kinase. Curr. Biol. 10 (2000) 1527-1530.

9. Cramer, E.M., Berger, G. and Berndt, M.C. Platelet-granule and plasma membrane share two components: CD9 and PECAM-1. Blood 84 (1994) 1722-1730.

10. Qbiogene Version 1.4 AdEasy Vector system manual. Carlsbad, CA.

11. Litwin, M., Clark, K., Noack, L., Furze, J., Berndt, M., Albelda, S., Vadas, M., and Gamble, J.R. Novel cytokine-independent induction of endothelial adhesion molecules regulated by platelet/endothelial cell adhesion molecule (CD31). J. Cell. Biol. 139 (1997) 219-228.

12. Pitson, S.M., Moretti, P.A.B., Zebl, J.R., Lynn, H.E., Xia, P., Vadas, M.A., Wattenberg, B.W. Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. EMBO J. 22 (2003) 5491-5500.

13. Xia, P., Gamble, J.R., Rye, K.A., Wang, L., Hii, C.S., Cockerill, P., Khew-Goodall, Y., Bert, A.G., Barter, P.J., and Vadas, M.A. Tumor necrosis factor-alpha induces adhesion molecule expression through the sphingosine kinase pathway. Proc. Natl. Acad. Sci. USA 95 (1998) 14196-14201.

14. Spiegel, S., Olivera, A., Zhang, H., Thompson, E.W., Su, Y. and Berger, A. Sphingosine-1-phosphate, a novel second messenger involved in cell growth regulation and signal transduction, affects growth and invasiveness of human breast cancer cells. Breast Cancer Res. Treat. 31 (1994) 337-348.
15. Pitson, S.M., Moretti, P.A., Zebol, J.R., Xia, P., Gamble, J.R., Vadas, M.A., D’Andrea, R.J. and Wattenberg, B.W. Expression of a catalytically inactive sphingosine kinase mutant blocks agonist-induced sphingosine kinase activation. A dominant-negative sphingosine kinase. J. Biol. Chem. 275 (2000) 33945-33950.

16. Limaye, V., Xia, P., Hahn, C., Smith, M., Vadas, M.A, Pitson, S.M. and Gamble, J.R. Chronic increases in sphingosine kinase-1 activity induce a pro-inflammatory, pro-angiogenic phenotype in endothelial cells. Cell. Mol. Biol. Lett. 14 (2009) in press, DOI: 10.2478/s11658-009-0009-1.