

Research article

CHRONIC INCREASES IN SPHINGOSINE KINASE-1 ACTIVITY INDUCE A PRO-INFLAMMATORY, PRO-ANGIOGENIC PHENOTYPE IN ENDOTHELIAL CELLS

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Abstract: Sphingosine kinase-1 (SK1) promotes the formation of sphingosine-1-phosphate (S1P), which has potent pro-inflammatory and pro-angiogenic effects. We investigated the effects of raised SK1 levels on endothelial cell function and the possibility that this signaling pathway is activated in rheumatoid arthritis. Human umbilical vein endothelial cells with 3- to 5-fold SK1 (EC^{SK}) overexpression were generated by adenoviral and retroviral-mediated gene delivery. The activation state of these cells and their ability to undergo angiogenesis was determined. S1P was measured in synovial fluid from patients with RA and OA. EC^{SK} showed an enhanced migratory capacity and a stimulated rate of capillary tube formation. The cells showed constitutive activation as evidenced by the induction of basal VCAM-1 expression, and

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Abbreviations used: DMS – N,N'-dimethylsphingosine; EC^{EV} – endothelial cells infected with EV; EC^{G82D} – endothelial cells over-expressing dominant-negative SK1; EC^{SK} – endothelial cells over-expressing SK1; E selectin – endothelial selectin; EV – empty vector; GPCR – G-protein coupled receptor; HUVEC – human umbilical vein endothelial cells; IL-1 – interleukin 1; PTX – pertussis toxin; S1P – sphingosine 1 phosphate; SK – sphingosine kinase 1; TNF – tumour necrosis factor; VCAM-1 – vascular cell adhesion molecule-1; VEGF – vascular endothelial growth factor

further showed a more augmented VCAM-1 and E selectin response to TNF compared with empty vector control cells (EC^{EV}). These changes had functional consequences in terms of enhanced neutrophil binding in the basal and TNF-stimulated states in EC^{SK}. By contrast, over-expression of a dominant-negative SK inhibited the TNF-induced VCAM-1 and E selectin and inhibited PMN adhesion, confirming that the observed effects were specifically mediated by SK. The synovial fluid levels of S1P were significantly higher in patients with RA than in those with OA. Small chronic increases in SK1 activity in the endothelial cells enhance the ability of the cells to support inflammation and undergo angiogenesis, and sensitize the cells to inflammatory cytokines. The SK1 signaling pathway is activated in RA, suggesting that manipulation of SK1 activity in diseases of aberrant inflammation and angiogenesis may be beneficial.

Key words: Inflammation, Angiogenesis, Endothelial cells, Sphingosine kinase, TNF

INTRODUCTION

The location of the endothelium at the interface between the blood and tissues endows upon it an obligatory role in the regulation of leukocyte trafficking and angiogenesis. The normal endothelium is in a non-activated state, evidenced by its inability to support leukocyte adhesion and angiogenesis. Angiogenesis and inflammation are to a large extent coordinately regulated, and a number of cytokines are known to induce endothelial activation resulting in leukocyte adhesion and the stimulation of angiogenesis. The inflammatory cytokines tumour necrosis factor (TNF), interleukin-1 (IL-1) [1] and the key angiogenic factor, vascular-endothelial growth factor (VEGF) [2] induce the expression of the adhesion molecules E selectin and VCAM-1, thus enhancing inflammation. Interestingly, E selectin, considered an inflammatory marker of the endothelium, has been detected in non-inflammatory angiogenic tissues including the human placenta and hemangiomas [3]. Moreover, antibodies to E selectin inhibit tube formation *in vitro* [4], while the addition of exogenous E selectin stimulates angiogenesis in the rat cornea [5].

The enzyme sphingosine kinase-1 (SK1) catalyzes the phosphorylation of membrane-associated sphingosine to form sphingosine-1-phosphate (S1P), a lipid signaling molecule which regulates cell proliferation, survival, and differentiation [6]. The cellular levels of S1P are regulated by SK1 activity, although S1P phosphatase is also important in determining these levels [7]. The cellular levels of SK are generally low, but can be rapidly increased by numerous agonists including TNF [8, 9], platelet-derived growth factor and serum [10], phorbol esters [11], nerve growth factor [12, 13], FcεRI [14], FcγRI [15], antigen receptor occupancy, and VEGF [16].

Sphingosine-1-phosphate is a ligand for the S1P family of G-protein-coupled receptors (GPCR) to regulate cell migration [17] and angiogenesis [18, 19]. In

endothelial cells (EC), exogenously added S1P is an important angiogenic regulator, by virtue of its mitogenic actions through the activation of the mitogen-activated protein kinase (MAPK) pathway [20, 21], the enhancement of cell survival by activating the phosphatidylinositol-3 kinase/Akt (PI-3K/Akt) pathway [22, 23] and the stimulation of cellular migration and remodeling [24]. In addition to regulating angiogenesis, S1P mediates the TNF-induced up-regulation of adhesion proteins in human umbilical vein endothelial cells (HUVEC) and SK1 is obligatory in mediating these effects. However, an intracellular second messenger role for S1P has also been postulated, since it can mobilize calcium from internal stores [25] and can stimulate mitogenic and anti-apoptotic effects [26]. We have also shown that modest increases in intracellular SK activity in the EC enhance cell survival in a receptor-independent manner through a novel pathway involving an interaction between the junctional molecule, platelet endothelial cell adhesion molecule-1 (PECAM-1) and the PI-3K/Akt pathway [27].

Since SK1 over-expression enhances EC survival, we sought to determine whether there are other functional consequences to the cell. In particular, we reasoned that the ability of the cells to support inflammation and angiogenesis would be altered. As endothelial activation is a key feature of rheumatoid arthritis, we then investigated whether the SK1/S1P signaling pathway was activated in RA.

MATERIALS AND METHODS

HUVEC culture

HUVEC were isolated and cultured as described [28], in a medium supplemented with 50 µg/ml EC growth supplement (Collaborative Research, MA, USA) and 50 µg/ml heparin (Sigma, St Louis, Missouri, USA).

Retrovirus production: stable cell lines

FLAG-epitope tagged SK [29] and SK^{G82D} [30] were cloned into vector PrufNeo [31]. Retroviral production was done via calcium phosphate transfection of PrufNeo-SK, PrufNeo-G82D, or empty PrufNeo into Bing cells. The retroviral supernatant was collected after 48 h. Stable cell lines were generated by infecting HUVEC with the retroviral supernatant carrying SK (to yield EC^{SK}), and empty vector (to yield EC^{EV}) or G82D (to yield EC^{G82D}), followed by selection with G418 (Promega, Madison, WI, USA) at 48 h.

Adenovirus production: transient cell lines

The recombinant adenovirus carrying SK, G82D, or an empty vector (EV) was produced according to the Qbiogene Version 1.4 AdEasyTM Vector system manual (<http://www.qbiogene.com/products/adenovirus/adeasy.shtml>). The viral titre was determined using the TCID₅₀ method according to the manufacturer's protocol. Transient transfection of HUVEC was achieved by infection with adenoviral preparations of SK (to yield EC^{SK}) or EV (to yield EC^{EV}), measured

using equivalent plaque-forming units (pfu)/cell, and this yielded a similar level of green fluorescent protein expression.

SK detection and activity, and flow cytometry

SK over-expression was detected by immunoblot analysis with anti-FLAG antibodies [27]. SK activity was determined using D-erythro-sphingosine and [γ - 32 P]ATP as substrates [32]. Flow cytometric analysis of the cell-surface expression of E-selectin and VCAM-1 was performed [8].

Cell migration

Cell migration was measured using transwells [33]. The lower surface of a 6.5-mm transwell (pore size 8 μ m; Corning Inc, NY, USA) was coated with fibronectin (50 μ g/ml in HBSS/ Ca^{2+} / Mg^{2+}) for 30 min at 37°C. HUVEC were re-suspended in HBSS/ Ca^{2+} / Mg^{2+} , and 1×10^5 cells in 100 μ l was added to the top well. To the lower well, 500 μ l of HBSS was added with 0.5% BSA/ Ca^{2+} / Mg^{2+} . The cells were incubated at 37°C for 24 h, then aspirated and washed with PBS, and adherent cells were removed by wiping the membrane with a PBS-soaked bud. Cells that had migrated to the lower surface of the membrane were fixed with methanol (15 min) and stained with 0.1% crystal violet in 0.1 M borate buffer, pH 9.0. The wells were washed three times with PBS. Stained cells in the lower transwells were eluted with 10% acetic acid (300 μ l) for 30 min, and the optical density was determined using an ELISA plate-reader and a single wavelength, 595 nm. The percentage of cells that had migrated was determined from a standard curve.

Tube formation

A 96-well microtitre tray was coated with Matrigel Basement Membrane Matrix (Beckton Dickinson Labware, Bedford, MA, USA). 3×10^5 EC^{EV} or EC^{SK} per ml in HUVE medium was prepared and 140 μ l was added to each well. Tube formation was observed visually at the start of the experiment and after 30 min, 60 min, 4 h and 24 h.

Neutrophil adhesion

HUVEC were seeded into fibronectin-coated Lab-Tek slides (3×10^4 cells/well) and incubated at 37°C for 24 h. The cells were washed and 1×10^5 neutrophils/well were added. Following incubation at 37°C for 30 min, non-adherent neutrophils were removed by washing three times. The EC were fixed with methanol. The number of adherent neutrophils in consecutive fields was determined by microscopy.

S1P measurements

S1P was quantitated using the SK-based method [34]. Briefly, S1P from synovial fluid was isolated (Bligh-Dyer extraction under alkaline conditions) and dephosphorylated with alkaline phosphatase. The resultant sphingosine was quantitatively converted to S1P by the addition of recombinant SK and

[γ - ^{32}P]ATP. The resultant $\text{S1}^{32}\text{P}$ was isolated (Bligh-Dyer extraction), subjected to thin-layer chromatography, and quantitated using phosphorimaging and comparison with an S1P standard.

Statistical analysis

Student's t-Test was used for parametric data with $p < 0.05$ considered significant. Significance testing for ratios was performed via ANOVA-style regression (Statistica Version 6.1, Statsoft, Inc.). The outcome measurements were log transformed enabling interpretation of the analysis as the median-fold change relative to a chosen baseline (MFI). The majority of the analyses were performed by normal linear regression; p-values were determined using the t-Test with appropriate degrees of freedom. Mean (μ) effects, relative to a specified baseline, and associated standard errors (s.e.) were determined by appropriate linear contrasts of the regression coefficients. For analyses of log transformed outcome data, approximate large sample 95% confidence intervals (CI) were obtained ($\mu \pm 1.96 \cdot \text{s.e.}$). MFI with approximately 95% CI were obtained by back-transformation.

RESULTS

Over-expression of SK1 alters adhesion molecule expression in HUVEC

As we previously reported, adenovirus-mediated SK1 over-expression was titrated to yield an approximately 5-fold increase in SK1 activity above EC^{EV} [27]. To determine whether chronic SK1 over-expression alters the activation state of EC, we measured their cell surface expression of adhesion molecules. EC^{SK} showed up-regulated basal VCAM-1 expression (Fig. 1A) although there was no consistent change in basal E selectin levels in four separate endothelial cell lines over-expressing SK ($p = 0.44$; not shown).

We next determined whether EC^{SK} exhibited an altered adhesion molecule response to stimulation with TNF. EC^{SK} generated by adenoviral-mediated delivery significantly augmented the normal TNF-induced up-regulation of VCAM-1 (Fig. 1B) and also showed a trend towards increasing the magnitude of the E selectin response to TNF (Fig. 1C), although this did not reach statistical significance ($p = 0.08$). It is noted however that in all four endothelial cell lines tested, over-expression of SK resulted in a consistent increase (15-80% above control) in the magnitude of the E selectin response to $\text{TNF}\alpha$. The lack of statistical significance needs to be interpreted in the context of the small sample size ($n = 4$), and the large sample confidence intervals used.

We confirmed our observations with a complementary method of SK1 over-expression in EC using the retrovirus, which yielded a 3.5-fold (95% CI 1.9-6.4; $p < 0.05$) increase in SK1 activity above that in the control. EC^{SK} generated using the retrovirus also showed higher basal VCAM-1 expression (Fig. 1D), and augmented the VCAM-1 and E selectin responses to stimulation with TNF (Fig. 1E, F).

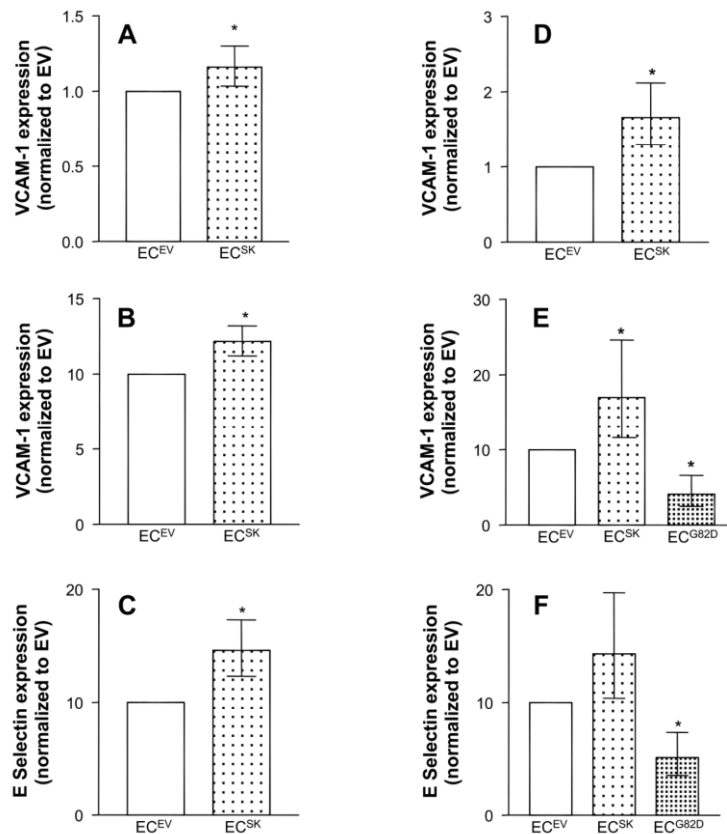


Fig. 1. Basal (A, D) and TNF-stimulated adhesion molecule expression (B, C, E, F) for EC^{SK}, EC^{G82D} and EC^{EV} obtained by infection with an adenovirus (A, B, C) or retrovirus (D, E, F). The cells were stimulated with TNF (0.5 ng/ml) for 4 h. VCAM-1 expression is given in A, B, D and E, and E selectin expression in C and F. The results are normalized to EC^{EV} = 1 for the basal, and EC^{EV} = 10 for the stimulated expression, and the bars represent 95% confidence intervals. A-F show the pooled data respectively from 3, 4, 4, 6, 5 and 6 separate experiments, using different isolates of endothelial cells. *p < 0.05 compared with EC^{EV}.

The mutant SK^{G82D} acts as a dominant-negative to block agonist-induced activation of SK1 without altering basal cellular activity [30]. In the EC, overexpression of SK^{G82D} significantly inhibited TNF-induced VCAM-1 and E selectin expression compared with EC^{EV} (Fig. 1E, F), confirming an important role of SK1 in TNF-induced adhesion molecules and substantiating an activity-dependent function of SK1 in mediating the adhesion protein expression.

Stimulating cells with TNF in very low doses that failed to up-regulate VCAM-1 or E selectin in EC^{EV} resulted in significant induction of both adhesion molecules in EC^{SK} (Fig. 2A, B). The induction of VCAM-1 expression by TNF

in EC^{SK} was 4.42-fold (95% CI 1.51-12.94) greater than in EC^{EV} ($p < 0.05$, $n = 3$); the induction of E selectin expression by TNF was 1.7- and 3.1-fold greater in EC^{SK} than in EC^{EV} in two separate EC lines. Thus, SK1 over-expression primes cells for a greater response to inflammatory cytokine.

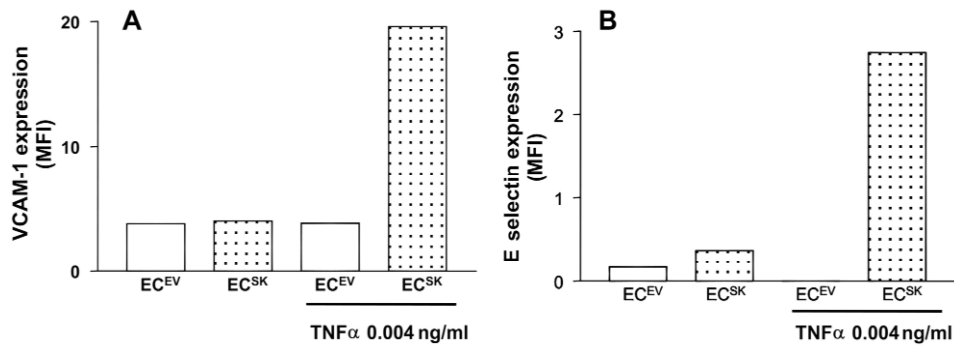


Fig. 2. The response of VCAM-1 (A) and E selectin (B) as reflected by the median fluorescence intensity (MFI) to very low doses of stimulation with TNF α (0.004 ng/ml) for 4 h in EC^{EV} and EC^{SK} generated by adenovirus-mediated gene delivery. The figure shows the data from a single experiment which is representative of two separate experiments in which the same trend was observed.

The induction of E selectin on EC by TNF peaks at 4-6 h and declines to near basal levels by 18-24 h [35, 36]. To determine whether the over-expression of SK1 altered this time course, EC^{SK} and EC^{EV} generated by retroviral infection were treated with TNF for 18 h. In four separate EC lines, the E selectin expression in EC^{SK} remained 2.01-fold (95% CI 1.14-3.53) higher than in EC^{EV} at 18 h post-TNF stimulation. EC^{G82D} showed a significant inhibition of this response (MFI above EC^{EV} 0.44, 95% CI 0.26-0.76, $p = 0.014$).

The results above show that retroviral and adenoviral delivery of SK1 generated similar phenotypes in EC. The adenoviral system enabled large numbers of cells to be rapidly generated, so this method was used for all subsequent experiments.

The effects of intracellular over-expression of SK1 are not mediated through S1P₁ receptors

The S1P₁ receptor, which is the major S1P receptor expressed in HUVEC, and is responsible for S1P-induced up-regulation of adhesion molecules, is sensitive to inhibition by pertussis toxin (PTx). To investigate whether our results could be explained by S1P acting on the S1P₁ receptor, we pre-treated cells with PTx and measured the adhesion molecule expression. PTx did not inhibit basal VCAM-1 expression in EC^{SK} or EC^{EV} (Fig. 3A); conversely, adhesion molecule expression was enhanced in two separate EC isolates. This is consistent with a previously reported inhibition mediated through the GPCR, which are PTx-sensitive [37].

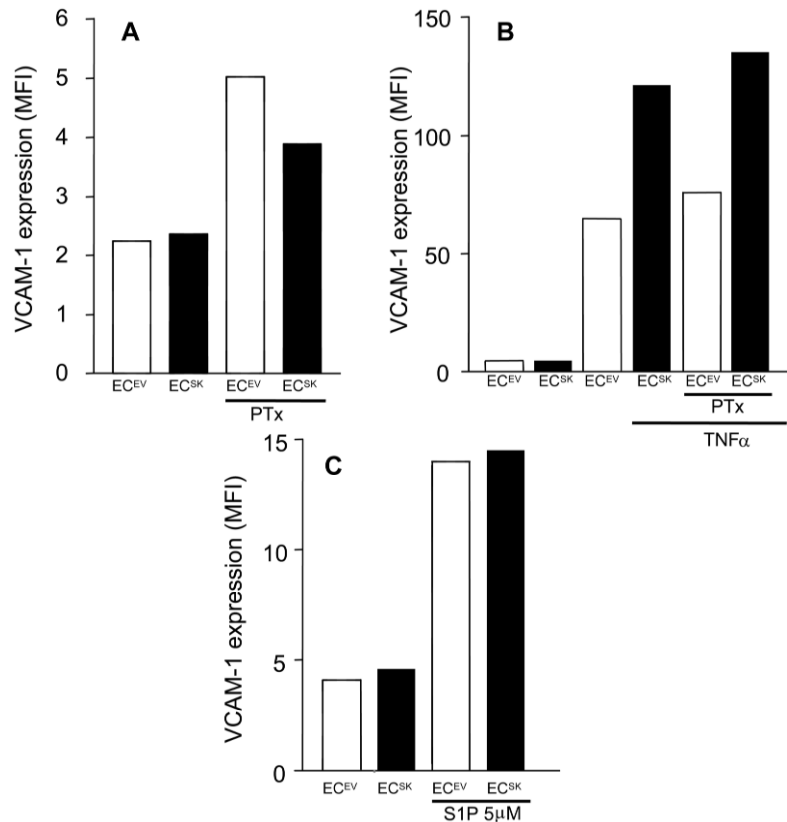


Fig. 3. The effect of 18 hours of treatment with 50 ng/ml pertussis toxin (PTx) on basal (A) and TNF α -stimulated (B) VCAM-1 expression, as reflected by the median fluorescence intensity (MFI) in EC^{SK} and control EC^{EV}. C – The effect of stimulation with 5 μ M S1P for four hours on VCAM-1 expression in EC^{SK} and EC^{EV}. The figure shows the data from a single experiment which is representative of two (A, B) or five (C) separate experiments using different endothelial cell isolates.

Likewise, pretreatment with PTx had no effect on the TNF-mediated induction of VCAM-1 expression in EC^{EV} or EC^{SK} (Fig. 3B), although PTx was functional on the cells in inhibiting their migratory capacity towards the chemoattractant fibronectin, a function dependent on the GPCR [38]. Similarly, basal and TNF-induced E selectin expression was not inhibited by pre-treatment with PTx (data not shown). Notably, EC^{EV} and EC^{SK} retained the capacity to respond to exogenous S1P, and showed a similar, significant up-regulation of VCAM-1 expression (Fig. 3C), indicating that the SIP₁ receptor pathway is intact in these cells. We further assessed if EC^{SK} shifted the dose-response curve to exogenous S1P stimulation (0.6, 1.2, 2.5 and 5.0 μ M). EC^{SK} consistently showed a greater VCAM-1 expression across all the doses of S1P tested, but there was no significant difference at any one dose. Overall, there was a tendency for the dose-response curve to shift to the left ($p = 0.08$; data not shown).

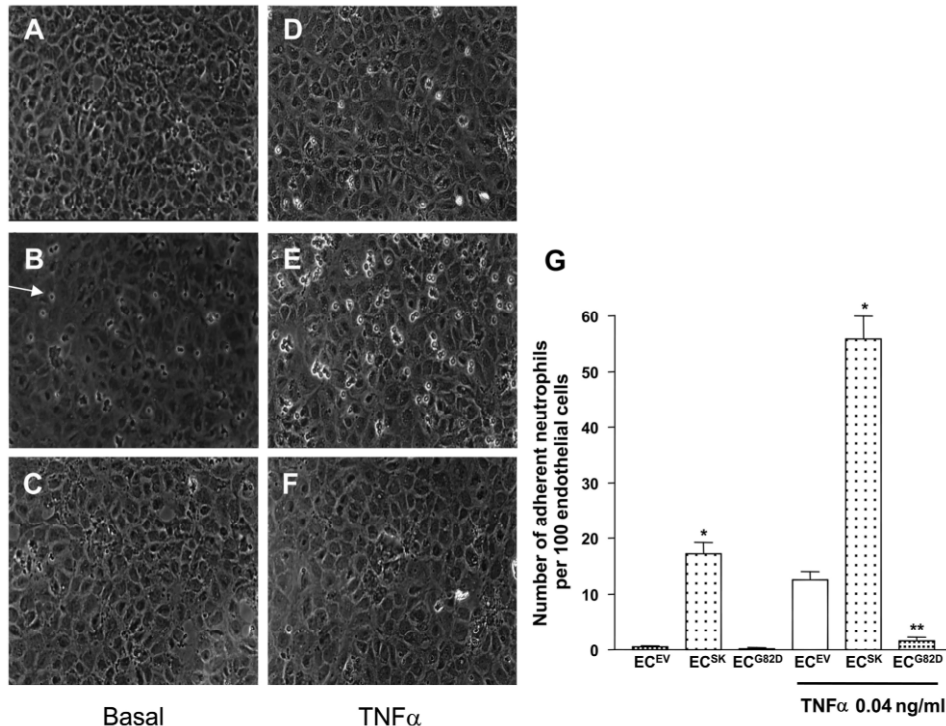


Fig. 4. Neutrophil adhesion to EC^{EV} (A, D), EC^{SK} (B, E) and EC^{G82D} (C, F) in the basal state (A-C) and when stimulated for four hours with 0.04 ng/ml TNF α (D-F) (80 x magnification). The white arrow indicates an adherent neutrophil. The figure shows the results from one experiment which is representative of two separate experiments. G – The number of adherent neutrophils per 100 endothelial cells, as determined from the pooled data of five separate microscopic fields obtained from four separate experiments for EC^{SK} and EC^{EV}, and three separate experiments for EC^{G82D}. The bars represent SEM. *p < 0.05 compared with the corresponding EC^{EV}, **p < 0.001 compared with the corresponding EC^{EV} using Student's t-Test.

SK enhances endothelial-neutrophil binding

We sought to determine whether the changes in the expression of the adhesion molecule observed in EC^{SK} had functional consequences in terms of neutrophil binding. In the basal state, EC^{EV} did not bind neutrophils, which is consistent with the behaviour of normal EC. However, EC^{SK} showed significant neutrophil adhesion (Fig. 4A, B). Stimulating EC with a low dose of TNF resulted in minimal neutrophil adhesion in EC^{EV} (Fig. 4D), but significantly greater adhesion in EC^{SK} (Fig. 4E). Consistent with a role for SK1 in mediating PMN adhesion, and with the TNF-activation of the SK1 signalling pathway, EC^{G82D} cells inhibited TNF-induced PMN adhesion (Fig. 4C, F, G).

SK1 promotes tube formation and cell migration

To determine whether SK1 over-expression alters the ability of EC to undergo capillary tube formation, cells were plated onto the complex basement membrane matrix, Matrigel. Equivalent numbers of EC^{SK} and EC^{EV} were seeded; the cells were visualized as single cell populations. After 30 min, EC^{SK} showed greater evidence of tube alignment than EC^{EV} (Fig. 5A). After one hour, tube formation by EC^{SK} was highly developed compared with EC^{EV} (Fig. 5B), although after 18 h, both EC^{SK} and EC^{EV} showed a similar pattern of tube formation (data not shown). The stimulated rate of tube formation in EC^{SK} suggests an alteration in their migratory capacity. Indeed, EC^{SK} showed enhanced migration towards the chemoattractant fibronectin (Fig. 5C). This SK1-induced migration was significantly attenuated by PTx, suggesting a role for G_{i/o}-linked GPCRs ($p < 0.05$). Even in the presence of PTx, EC^{SK} maintained an enhanced rate of cell migration compared with EC^{EV} ($p < 0.05$), indicating that a component of SK1-stimulated cell migration to fibronectin is independent of a G_{i/o}-linked GPCR.

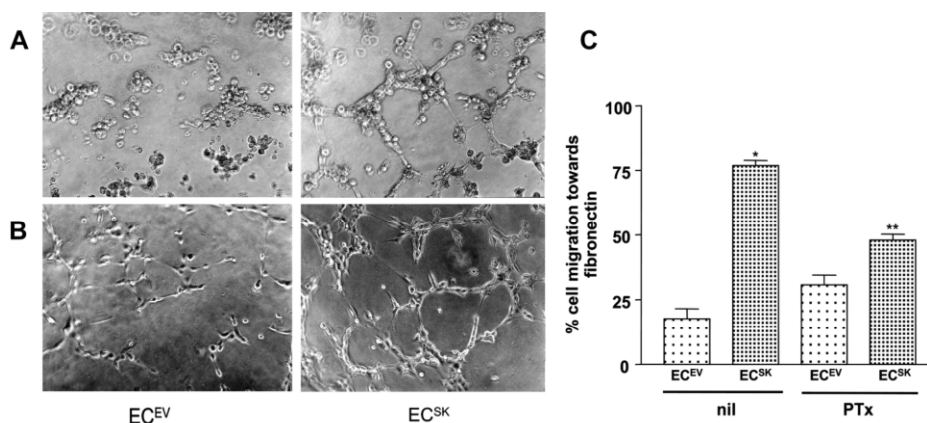


Fig. 5. Tube formation by EC^{SK} and control EC^{EV} in Matrigel at 30 min (A) and at one hour (B). C –The percentage of EC^{SK} and EC^{EV} which migrated towards a gradient of fibronectin (migration towards transwells not coated with fibronectin was subtracted). The effect of pretreatment with 50 ng/ml pertussis toxin (PTx) for 18 hours is shown. The figure shows the pooled data of two separate experiments performed in duplicate, and bars represent SEM. * $p < 0.001$ untreated EC^{SK} compared with untreated EC^{EV}, and EC^{SK} treated with pertussis toxin compared with untreated EC^{SK}. ** $p < 0.05$ EC^{SK} compared with EC^{EV} in the presence of pertussis toxin.

The SK pathway is activated in RA

As rheumatoid arthritis (RA) is characterized by chronic inflammation and enhanced angiogenesis, we sought evidence for the enhanced activity of SK1 in RA compared with the traditionally accepted control of osteoarthritis (OA). The levels of S1P were higher in the synovial fluid from patients with RA ($n = 14$)

compared with the levels from patients with OA (n = 8; Fig. 6) indicating activation of the SK pathway in RA.

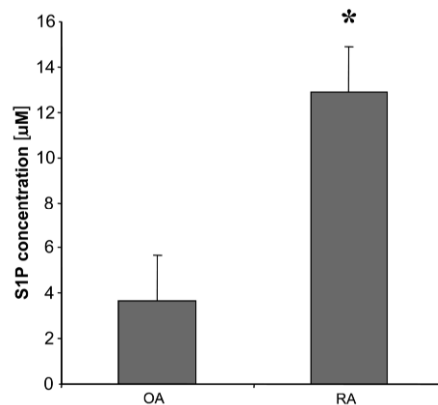


Fig. 6. The S1P levels from 14 patients with RA (10 F, mean age 57.9y; mean level $10.38 \mu\text{M} \pm 1.07$) compared with the levels from 8 patients with OA (5 F, mean age 69.5y; mean level $3.66 \mu\text{M} \pm 0.23$), $p = 0.00005$.

DISCUSSION

The results of this study demonstrate that the levels and activity of SK1 are a major regulator of endothelial function. We show that minor but lasting increases in SK1 activity in the EC can have major functional consequences in the vasculature with resetting of the angiogenic and inflammatory potential of the endothelium, and sensitization to inflammatory cytokines. The significance of our findings was corroborated by our demonstration that the SK pathway is activated in RA, implicating SK as a potential therapeutic target.

Using two complementary methods of SK1 over-expression in EC, we generated cells with a 2- to 5-fold increase in SK1 activity, which is of similar magnitude to the endogenous SK1 activity induced following TNF-stimulation of normal EC [8]. Thus, we were able to investigate the effects of chronic SK1 over-activity at what we estimate to be “normal” physiological levels. In contrast to TNF-stimulated SK1 activity, which is extremely transient, peaking at 10 min and returning to basal levels within 30 min [8], our system enabled us to investigate the effects of chronic sustained increases in SK1 activity. Although chronic activation of SK1 activates the endothelium in the absence of cytokine, as evidenced by basal VCAM-1 induction (Fig. 1), by far the most dramatic changes were in the sensitization of EC^{SK} to subliminal doses of TNF (Fig. 2), suggesting that SK1 over-expression resets the threshold of endothelial responsiveness. Although the magnitude of the changes in adhesion molecule expression was small, the effect on neutrophil adhesion was large (Fig. 4), suggesting that either the levels of adhesion molecules are synergistic in their regulation of neutrophil adhesion and/or that SK1 alters other pathways which

influence the endothelial binding of neutrophils. Our findings are consistent with our previous demonstration that SK1 is an obligatory enzyme in mediating the adhesion molecule up-regulation induced by TNF [8]. This role is substantiated by the over-expression of the dominant-negative SK1, which blocked TNF-induced VCAM-1 and E selectin expression (Fig. 1) and inhibited TNF-induced neutrophil adhesion (Fig. 4). This inactive mutant also confirms that the catalytic activity of SK1 is critical in mediating the TNF-induced effects in EC.

In addition to altering the inflammatory potential of the endothelium, we found that SK1 over-expression sensitizes the cells to angiogenic factors, as evidenced by the induction of capillary tube formation (Fig. 5). Receptor regulation is unlikely to be the mechanism, as changes in the levels of the VEGF receptors, Flk-1 or Flt-1, were not seen in the EC^{SK} (data not shown). However, the EC^{SK} did show enhanced migration to a chemoattractant (Fig. 5), which was at least partially mediated through a PTx-insensitive mechanism. Exogenous S1P induces cell spreading on Matrigel and enhances migration [39], which are effects mediated via binding to S1P₁ and S1P₃ receptors [19, 40] resulting in the activation of the Rho family of GTP-binding proteins [39]. Rho is involved in the organization of the actin cytoskeleton, and its activity is essential in regulating EC organization during angiogenesis [41, 42]. Although our studies cannot dismiss a role for extracellular S1P in stimulating migration and angiogenesis, it is noteworthy that even in the presence of PTx, EC^{SK} maintained a migratory advantage (Fig. 5) suggesting that at least a component of the response is mediated independently of the G_{i/o}-linked S1P₁, the most abundant S1P receptor in the EC. Indeed, previous studies have suggested intracellular roles for S1P. For example, microinjection of S1P into Swiss 3T3 cells stimulates mitogenesis and anti-apoptosis, two additional features of angiogenesis, along with the activation of phospholipase D and the phosphorylation of p125^{FAK} [43]. Phospholipase D is involved in MAPK activation and EC migration [44], while p125^{FAK} promotes angiogenesis, largely through enhancing cell migration [45]. Thus, it is reasonable to speculate that the effects of SK1 over-expression in HUVEC described here are mediated by intracellular S1P. Notably, PTx failed to inhibit adhesion molecule expression in HUVEC by SK1 over-expression (Fig. 3), suggesting an S1P₁-independent role for SK1. It is possible that S1P₂ and S1P₃, also expressed in HUVEC, albeit at considerably lower levels than S1P₁, may mediate the effects of SK1 expression observed here, although micromolar concentrations of S1P are required to induce adhesion molecule expression [8], whereas the activation of S1P receptors requires only nanomolar S1P concentrations [18]. It is acknowledged that S1P₁ signaling was inhibited only with the use of pertussis toxin, and that the use of S1P₂ antagonists such as JTE103 and S1P₁/S1P₃ antagonists such as VPC2309 would further help clarify the role of S1P receptors in the observed responses.

The recent finding that raised SK1 activity in HUVEC inhibits basal permeability [46] is out of keeping with the pro-angiogenic phenotype observed.

PECAM-1 has a well documented role in cell survival, migration and permeability. It is to be noted that TNF and VEGF, which stimulate SK1 activity, enhance endothelial leakiness, and stimulate PECAM-1 phosphorylation [47, 48], whilst angiopoietin, which also stimulates SK1 activity, inhibits EC permeability [46]. Thus, SK1 appears to have dichotomous effects, i.e. a pro-inflammatory pro-angiogenic phenotype, but also the ability to exert anti-permeability effects. At this stage, we would suggest that this may reflect agonist and context-specific differences in the level of PECAM-1 phosphorylation.

Our finding of raised S1P levels in RA synovial fluid indicates activation of the SK signaling pathway in RA. Indeed our findings are consistent with those of Kitano *et al.*, who, using high performance liquid chromatography, also found elevated levels of S1P in synovial fluid from patients with RA compared with the levels for patients with OA [49]. This same group demonstrated that S1P₁ expression is up-regulated in RA synovium and that the binding of S1P to its receptor regulates synovial proliferation and enhances the COX-2 and PGE2 production induced by inflammatory cytokine [49]. Furthermore, the resistance to Fas-induced death of lymphoblastoid B-cell lines from patients with RA [50] was shown to be mediated by extracellular S1P acting through GPCR to enable a PI-3K-dependent activation of SK1 [51]. Hence, the recent demonstration that SK1-knockout mice are not protected from collagen-induced arthritis [52] is surprising. Certainly the role of SK1 in inflammation was supported in a murine model of ulcerative colitis wherein the inhibition of SK ameliorated the disease [53].

This study not only broadens our understanding of the function of SK1 in EC, but also suggests a role for SK1 in the pathogenesis of conditions characterized by aberrant angiogenesis and inflammation, such as RA. Of note, TNF, IL-1, and VEGF are found in greater abundance in patients with RA, and clinical interest has thusfar been directed towards targeting these cytokines. As each of these may also activate SK1 [54], a rationale exists for manipulating the intracellular SK1 activity to create the desired therapeutic effects.

CONCLUSIONS

Modestly raised intracellular SK1 activity in the EC enhances the expression of adhesion molecules, sensitizes the cells to inflammatory cytokines, and results in enhanced leukocyte adhesion in the basal and stimulated states. These pro-inflammatory changes are accompanied by the stimulation of tube formation and migration. The effects on endothelial cell function of more marked increases in SK1 activity have recently been described [55]. Evidence for the activation of the SK/S1P signaling pathway in RA, a disease characterized by aberrant inflammation and angiogenesis was found, suggesting the possibility of manipulating this pathway for therapeutic purposes.

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REFERENCES

1. Prendergast, R.A., Luty, G.A. and Dinarello, C.A. Interleukin-1 induces corneal neovascularisation. **Fed. Proc.** 46 (2002) 1200.
2. Koch, A.E., Harlow, L.A., Haines, G.K., Amento, E.P., Unemori, E.N., Wong, W.L., Pope, R.M. and Ferrara, N. Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. **J. Immunol.** 152 (1994) 4149-4156.
3. Kraling, B.M., Razon, M.J., Boon, L.M., Zurakowski, D., Seachord, C., Darveau, R.P., Mulliken, J.B., Corless, C.L. and Bischoff, J. E-selectin is present in proliferating endothelial cells in human hemangiomas. **Am. J. Pathol.** 148 (1996) 1181-1191.
4. Nguyen, M., Strubel, N.A. and Bischoff, J. A role for sialyl Lewis-X/A glycoconjugates in capillary morphogenesis. **Nature** 365 (1993) 267-269.
5. Koch, A.E., Halloran, M.M., Haskell, C.J., Shah, M.R. and Polverini, P.J. Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. **Nature** 376 (1995) 517-519.
6. Pyne, S. and Pyne, N.J. Sphingosine 1-phosphate signalling in mammalian cells. **Biochem. J.** 349 (2000) 385-402.
7. Johnson, K.R., Johnson, K.Y., Becker, K.P., Bielawski, J., Mao, C. and Obeid, L.M. Role of human sphingosine-1-phosphate phosphatase 1 in the regulation of intra- and extracellular sphingosine-1-phosphate levels and cell viability. **J. Biol. Chem.** 278 (2003) 34541-34547.
8. 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- α induces adhesion molecule expression through the sphingosine kinase pathway. **Proc. Natl. Acad. Sci. USA** 95 (1998) 14196-14201.
9. Xia, P., Wang, L., Gamble, J.R. and Vadas, M.A. Activation of sphingosine kinase by tumor necrosis factor- α inhibits apoptosis in human endothelial cells. **J. Biol. Chem.** 274 (1999) 34499-34505.

10. Olivera, A. and Spiegel, S. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. **Nature** 365 (1993) 557-560.
11. Mazurek, N., Megidish, T., Hakomori, S. and Igarashi, Y. Regulatory effect of phorbol esters on sphingosine kinase in BALB/C 3T3 fibroblasts (variant A31): demonstration of cell type-specific response – a preliminary note. **Biochem. Biophys. Res. Commun.** 198 (1994) 1-9.
12. Edsall, L.C., Pirianov, G.G. and Spiegel, S. Involvement of sphingosine 1-phosphate in nerve growth factor-mediated neuronal survival and differentiation. **J. Neurosci.** 17 (1997) 6952-6960.
13. Rius, R.A., Edsall, L.C. and Spiegel, S. Activation of sphingosine kinase in pheochromocytoma PC12 neuronal cells in response to trophic factors. **FEBS Lett.** 417 (1997) 173-176.
14. Choi, O.H., Kim, J.H. and Kinet, J.P. Calcium mobilization via sphingosine kinase in signalling by the Fc epsilon RI antigen receptor. **Nature** 634 (1996) 634-636.
15. Melendez, A., Floto, R.A., Gillooly, D.J., Harnett, M.M. and Allen, J.M. Fc gamma RI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. **J. Biol. Chem.** 273 (1998) 9393-9402.
16. Wu, W., Shu, X., Hovsepian, H., Mosteller, R.D. and Broek, D. VEGF receptor expression and signaling in human bladder tumors. **Oncogene** 29 (2003) 3361-3370.
17. 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.
18. 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.
19. Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I. and Hla, T. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. **Cell** 99 (1999) 301-312.
20. An, S., Zheng, Y. and Bleu, T. Sphingosine 1-phosphate-induced cell proliferation, survival, and related signaling events mediated by G protein-coupled receptors Edg3 and Edg5. **J. Biol. Chem.** 275 (2000) 288-296.
21. Kimura, T., Watanabe, T., Sato, K., Kon, J., Tomura, H., Tamama, K., Kuwabara, A., Kanda, T., Kobayashi, I., Ohta, H., Ui, M. and Okajima, F. Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3. **Biochem. J.** 348 (2000) 71-76.
22. 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)10627-10633.
23. Morales-Ruiz, M., Lee, M.J., Zollner, S., Gratton, J.P., Scotland, R., Shiojima, I., Walsh, K., Hla, T. and Sessa, W.C. Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. **J. Biol. Chem.** 276 (2001) 19672-19677.
 24. Spiegel, S. Sphingosine 1-phosphate: a ligand for the EDG-1 family of G-protein- coupled receptors. **Ann. N. Y. Acad. Sci.** 905 (2000) 54-60.
 25. Ghosh, T.K., Bian, J. and Gill, D.L. Intracellular calcium release mediated by sphingosine derivatives generated in cells. **Science** 248 (1990) 1653-1656.
 26. 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.
 27. 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 Bcl-2 family members. **Blood** 105 (2005) 3169-3177.
 28. 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.
 29. Pitson, S.M., D'andrea, R.J., Vandeleur, L., Moretti, P.A., Xia, P., Gamble, J.R. Vadas, M.A. and Wattenberg, B.W. Human SK: purification, molecular cloning and characterization of the native and recombinant enzymes. **Biochem. J.** 350 (2000) 429-441.
 30. 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.
 31. Zannettino, A.C., Rayner, J.R., Ashman, L.K., Gonda, T.J. and Simmons, P.J. A powerful new technique for isolating genes encoding cell surface antigens using retroviral expression cloning. **J. Immunol.** 156 (1996) 611-620.
 32. Roberts, J.L., Moretti, P.A.B., Darrow, A.L., Derian, C.K., Vadas, M.A. and Pitson, S.M. An assay for sphingosine kinase activity using biotinylated sphingosine and streptavidin-coated membranes. **Anal. Biochem.** 331 (2004)122-129.
 33. Leavesley, D.I., Schwartz, A., Rosenfeld, M. and Cheresch, D.A. Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. **J. Cell. Biol.** 121 (1993) 163-170.
 34. Edsall, L.C. and Spiegel, S. Enzymatic measurement of sphingosine 1-phosphate. **Anal. Biochem.** 272 (1999) 80-86.

35. Gamble, J.R., Harlan, J.M., Klebanoff, S.J. and Vadas, M.A. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. **Proc. Natl. Acad. Sci. USA** 82 (1985) 8667-8671.
36. Gamble, J.R., Khew-Goodall, Y. and Vadas, M.A. Transforming growth factor-beta inhibits E-selectin expression on human endothelial cells. **J. Immunol.** 150 (1993) 4494-4503.
37. Sadeghi, M.M., Collinge, M., Pardi, R. and Bender, J.R. Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. **J. Immunol.** 165 (2000) 2712-2718.
38. Yamauchi, J., Miyamoto, Y., Kokubu, H., Nishii, H., Okamoto, M., Sugawara, Y., Hirasawa, A., Tsujimoto, G. and Itoh, H. Endothelin suppresses cell migration via the JNK signaling pathway in a manner dependent upon Src kinase, Rac1, and Cdc42. **FEBS Lett.** 11 (2002) 284-288.
39. Okamoto, H., Yatomi, Y., Ohmori, T., Satoh, K., Matsumoto, Y. and Ozaki, Y. Sphingosine 1-phosphate stimulates G(i)- and Rho-mediated vascular endothelial cell spreading and migration. **Thromb. Res.** 99 (2000) 259-265.
40. Ancellin, N., Colmont, C., Su, J., Li, Q., Mittereder, N., Chae, S.S., Stefansson S, Liao G, and Hla T. Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. **J. Biol. Chem.** 277 (2002) 6667-6675.
41. Hoang, M.V., Whelan, M.C. and Senger, D.R. Rho activity critically and selectively regulates endothelial cell organization during angiogenesis. **Proc. Natl. Acad. Sci. USA** 101 (2004) 1874-1879.
42. Bayless, K.J. and Davis, G.E. Sphingosine-1-phosphate markedly induces matrix metalloproteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional collagen and fibrin matrices. **Biochem. Biophys. Res. Commun.** 26 (2003) 903-913.
43. Van Brocklyn, J.R., Lee, M.J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D.M., Coopman, P.J., Thangada, S., Liu, C.H., Hla, T. and Spiegel, S. Dual actions of sphingosine-1-phosphate: extracellular through the Gi- coupled receptor Edg-1 and intracellular to regulate proliferation and survival. **J. Cell. Biol.** 142 (1998) 229-240.
44. Yoon M.J., Cho, C.H., Lee, C.S., Jang, I.H., Ryu, S.H. and Koh, G.Y. Localization of Tie2 and phospholipase D in endothelial caveolae is involved in angiopoietin-1-induced MEK/ERK phosphorylation and migration in endothelial cells. **Biochem. Biophys. Res. Commun.** 308 (2003) 101-105.
45. Haskell, H., Natarajan, M., Hecker, T.P., Ding, Q., Stewart, J. Jr., Grammer, J.R. and Gladson, C.L. Focal adhesion kinase is expressed in the angiogenic blood vessels of malignant astrocytic tumors *in vivo* and promotes capillary tube formation of brain microvascular endothelial cells. **Clin. Cancer Res.** 9 (2003) 2157-2169.

46. Li, X., Stankovic, M., Bonder, C.S., Hahn, C.N., Parsons, M., Pitson, S.M., Proia, R.L., Vadas, M.A. and Gamble, J.R. Basal and angiopoietin-1-mediated endothelial permeability is regulated by sphingosine kinase-1. **Blood** 111 (2008) 3489-3497.
47. Esser, S., Lampugnani, M.G., Corada, M., Dejana, E. and Risau, W. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. **J. Cell. Sci.** 111 (1998) 1853-1865.
48. Ferrero, E., Villa, A., Ferrero, M.E., Toninelli, E., Bender, J.R., Pardi, R. and Zocchi, M.R. Tumor necrosis factor -induced vascular leakage involves PECAM1 phosphorylation. **Cancer Res.** 56 (1996) 3211-3215.
49. Kitano, M., Hla, T., Sekiguchi, M., Kawahito, Y., Yoshimura, R., Miyazawa, K., Iwasaki, T., Sano, H., Saba, J.D. and Tam, Y.Y. Sphingosine 1-phosphate/sphingosine 1-phosphate receptor 1 signaling in rheumatoid synovium: regulation of synovial proliferation and inflammatory gene expression. **Arthritis Rheum.** 54 (2006) 742-753.
50. Pi, X., Tan, S.Y., Hayes, M., Xiao, L., Shayman, J.A., Ling, S. and Holoshitz, J. Sphingosine kinase 1-mediated inhibition of Fas death signaling in rheumatoid arthritis B lymphoblastoid cells. **Arthritis Rheum.** 54 (2006) 754-764.
51. Tan, S.Y., Xiao, L., Pi, X. and Holoshitz, J. Aberrant Gi protein coupled receptor-mediated cell survival signaling in rheumatoid arthritis B cell lines. **Front Biosci.** 12 (2007) 1651-1660.
52. Michaud, J., Kohno, M., Proia, R.L. and Hla, T. Normal acute and chronic inflammatory responses in sphingosine kinase 1 knockout mice. **FEBS Lett.** 580 (2006) 4607-4612.
53. Maines, L.W., Fitzpatrick, L.R., French, K.J., Zhuang, Y., Xia, Z., Keller, S.N., Upson, J.J. and Smith, C.D. Suppression of Ulcerative Colitis in Mice by Orally Available Inhibitors of Sphingosine Kinase. **Dig. Dis. Sci.** 53 (2008) 997-1012.
54. Shu, X., Wu, W., Mosteller, R.D. and Broek, D. Sphingosine kinase mediated vascular endothelial growth-factor-induced activation of ras and mitogen-activated protein kinases. **Mol. Cell Biol.** 22 (2002) 7758-7768.
55. Limaye, V., Vadas, M.A., Pitson, S.M. and Gamble, J.R. The effects of markedly raised intracellular sphingosine kinase-1 activity in endothelial cells. **Cell. Mol. Biol. Lett.** 14 (2009) in press, DOI: 10.2478/s11658-009-0008-2.