Stable Knock-Down of the Sphingosine 1-Phosphate Receptor S1P₁ Influences Multiple Functions of Human Endothelial Cells

Vera Krump-Konvalinkova, Satoshi Yasuda, Tina Rubic, Natalia Makarova, Jörg Mages, Wolfgang Erl, Claudia Vosseler, C. James Kirkpatrick, Gabor Tigyi, Wolfgang Siess

Objectives—Sphingosine 1-phosphate (S1P) is a bioactive phospholipid acting both as a ligand for the G protein–coupled receptors S1P₁,₃ and as a second messenger. Because S1P₁ knockout is lethal in the transgenic mouse, an alternative approach to study the function of S1P₁ in endothelial cells is needed.

Methods and Results—All human endothelial cells analyzed expressed abundant S1P₁ transcripts. We permanently silenced (by RNA interference) the expression of S1P₁ in the human endothelial cell lines AS-M.5 and ISO-HAS.1. The S1P₁ knock-down cells manifested a distinct morphology and showed neither actin ruffles in response to S1P nor an angiogenic reaction. In addition, these cells were more sensitive to oxidant stress–mediated injury. New S1P₁-dependent gene targets were identified in human endothelial cells. S1P₁ silencing decreased the expression of platelet–endothelial cell adhesion molecule-1 and VE-cadherin and abolished the induction of E-selectin after cell stimulation with lipopolysaccharide or tumor necrosis factor-α. Microarray analysis revealed downregulation of further endothelial specific transcripts after S1P₁ silencing.

Conclusions—Long-term silencing of S1P₁ enabled us for the first time to demonstrate the involvement of S1P₁ in key functions of endothelial cells and to identify new S1P₁-dependent gene targets. (Arterioscler Thromb Vasc Biol. 2005; 25:546-552.)

Key Words: S1P₁  ■ functional analysis  ■ siRNA  ■ permanent inhibition  ■ endothelial cells

The bioactive sphingolipid metabolite, sphingosine 1-phosphate (S1P), is an important component of serum that is released primarily from activated platelets. S1P is a multifunctional physiologic mediator implicated in the regulation of a broad spectrum of biologic processes, including proliferation, survival, regulation of cytoskeletal reorganization, motility, and differentiation in many cell types.¹⁻⁵

The response of cells to S1P has been shown to be mediated predominantly by G protein–coupled receptors. Five receptors encoded by the endothelial differentiation gene family that bind S1P with high affinity have been described.⁶⁻¹⁰ The receptors couple to multiple G proteins that activate different intracellular signaling pathways. Several of these receptors are simultaneously coexpressed on the same cell.

In addition to acting as a ligand of cell surface receptors, S1P can function as a second messenger.³⁻⁴,¹¹ A variety of external stimuli, particularly growth factors and chemottractants, as well as lysophosphatidic acid and S1P, have been reported to strongly stimulate S1P kinase (SphK) to generate intracellular S1P, which can mobilize calcium from internal stores,¹² regulate cell survival by activating the transcription factor nuclear factor-κB,¹³ and control cell proliferation by mediating Ras and extracellular signal–regulated kinase 1/2 activation in cells stimulated with vascular endothelial growth factor (VEGF).¹⁴

Stimulation of the S1P₁ receptor (previously known as endothelial differentiation gene-1, edg-1) activates a G₁-linked pathway leading to cell growth, survival, and migration.¹⁵,¹⁶ The signaling pathways mediating these responses include activation of the Ras and Rho GTPases, which direct mitogenesis and cytoskeletal remodeling, respectively. S1P₁ receptor–knockout mice are not viable and die in utero because of defects in vascular maturation.¹⁷ Coordinate endothelial expression of S1P₁, S1P₂, and S1P₃ is essential for embryonic development.¹⁸,¹⁹
The cytoskeletal and morphogenetic response of human umbilical vein endothelial cells (HUVECs) to S1P has been evaluated with the use of antisense oligonucleotides directed against S1P1 and S1P2. Receptor expression inhibition studies indicated that S1P1 couples to Rac, regulating cortical actin assembly, whereas S1P2 couples to Rho, involved in stress fiber formation. However, measurements of Rac and Rho activity by biochemical assays indicated that both receptors are capable of activating both Rac and Rho pathways involved in migration and morphogenetic differentiation of endothelial cells into capillary-like structures. Both receptors mediate the activation of membrane type 1-matrix metalloproteinase by S1P.

To analyze the function of S1P1 in human endothelial cells, we generated human endothelial cell lines with a permanently reduced expression of S1P1 by introducing plasmids encoding small interfering RNA (siRNA) targeted to S1P1 into the cell lines AS-M.5 and ISO-HAS.1. The use of established human endothelial cell lines enabled us to study the long-term functional consequences of S1P1 silencing.

Methods

Please see the expanded online Methods section at http://atvb.ahajournals.org.

Cell Culture

HUVECs were isolated as described and propagated in ECG medium (PromoCell). The human cell lines derived from human pulmonary microvascular endothelial cells (HPMEC-ST1.6R) and from patients with angiosarcoma (AS-M.5 and ISO-HAS.1) were cultured in ECG medium-MV (PromoCell).

Cloning of siS1P1 Sequences

The S1P1-specific siRNA expression vectors were generated by cloning the sequences encoding the hairpin siRNA targeted to S1P1 into the siRNA expression vector pSilencer 1.0-U6 (Ambion, Austin, Tex). The targeted sequences were as follows: #1, 5'-GGAGATGCGTCGGGCCTTC-3'; #2, 5'-CTGCACTAGTGCGCTGCC-3'; and #3, 5'-TGACGATCTATATAGCA-3', located at bp 1196 to 1214, 797 to 816, and 1940 to 1958, respectively (NCBI accession number BC018650). The control plasmid was pSilencer negative control (Silco) that encodes siRNA having no significant sequence similarity to human gene sequences (Ambion). The secondary structure was determined by using the Vienna RNA secondary structure prediction program.

Generation of S1P1–Knock-Down Endothelial Cell Lines

Plasmids encoding siRNA targeted to the S1P1 transcript were introduced into endothelial cells, and stably transfected cells were selected as described at www.ahajournals.org.

Results

Expression Profile of S1P Receptors in Cultured HUVECs

Transcripts encoding S1P1 were abundant in all cultures of endothelial cells tested. Transcripts encoding S1P1 were detected by reverse transcription–polymerase chain reaction (RT-PCR) in all but ISO-HAS.1 cells (Figure 1). By quantitative RT-PCR, the S1P1 transcripts were less abundant than S1P1 transcripts, representing 4.3%, 3.5%, and 0.75% of S1P1 expression levels in HUVEC, AS-M.5, and ISO-HAS.1, respectively. A very weak expression of S1P2 was observed in HUVECs, ISO-HAS, and a subclone of HPMEC-ST1.6R, designated HPMEC-ST1.6R.D. Transcripts encoding either S1P2 or S1P3 were not detected (Figure 1).

siRNA Efficiently Reduces the Concentration of S1P1 Transcripts

To gain insight into the biologic function of S1P1 in human endothelial cells, we introduced into the cell lines AS-M.5 and ISO-HAS.1 plasmids encoding siRNA targeted to 3 different sequences in the S1P1 transcript (Sil#1, Sil#2, Sil#3) and isolated stably transfected cells. We measured by quantitative RT-PCR the levels of S1P1 transcripts in the transfected cells and compared these with unmanipulated cells and with cells stably transfected with a control plasmid (Silco; Figure 2). The levels of S1P1 transcripts differed substantially between the different cells. In the AS-M.5 cells transfected with Sil#1 (AS-M.5-Sil#1), S1P1 transcripts were repeatedly reduced to ∼20% of levels detected in either AS-M.5 or AS-M.5-Silco cells (Figure 2). We isolated single cell–derived clones of AS-M.5-Sil#1 and analyzed them for expression of S1P1. Individual clones (cl.) differed in the levels of S1P1 expression, reaching average values of 15% to 70% of S1P1 levels detected in the control pool of cells (Figure 2). The low S1P1 expression level remained stable for at least 6 months in a selected clone, AS-M.5-Sil#1, T2, cl.3. In contrast, expression of S1P1 in the cells containing the construct Sil#2 and Sil#3 was reduced to only 60% of control or not affected, respectively (Figure 2).

The 3 pSil-derived plasmids encoding siRNA sequences targeted to S1P1 transcript also were stably introduced into

Figure 1. Expression of S1P receptors in human endothelial cells. Total RNA from the endothelial cell lines ISO-HAS.1, HPMEC-ST1.6R, designated HPMEC-ST1.6R.D. Transcripts encoding either S1P1 or S1P2, S1P3, S1P4, or β-actin. RT-PCR products were separated on agarose gel and stained with ethidium bromide. Abbreviations are as defined in text.
the ISO-HAS.1 cell line. In this cell line, only the pSil#1 construct was efficient in reducing the expression of S1P1 (Figure 2, dark gray bars). S1P1 expression in single cell–derived clones of these cells was stably reduced by 50% to 80%.

The low expression of S1P1 transcripts, as measured by quantitative RT-PCR, was unchanged in the clones of S1P1-knockdown AS-M.5 clones (T2, Sil#1, cl.2, 3, and 6) and also not affected by transfection of ISO-HAS.1 with Sil#1 plasmid (not shown), suggesting that S1P1 downregulation had no effect on expression of S1P1.

Cells With Reduced S1P1 Expression Display Altered Morphology and Have Altered Proliferation Characteristics

The S1P1-knockdown AS-M.5 cells exhibited a distinctive morphology (Figure Ia, A and B, available online at http://atvb.ahajournals.org) compared with either the nonmanipulated cells (Figure Ia, D) or the cells transfected with Silco (Figure Ia, C). S1P1-knockdown cells contained vesicles on their surface, a feature that was more prominent in low-density cultures (Figure Ia, B). These profound changes in cellular morphology were observed in cultures of AS-M.5 cells transfected with the construct Sil#1 before the clonal selection in 2 independent experiments. After single-cell cloning, 3 single cell–derived clones with very low S1P1 expression levels (T2, cl.2, 3, and 6) displayed this morphology, suggesting that S1P1 downregulation had no effect on expression of S1P1.

Figure 2. Knockdown of S1P1 by RNA interference. AS-M.5 (gray) and ISO-HAS.1 (dark gray) cells were transfected with pSilencer negative control vector (Silco) and pSilencer encoding siRNA targeted to sequence #1 and #2 in the S1P1 transcript. Two independent transfections (T1, T2) were performed. Pools of transfected cells (Silco, Sil#1, and Sil#2) as well as single cell–derived clones (cl.) isolated from the pools of transfected cells were analyzed for S1P1 expression by real-time RT-PCR. Values (% of control) are mean±SEM. All other abbreviations are as defined in text.

In low-density cultures, AS-M.5-pSil#1 cells grew considerably slower than did the control AS-M.5 cells (not shown). However, no major differences in growth characteristics were observed in high-density cultures, suggesting that the AS-M-

S1P1-Knockdown Cells Do Not Show S1P-Induced Reorganization of Actin

S1P-induced remodeling of the actin cytoskeleton is a characteristic response in many cell types. To verify the role of S1P1 in this reaction, we evaluated cytoskeletal responses in serum-starved cells. Confluent monolayers of both AS-M.5 and AS-M.5-Silco cells showed F-actin fibers distributed throughout the cytoplasm (Figure Ib, A and C). Treatment with S1P for 2 minutes caused spectacular reorganization of the actin cytoskeleton: fibers of F-actin were concentrated at cell-to-cell contacts in confluent cultures (Figure Ib, D and F) and into a subcortical network in subconfluent cultures (not shown). In contrast, neither actin fibers nor a response to S1P treatment was observed in AS-M.5-Sil#1 cells (Figure Ib, B and E). These observations demonstrate that the S1P1 receptor is essential for the reorganization of the actin cytoskeleton induced by S1P in human endothelial cells.

Figure 3. S1P1-knockdown endothelial cells show no angiogenic response. AS-M.5 (A, D), AS-M.5 S1P1 knockdown cells (AS-M.5-Sil#1; B, E), and AS-M.5 cells containing the control plasmid (AS-M.5-Silco; C, F) were plated on Matrigel and incubated for 6 (A–C) or 24 (D–F) hours. Phase-contrast micrographs of capillary structures. Bar represents 100 μm. Abbreviations are as defined in text.

SiIl#1 cells may depend on substances released by neighboring cells available in high-density cultures.

S1P1 Is Indispensable for the Angiogenic Response of Endothelial Cells

To evaluate the effect of reduced expression of S1P1 on the angiogenic reaction, we examined endothelial morphogenesis into capillary-like networks. Capillary formation was evaluated after 6 (Figure 3A through 3C) and 24 (Figure 3D through 3F) hours. The AS-M.5 (Figure 3A and 3D) and control AS-M.5-Silco (Figure 3C and 3F) cells showed the characteristic formation of capillary structures. In contrast, no angiogenic reaction was observed in AS-M.5-Sil#1 cultures (Figure 3B and 3E). Therefore, S1P1 is required for the angiogenic reaction of endothelial cells. A similar angiogenic reaction was observed in ISO-HAS.1 cells expressing S1P1 but very little S1P3 (not shown).
Responses to Oxidative Stress in S1P1-Knockdown Cells

The response to oxidative stress induced by 4-hydroxynonenal (4-HNE; 10 to 20 μmol/L, 6 hours, 37°C) was evaluated by quantifying the ratio of necrotic to apoptotic cells. The percentage of both apoptotic and necrotic cells increased dependent on the concentration of 4-HNE and reached ~10% in ISO-HAS.1 (not shown) and AS-M.5 cells. Whereas apoptosis was the same in control and AS-M.5-sil#1 cells, the proportion of necrotic cells in AS-M.5-Sil#1 cultures increased dramatically after 4-HNE treatment, attaining 30% (Figure II, available online at http://atvb.ahajournals.org).

Reduced S1P1 Expression Abolishes the Expression of Cell Adhesion Molecules

S1P1 silencing led to a reduction in expression of both VE-cadherin and platelet–endothelial cell adhesion molecule-1 (PECAM-1). The degree of S1P1 knockdown was correlated with the expression levels of these cell surface proteins in both AS-M.5 and ISO-HAS.1 cells (Figure 4a). The typical cell surface expression pattern of VE-cadherin and PECAM-1 was observed in AS-M.5 cells (Figure 4b, A and D) and in HUVECs (Figure 4b, C and F). In contrast, no expression of VE-cadherin or PECAM-1 could be detected at cell-to-cell contacts in monolayers of AS-M.5-Sil#1 cells (Figure 4b, B and E). Gene expression analyses by microarrays showed downregulation of transcripts encoding PECAM-1 and VE-cadherin in 2 different clones of S1P1-silenced AS-M.5 cells (Table I, available online at http://atvb.ahajournals.org). These observations indicate that S1P1 is required for gene transcription and expression of VE-cadherin and PECAM-1 in human endothelial cells.

S1P1 Modulates the Endothelial Cell Response to Proinflammatory Stimuli

Similar to freshly isolated HUVECs that respond to proinflammatory stimuli by the transcriptional upregulation of cell adhesion molecules, both AS-M.5 (not shown) and AS-M.5-Silco cells responded to stimulation by tumor necrosis factor-α (TNF-α) (100 ng/mL, 6 hours) and lipopolysaccharide (LPS; 1 μg/mL, 6 hours) by a transient induction of transcription of E-selectin and intercellular adhesion molecule-1 (ICAM-1) on the cell surface (Figure 4c). Albeit diminished in comparison with the control cells, induction of ICAM-1 expression in response to TNF-α and LPS was clearly detectable in the knockdown AS-M.5 cells. However, in contrast to either the AS-M.5 or AS-M.5-Silco cells, no induction of E-selectin production was observed in AS-M.5-Sil#1 (Figure 4c). Analysis of E-selectin transcripts by real-time RT-PCR showed that E-selectin transcripts were highly reduced in the LPS-stimulated, S1P1-knockdown cells compared with the control cells, suggesting that E-selectin expression was regulated at the transcriptional level (not shown). Thus, we conclude that S1P1, is involved in the regulation of the inflammatory response of endothelial cells.

Microarray Analysis of Genes Expressed in S1P1-Silenced Endothelial Cells

Searching for gene targets that are dependent on S1P1 expression, we compared gene expression patterns of 2 clones of S1P1-knockdown AS-M.5 cells (T2, cl.2 and cl.3) with those of AS-M.5-Silco and AS-M.5. Among the 22 238 gene probes that the microarrays contained, we identified 82 genes with increased expression and 220 downregulated transcripts in the S1P1-knockdown cells (2-fold decrease and increase; error probability <5%). Many endothelial specific transcripts were downregulated (Table II, available online at http://atvb.ahajournals.org), but the expression of other genes was not affected in the S1P1-knockdown AS-M.5 cells. The latter genes include angiotensin II receptor (Hs.405348), endothe-
S1P1 transcripts. A similar observation was reported for than were the less exposed sequences (Sil#2, Sil#3) in the barrier function. Our results demonstrate that the S1P1 receptor is required for S1P-induced actin reorganization in human endothelial cells. Long-term silencing of S1P1 enabled us to identify new S1P1-dependent gene targets in endothelial cells. Down-regulation of S1P1 led to a reduced expression of the adhesion molecules PECAM-1, expressed predominantly at the endothelial cell contacts, and VE-cadherin, usually expressed at intercellular junctions. The degree of S1P1 knockdown was correlated with the extent of suppression of PECAM-1 and VE-cadherin. This suggests that the expression of these surface molecules is controlled by S1P1. VE-cadherin expression was previously reported to be reduced after injection of antisense oligonucleotides directed against S1P1 into HUVECs. Because functional VE-cadherin was demonstrated to be required for endothelial cell morphogenesis and PECAM-1–null endothelial cells failed to migrate in response to S1P, the lack of expression of both VE-cadherin and PECAM-1 in S1P1-knockdown endothelial cells may impede their morphogenetic reaction on Matrigel.

The S1P1-knockdown endothelial cells differed from the control cells also in their response to proinflammatory stimuli. Silencing of S1P1 had no major effect on LPS-induced expression of ICAM-1 but reduced the TNF-α–stimulated expression of ICAM-1 by 60%. No expression of E-selectin in response to stimulation with either LPS or TNF-α was observed in the S1P1-knockdown endothelial cells, implicating involvement of S1P1 in the regulation of E-selectin expression. Several lines of evidence suggest a possible participation of S1P in the inflammatory reaction of endothelial cells. TNF-α was found to induce rapid activation of SphK and subsequent generation of intracellular S1P that resulted in strong stimulation of E-selectin expression in HUVECs. The defective control of E-selectin expression in the S1P1-knockdown cells suggests that a functional S1P1 receptor is required to mediate expression of E-selectin in stimulated endothelial cells and implies that the intracellularly produced S1P does not function as a second messenger but rather is released from the cells and then activates S1P1. S1P treatment of both HUVEC and AS-M.5 cultures (not

Discussion

The analysis of expression profiles of S1P receptors showed that all human endothelial cell lines (AS-M.5, ISO-HAS.1, HPMEC-ST1, and HUVECs) expressed transcripts encoding S1P1, suggesting that the S1P1 receptor may have a ubiquitous function in endothelial cells. All human endothelial cells tested also contained transcripts encoding S1P1, at a much lower level, however, as measured by quantitative RT-PCR. Expression of both S1P1 and S1P3 characterizes the typical expression pattern of S1P receptors in cultured human endothelial cells.

To examine the function of S1P1 in human endothelial cells, we permanently silenced by RNA interference the expression of S1P1 in the established endothelial cell lines AS-M.5 and ISO-HAS.1, previously shown to possess many characteristics of freshly isolated endothelial cells. The siRNA targeted to a large loop in the secondary structure of the S1P1 transcript (Sil#1) was more efficient in silencing than were the less exposed sequences (Sil#2, Sil#3) in the S1P1 transcripts. A similar observation was reported for silencing of the insulin-like growth factor receptor, suggesting that the secondary structure of the targeted transcripts may be important for efficient silencing by siRNA. The reduction of S1P1 expression in the cells transfected with plasmids Sil#1 did not affect the already very low level of S1P3 expression and was stable for at least 6 months of continuous culturing.

Silencing of S1P1 transcripts to <20% of control profoundly altered the phenotype of endothelial cells. The S1P1-knockdown endothelial cells (AS-M.5-Sil#1) manifested an altered morphology and proliferated slowly in low-density cultures. In contrast to the control cells showing spectacular reorganization of the actin cytoskeleton in response to S1P, the S1P1-knockdown AS-M.5 cells showed no reorganization of actin after S1P treatment. Actin reorganization is an immediate response triggered by S1P. It has been reported to be regulated through balance between Rho and Rac activity in HUVECs and to be correlated with an enhancement of endothelial cell barrier function. Our results demonstrate that the S1P1 receptor is required for S1P-induced actin reorganization in human endothelial cells.

The S1P1-knockdown cells exhibited altered angiogenic properties. No formation of typical capillary-like networks of differentiated endothelial cells on Matrigel could be observed with AS-M.5-Sil#1, consistent with the reports on the involvement of S1P1 in angiogenesis. Both the lack of angiogenic response of AS-M.5-Sil#1 and the capacity to form capillary-like structures of ISO-HAS.1 expressing S1P1, but almost no S1P1, suggest that S1P1 but not S1P3 is essential for endothelial cell morphogenesis. However, we cannot exclude a cooperative effect of S1P and S1P3 previously observed in short-term inhibition studies with antisense oligonucleotides. In a recent study, downregulation of S1P1 expression effectively suppressed tumor angiogenesis in a murine model in vivo.

We observed an increased proportion of both apoptotic and necrotic cells in both control and AS-M.5-Sil#1 cells after treatment with 4-HNE, a mediator of oxidative stress shown to induce apoptotic death in HUVECs. Whereas no major difference between control and AS-M.5-Sil#1 cells was observed in the proportion of apoptotic cells, the proportion of necrotic cells in AS-M.5-Sil#1 increased significantly. S1P has been previously reported to activate SphK, which results in the intracellular production of S1P and has an anti-apoptotic effect in endothelial cells. However, our observations indicate that S1P1 is not involved in the antiapoptotic activity of S1P but may rather protect endothelial cells from necrosis.

Long-term silencing of S1P1 enabled us to identify new S1P1-dependent gene targets in endothelial cells. Reduction of S1P1 led to a reduced expression of adhesion molecules PECAM-1, expressed predominantly at the endothelial cell contacts, and VE-cadherin, usually expressed at intercellular junctions. The expression of these surface molecules is controlled by S1P1. VE-cadherin expression was previously reported to be reduced after injection of antisense oligonucleotides directed against S1P1 into HUVECs. Because functional VE-cadherin was demonstrated to be required for endothelial cell morphogenesis and PECAM-1–null endothelial cells failed to migrate in response to S1P, the lack of expression of both VE-cadherin and PECAM-1 in S1P1-knockdown endothelial cells may impede their morphogenetic reaction on Matrigel.

The S1P1-knockdown endothelial cells differed from the control cells also in their response to proinflammatory stimuli. Silencing of S1P1 had no major effect on LPS-induced expression of ICAM-1 but reduced the TNF-α–stimulated expression of ICAM-1 by 60%. No expression of E-selectin in response to stimulation with either LPS or TNF-α was observed in the S1P1-knockdown endothelial cells, implicating involvement of S1P1 in the regulation of E-selectin expression. Several lines of evidence suggest a possible participation of S1P in the inflammatory reaction of endothelial cells. TNF-α was found to induce rapid activation of SphK and subsequent generation of intracellular S1P that resulted in strong stimulation of E-selectin expression in HUVECs. The defective control of E-selectin expression in the S1P1-knockdown cells suggests that a functional S1P1 receptor is required to mediate expression of E-selectin in stimulated endothelial cells and implies that the intracellularly produced S1P does not function as a second messenger but rather is released from the cells and then activates S1P1. S1P treatment of both HUVEC and AS-M.5 cultures (not
shown). The latter observations suggest that a synergistic interaction of the signal transduction pathways induced by TNF-α and S1P, receptor activation is required for robust E-selectin expression observed after treatment of endothelial cells with TNF-α.

Further S1P1-dependent gene targets were identified by microarray analysis of gene expression. Many endothelial specific transcripts, such as the VEGF-receptor KDR, were downregulated in S1P1-silenced endothelial cells, but other endothelial specific genes, such as the VEGF-receptor Flt-1 and the fms-related kinase Flt-3, were not affected.

In conclusion, our results demonstrate that long-term silencing of S1P1 profoundly alters gene expression and key functions of cultured human endothelial cells. This observation, if confirmed in vivo, could have important therapeutic consequences for chronic inhibition of the S1P1 receptor with receptor antagonists or low concentrations of agonists leading to S1P downregulation. The immunosuppressive drug FTY720, an agonist of S1P-receptors currently used in clinical trials, at therapeutic concentrations has been recently reported to block S1P signaling through downregulation of S1P1,2,5. Our results imply that FTY720 could be harmful if it induced downregulation of S1P1 on vascular endothelium in vivo.

Acknowledgments

The study was supported by the August-Lenz-Stiftung and the Deutsche Forschungsgemeinschaft (graduate program Vascular Biology in Medicine GRK 438: T.R. and C.V., SFB 413 and Si 247/9). We thank Dr Reinhard Hoffmann for his help with the microarray analyses and Nicole Wilke for technical assistance.

References

Stable Knock-Down of the Sphingosine 1-Phosphate Receptor S1P1 Influences Multiple Functions of Human Endothelial Cells
Vera Krump-Konvalinkova, Satoshi Yasuda, Tina Rubic, Natalia Makarova, Jörg Mages, Wolfgang Erl, Claudia Vosseler, C. James Kirkpatrick, Gabor Tigyi and Wolfgang Siess

Arterioscler Thromb Vasc Biol. 2005;25:546-552; originally published online December 23, 2004;
doi: 10.1161/01.ATV.0000154360.36106.d9
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/3/546

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/02/27/01.ATV.0000154360.36106.d9.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at: http://atvb.ahajournals.org//subscriptions/
Figure I online
Morphology of S1P₁-knock-down endothelial cells (a) and absent reorganization of the actin cytoskeleton in S1P₁-knock-down endothelial cells (b).
(a) The morphology of AS-M.5-Sil#1, cl.3 cultures (A: confluent, B: subconfluent cells) was compared to confluent cells of both unmanipulated AS-M.5 cells (D) and AS-M.5 cells stably transfected with a control plasmid (AS-M.5-Silco) (C). Phase contrast, bar indicates 50 µm.
(b) Confluent cultures of AS-M.5 cells (A, D), AS-M.5 S1P₁-knock-down cells (AS-M.5-Sil#1, B, E) and AS-M.5 cells containing the control plasmid (AS-M.5-Silco, C, F) were serum starved for 12 hours. Cells were untreated (A, B, C) or treated with 5µM S1P for 2 min. (D, E, F). Fixed cells were stained with phalloidin-Alexa 546 and F-actin was visualized by fluorescence microscopy. Bar indicates 100 µm.
**Figure II online.** Increased sensitivity S1P₁-knock-down endothelial cells to oxidative stress.
Response of cells to 4-HNE (5h, 37°C, 10µM, 20µM) compared to controls incubated in the buffer containing 0.1% EtOH, the same amount as the HNE-20 µM sample. The proportion of apoptotic (diamonds) and necrotic (squares) cells determined by flow cytometry after AnnexinV/propidium iodide staining. AS-M.5-Sil#1 (full lines) and AS-M.Silco (interrupted lines).
Stable knock-down of the sphingosine-1-phosphate receptor S1P₁ influences multiple functions of human endothelial cells

V. Krump-Konvalinkova, et al.

Supplementary Information

Methods-Online

Cloning of siRNA Sequences targeted to S1P₁

The S1P₁-specific siRNA expression vectors were generated by cloning the sequences encoding the hairpin siRNA targeted to S1P₁ into pSilencer™ 1.0-U6 (Ambion, Austin, Tx). For this, DNA oligonucleotides having the sequence: 5′- sense strand of the targeted sequence - TTCAAGAGA (spacer) - reverse complementary strand of the targeted sequence - stretch of five T (RNA polymerase III terminator sequence) - 3′ were annealed to the complementary oligonucleotides provided of adaptor sequence for ApaI and EcoRI sites at the 3’and 5’ terminus respectively. The targeted sequences in the S1P₁ mRNA were: #1: 5´-GGAGATGCCTGGGCCTTC-3´, #2: 5´-CTGCATCAGTGCGCTGTCC-3´, and #3: TGATCGATCATCTATAGCA-3´, located at bp 1196-1214, 797-816, and 1940-1958 respectively (NCBI accession number BC018650). These sequences were evaluated against the database using the NIH blast program to test for specificity. The annealed oligonucleotides were inserted between the ApaI and EcoRI sites of the polylinker in the siRNA expression vector pSilencer™ 1.0-U6 and transformed into competent DH5α (Invitrogen, Karlsruhe, Germany). The correctness of the inserts was confirmed by sequencing using primers P1 (5´- GAGACTATAAATATCCCTTGG-3´) and P4 (5´- GACCATGATTACGCAAAGCGCG-3´). The control plasmid was pSilencer Negative Control that encodes siRNA having no significant sequence similarity to human gene sequences (Ambion).
**Transfection, Selection of Transfected Cells**

Cells were transfected using Lipofectin (Invitrogen, Carlsbad, Tx) according to the instructions of the manufacturer. Briefly, subconfluent overnight monolayers (5 x 10⁴ cells/well in a 6-well plate) were exposed to complexes of plasmid (1-10µg) and Lipofectin 10µl in Opti-MEM® medium (Invitrogen) for 3 h. Transfection conditions were optimized in transient transfections using a plasmid encoding Green Fluorescent Protein (GFP), pGFP-C1 (Becton Dickinson, Heidelberg, Germany). Approximately 40% of treated cells routinely expressed GFP after 24 h. In cotransfection experiments, a 10-fold excess of the pSilencer-derived plasmids encoding the siRNA as compared to the pcDNA3 (Invitrogen) encoding the aminoglycoside phosphotransferase gene that confers cells resistance to G418, was used. Stably transfected cells were selected in media containing 75µg/ml G418 (Sigma-Aldrich, Saint Louis, MO), beginning 3 days after transfection. The untransfected cells died in the selection medium within 7 days. The selection was maintained throughout the culture of transfected cells. The cells were cloned by limiting dilution in 96-well plates. Genomic DNA of stably transfected cells was isolated using the Genomic Prep Blood DNA Isolation Kit (Amersham Biosciences, Freiburg, Germany) and subjected to PCR using puReTaq™ PCR Beads (Amersham Biosciences). The presence of the si-RNA-encoding sequences in the genomic DNA of the G418-resistant cells was confirmed by PCR using primers P1 and P4 (see above).

**PCR and semi-quantitative RT-PCR of S1P Receptors**

Total cellular RNA was isolated using TRIZOL Reagent (Invitrogen) followed by treatment with deoxyribonuclease I (Invitrogen) and then reverse-transcribed using ThermoScrip-RT-PCR System (Invitrogen). cDNA was synthesized from 250 ng total RNA and amplified with TaKaRaEx polymerase (TaKaRa, Shiga, Japan) and PCR was performed for 30 cycles at
94°C for 30s, 59°C for 45s, and 72°C for 1 min. The quantity of cDNA subjected to PCR was normalized to β-actin PCR products. Primers used for amplification of human S1P receptor sequences are indicated in supplementary Table 1. Primers used for amplification of E-selectin were: 5´-ATCAACATGAGCTGCAGTGG-3´ and 5´-AGCTTCCGTCTGATTCAA GG-3´, giving a product of 304 bp.

Quantitative RT-PCR of S1P$_1$ and S1P$_3$

Total cellular RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany). Real-time RT-PCR of S1P$_1$ and actin was performed using Ready-To-Go RT-PCR beads (Amersham Biosciences Europe GmbH, Freiburg) in the iCycler iQ$^\text{TM}$ (BioRad, Munich, Germany). Hundred ng of total cellular RNA were used in a 50 µl reaction. Reverse transcription was done at 42°C for 30 min using random hexamer according to the instructions of the manufacturer. PCR primers were: β-actin (5´-CACCACACCTTCTACAATGAGC-3´ and 5´-CAGAGGCGTACAGGGATAGC-3´), S1P$_1$ (5´-GACTCTGCTGGCGAATGAGCC-3´ and 5´-GCCCTTCCAGTGATGTTTCACAG-3´), and S1P$_3$ (5´-CTCAGGGAGGGCAGTCATG-3´ and GGCAGGATGGTAGAGCAGTC-3´) yielding amplification products with 153 bp, 350 bp, and 233 bp size, respectively. PCR primers for amplification of E-selectin were: 5´-ATCAACATGAGCTGCAGTGG-3´ AND 5´-AGCTTCCGTCTGATTCAAAGG-3´ and yielded products of 304 bp. Quantitative RT-PCR were optimized with regard to MgCl$_2$ concentration and annealing temperature by gradient PCR. The optimized annealing temperature for S1P$_1$, and actin primer pairs was 63°C, each RT-PCR reaction contained 3.5 mM MgCl$_2$ and 100 nM of each primer. The quantitative RT-PCR for S1P$_3$ was performed at annealing temperature of 60°C and the reaction contained 2.5 mM MgCl$_2$ and 100 nM of each primer. The amplified DNA was detected using SYBR Green (Molecular Probes, Leiden, The Netherlands). The following protocol was used for amplification of cDNA: denaturation at 95°C for 10 min; amplification and quantification cycles repeated 40 times (95°C for 15 s,
annealing at 63°C/ 60°C for 15 s, elongation at 72°C for 60 s) with a single fluorescence measurement and melting point analysis (60-95°C in 0.5 °C steps) with a continuous fluorescence measurement and a cooling step to 10°C.

The cDNA sequences were obtained from GenBank, the PCR primers were designed using the PRIMER3 software from the Whitehead Institute of Biomedical Research. To confirm the specificity of primers, the RT-PCR products were tested by melting point analysis (dissociation melting curve) as well as by examination of molecular weight of RT-PCR products after electrophoresis on agarose gels stained with ethidium bromide and sequencing. No primer-dimers were generated during the applied 40 real-time PCR amplification cycles.

The real-time PCR efficiencies for reference and target molecules were calculated using the iCyclerIQ™ software. Dilution series over four orders of magnitude (from 1µg to 1 ng/reaction) were used for each primer pair to determine the real-time PCR efficiency for one cycle in the exponential phase, calculated according to the equation: $E = 10^{[\frac{-1}{\text{slope}}]}$. The RT-PCR efficiencies for β-actin and S1P₁ were ~100% with high linearity (correlation coefficient >0.995). The relative expression ratio of a target gene was calculated based on the real-time PCR Expression ($E$) and the threshold cycle ($C_T$) deviation of an unknown sample versus control, and expressed in comparison to a reference gene using the equation: fold change $= 2^{\Delta(\Delta C_T)}$, where $\Delta C_T = C_T$ (target transcript) - $C_T$ (reference housekeeping transcript) and $\Delta(\Delta C_T) = \Delta C_T$ (treated sample) – $\Delta C_T$ (untreated control) [Applied Biosystems, ABI PRISM 7700 sequence detection system user bulletin, 1997, #2]. The β-actin gene was used as the reference gene. Each sample was analyzed in duplicate. Inter-assay variation was investigated both in different experimental runs performed with the same RNA and with RNA preparations isolated from related cultures.

**Gene Expression Analysis by Microarray**
The GeneChip® expression array analyses were performed using Affymetrix Human Genome U133A GeneChips (Affymetrix, Santa Clara, Ca) according to the instructions of the manufacturer. Total cell RNA (20µg) was biotin-labeled in one cycle T7-driven in vitro transcription. Amplified cRNA (15 µg) was hybridized at 45°C overnight, washed and stained as recommended by the manufacturer. The gene chips were subsequently scanned and analyzed using Affymetrix Microarray Suite 5.0 software (MAS 5.0). Each array was checked for general assay quality (average background noise of 91, mean scaling factor of 0.95 with a standard deviation of 0.16, 3´-5´ ratios for Gapdh and β-actin < 1.3).

Two independent experiments were performed. For global normalization and to generate expression values, Affymetrix CEL files were imported into the program RMA Express (V0.3 alpha 64, © 2003-2004 by B. M. Bolstad). As settings we activated the background correction algorithm and used quantile normalization. A statistic analysis using SAM algorithm V1.15 (two class, unpaired data) was performed to detect genes with significant differences in signal intensities between controls and S1P₁-knock down cells.

Monitoring Actin Reorganization

The cells were seeded on glass well chamber-slides (Becton Dickinson) coated with 5µg/ml fibronectin (Roche, Mannheim, Germany). The cultures had been serum-starved overnight before being used for experiments to exclude the effects of residual S1P in FCS. The cells were exposed to 5µM S1P (Biomol, Hamburg, Germany) for 2 min, fixed with paraformaldehyde (3.7%) in cytoskeleton-stabilization (CS) buffer (PIPES 0.1M, EGTA 1mM, polyethylene glycol 800, 4%) for 15 min at room temperature, washed 4 x with PBS, permeabilized with 0.1 % Triton-X 100 in PBS for 3 min, and washed 4 x with PBS. Staining for F-actin was performed with fluorochrome-conjugated phalloidin (AlexaFluor®546 phalloidin; Molecular Probes), diluted in PBS containing 1% BSA for 20 min in dark. The cells were then washed twice with PBS, rinsed with distilled water, and mounted with
Gel/Mount (Biomeda/Natutec, Germany). The stained cells were examined using a Leica fluorescence microscope (Leica DMRBE, Wetzlar, Germany).

**PECAM-1 and VE-cadherin Expression**

The cells were grown on fibronectin-coated glass-chamber slides (Becton Dickinson). After reaching confluence, the cells were fixed with buffered paraformaldehyde (3.7%) for 15 min at room temperature, washed with PBS, and exposed to primary antibodies diluted in PBS containing 3% BSA for 1h at room temperature, followed by an overnight incubation at 4°C. PECAM-1 was detected using monoclonal mouse anti-human CD31 from DAKO (Hamburg, Germany) and VE-cadherin was visualized with monoclonal mouse anti-human CD144 from Becton Dickinson (Heidelberg, Germany). After extensive washing, the cells were exposed to Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes) for 1 h at room temperature, washed with PBS, rinsed with distilled water, and mounted with Gel/Mount (Biomeda/Natutec, Germany). PECAM-1 and VE-cadherin expression was visualized by fluorescence microscopy (see above).

**Angiogenesis Assay/ Morphogenic Differentiation Assay**

Matrigel (Becton Dickinson, Bedford, MA) was diluted 1:3 in cell culture medium (Endothelial Cell Growth Medium, PromoCell), layered onto prechilled wells of a 24-well plate, and incubated at 37°C for 30 minutes, until solidified. The cells (1 x 10^5) were resuspended in complete medium, deposited onto the solidified matrix, and incubated at 37°C in a 5% CO₂ environment. For evaluation of effects of S1P on the angiogenic reaction, serum-starved cells in serum-free medium were plated on solidified matrigel and S1P(100 nM) was added immediately after plating the cells. The plates were examined by phase contrast microscopy using an inverted microscope (Olympus 1x50) and photographed with digital camera (Nikon Coolpix 4500).
Quantification of E-selectin, ICAM-1, PECAM-1 and VE-cadherin

The cells were incubated in the presence of 100 ng/ml of tumor necrosis factor-α (TNF–α) or 1 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich) at 37°C for 5 h. After stimulation, the cells were detached using trypsin/EDTA, diluted in Medium 199 (Sigma-Aldrich) containing 10% FCS, centrifuged at 250 x g for 10 minutes. The pellet was suspended in PBS containing 1% FCS and incubated with primary antibodies to CD54 (Dianova, Hamburg, Germany) or to CD62E (Seromed, Berlin, Germany) on ice for 60 min. Expression of PECAM-1 and VE-cadherin was analyzed in subconfluent cultures of unstimulated cells using monoclonal mouse anti-human CD31 from DAKO (Hamburg, Germany) and monoclonal mouse anti-human CD144 from Becton Dickinson (Heidelberg, Germany) respectively. After extensive washing with PBS containing 1% FCS, the cell suspensions were incubated with FITC-labeled goat F(ab)2 to mouse immunoglobulin (DAKO) secondary antibody for 45 min, extensively washed with PBS containing 1% FCS, and fixed with 1% parafolmaldehyde. The fluorescence was quantified by flow cytometry using a FACScan instrument (Becton Dickinson) and 5000 events were captured for every sample.

Evaluation of 4-Hydroxy-2-nonenal-induced Oxidative Stress

The apoptotic/necrotic cells induced by 4-Hydroxy-2-nonenal (4-HNE) (Cayman Chemicals, Ann Arbor, MI) were identified using combined Annexin V-FITC (BD PharMingen, SanDiego, CA) and propidium iodide (PI) staining. 4-HNE was dissolved in ethanol. Cells from confluent cultures were harvested and incubated with FITC-labeled annexin in binding buffer (PharMingen) for 30 min at 37°C in the dark. Counterstaining was done by adding 10 µl of PI solution (1 mg/ml) (Sigma-Aldrich). The cells were analyzed by flow cytometry on a FACScan (Becton Dickinson) using CellQuest software with single cell gating. After setting
markers for negative and positive populations, the percentage of Annexin V-positive/PI-negative cells was determined.
Table I online. PCR primers for detection of transcripts encoding human S1P receptors

<table>
<thead>
<tr>
<th>target transcript</th>
<th>primer sequence</th>
<th>size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S1P₁</strong></td>
<td>5´- GCC CAG TGG TTT CTG CGG GAA-3´  &lt;br&gt; 5´- ACC AAG GAG TAG ATC CTG CAG TA-3´</td>
<td>341</td>
</tr>
<tr>
<td><strong>S1P₂</strong></td>
<td>5´- AGG TGG CCT CCG CCT TCA TC-3´ &lt;br&gt; 5´- GCT TGA CCT TGG CGA TG GCC-3´</td>
<td>315</td>
</tr>
<tr>
<td><strong>S1P₃</strong></td>
<td>5´- CTC AGG GAG GGC AGT ATG TTC – 3´ &lt;br&gt; 5´- GGA CTT GAC CAG GAA GTA GAT GCG-3´</td>
<td>336</td>
</tr>
<tr>
<td><strong>S1P₄</strong></td>
<td>5´- TCC AGC CTT CTG CCC CTC TAC-3´ &lt;br&gt; 5´- CAG GGC CAG GAT CCA GTC CAT-3´</td>
<td>306</td>
</tr>
<tr>
<td><strong>S1P₅</strong></td>
<td>5´- GCC GGT GAG CGA GGT CAT CGT-3´ &lt;br&gt; 5´- TAG GCC TTG GCG TAG AGC GG -3´</td>
<td>552</td>
</tr>
</tbody>
</table>
Table II online. Stable knock-down of S1P$_1$ leads to reduced expression of endothelial specific transcripts.

The table lists transcripts characteristic for endothelial cells that were downregulated at least two-fold in S1P$_1$-knock-down AS-M.5 cells, as detected by microarray analyses using 22,238 human gene probes. The values indicate signal intensities of two independent analyses for AS-M.5 and AS-M.5, T2, Sil#1, cl.3 and one analysis for AS-M.5, Silco and AS-M.5, T2, Sil#1, cl.2. Where appropriate (genes detected by several probes) values are given as mean (SEM).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>UniGene ID</th>
<th>AS-M.5</th>
<th>AS-M.5</th>
<th>AS-M.5</th>
<th>AS-M.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T2, Sil#1, cl.3</td>
<td>T2, Sil#1, cl.2</td>
<td>Silco</td>
</tr>
<tr>
<td>angiopoietin 2</td>
<td>Hs.115181</td>
<td>738 (85)</td>
<td>37 (6)</td>
<td>43 (8)</td>
<td>1251 (8)</td>
</tr>
<tr>
<td>endoglin</td>
<td>Hs.76753</td>
<td>2292</td>
<td>413</td>
<td>489</td>
<td>1508</td>
</tr>
<tr>
<td>EDNRB</td>
<td>Hs.82002</td>
<td>147</td>
<td>37</td>
<td>32</td>
<td>420</td>
</tr>
<tr>
<td>endothelin 1</td>
<td>Hs.511899</td>
<td>1034</td>
<td>161</td>
<td>183</td>
<td>1573</td>
</tr>
<tr>
<td>ESM1</td>
<td>Hs.410668</td>
<td>146</td>
<td>38</td>
<td>39</td>
<td>687</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>Hs.433303</td>
<td>2317 (454)</td>
<td>129 (25)</td>
<td>204 (69)</td>
<td>2403 (299)</td>
</tr>
<tr>
<td>JAM3</td>
<td>Hs.419149</td>
<td>812</td>
<td>209</td>
<td>286</td>
<td>652</td>
</tr>
<tr>
<td>KDR</td>
<td>Hs.12337</td>
<td>564</td>
<td>92</td>
<td>124</td>
<td>675</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Hs.78146</td>
<td>1998 (504)</td>
<td>116 (27)</td>
<td>164 (62)</td>
<td>3239 (690)</td>
</tr>
<tr>
<td>PCDH1</td>
<td>Hs.79769</td>
<td>566</td>
<td>144</td>
<td>223</td>
<td>611</td>
</tr>
<tr>
<td>PCRE</td>
<td>Hs.82353</td>
<td>489</td>
<td>177</td>
<td>173</td>
<td>434</td>
</tr>
<tr>
<td>S1P$_1$</td>
<td>Hs.154210</td>
<td>268</td>
<td>88</td>
<td>110</td>
<td>296</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Hs.76206</td>
<td>1210</td>
<td>29</td>
<td>33</td>
<td>1955</td>
</tr>
</tbody>
</table>
Endoglin (CD105); EDNRB, endothelin receptor type B; ESM1: endothelial cell-specific molecule 1; ICAM-2: intercellular cell adhesion molecule-2 (CD102); JAM3: junctional adhesion molecule 3; KDR: vascular endothelial growth factor (VEGF) receptor; PCDH1: protocadherin 1(cadherin-like 1); PCRE, Protein C receptor-endothelial; PECAM-1: platelet endothelial cell adhesion molecule-1(CD31); VE-cadherin: vascular endothelial cadherin 5.