Sphingosine 1-Phosphate Receptor 2 Negatively Regulates Neointimal Formation in Mouse Arteries

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Abstract—Neointimal lesion formation was induced in sphingosine 1-phosphate (S1P) receptor 2 (S1P2)-null and wild-type mice by ligation of the left carotid artery. After 28 days, large neointimal lesions developed in S1P2-null but not in wild-type arteries. This was accompanied with a significant increase in both medial and intimal smooth muscle cell (SMC) replication between days 4 to 28, with only minimal replication in wild-type arteries. S1P2-null SMCs showed a significant increase in migration when stimulated with S1P alone and together with platelet-derived growth factor, whereas both wild-type and null SMCs migrated equally well to platelet-derived growth factor. S1P increased Rho activation in wild-type but not in S1P2-null SMCs, and inhibition of Rho activity promoted S1P-induced SMC migration. Plasma S1P levels were similar and did not change after surgery. These results suggest that activation of S1P2 normally acts to suppress SMC growth in arteries and that S1P is a regulator of neointimal development. (Circ Res. 2007;101:995-1000.)

Key Words: sphingosine 1-phosphate receptors ■ smooth muscle cells ■ neointima

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid formed by activation of sphingosine kinases.1 It exerts pleiotropic effects on many cells by regulating cytoskeletal rearrangement, cell survival, cell migration, cell proliferation, angiogenesis, and vascular development.2-5 Recently S1P has received attention as a regulator of the cardiovascular system. In part, this is because there are high levels of S1P in plasma, and a recent report showed that they correlate well with the reoccurrence of vascular events.6-10 Further platelets release S1P during their activation, and consequently S1P levels are likely to be high at sites of arterial injury.11,12 S1P acts through 5 G protein–coupled receptors (S1P1 to S1P5), although arterial smooth muscle cells (SMCs) express only S1P1, S1P2, and S1P3.4,13 Initially these receptors were called endothelial cell differentiation gene receptors.14 Activation of S1P receptors induces coupling to a variety of G proteins, which in turn leads to activation of multiple pathways. In SMCs most work has concentrated on S1P1 and S1P3 because they have opposing actions. S1P1 couples to Gi and leads to activation of extracellular signal-regulated kinase, phosphatidylinositol 3-kinase, and Rac.13,15,16 Adult SMCs only weakly express S1P2, although it is more highly expressed in pax cells, and this has been linked to their increased ability to migrate and proliferate in response to S1P.17 S1P2 is also strongly expressed in SMCs from rat intimal lesions as well as in human atherosclerotic lesions.17,18 These data have been used to suggest that activation of S1P1 may induce events leading to restenosis and the formation of arterial lesions. S1P2 is the main receptor expressed by most adult medial SMCs and couples to Gi, Gq, and G12/13, and its activation by S1P is associated with inhibition of SMC migration.13,19 This is thought to occur via coupling to G12/13, leading to Rho activation and suppression of Rac activity.19,20

Relatively little is known about the function of S1P receptors in arteries. The most striking data come from studies in which S1P receptors have been deleted. S1P1−/− mice exhibit embryonic hemorrhage, have poorly developed blood vessels, and die in utero.21 S1P1−/− embryonic fibroblasts show a defect in migration and in the activation of the small GTPase Rac. The S1P2−/− mice are viable and show no obvious phenotype.22,23 In this study, we proposed to determine whether S1P2 plays any role in the growth of neointimal lesions in mouse arteries. Our data show that S1P2−/− arteries develop significantly larger neointimal lesions than wild-type arteries and that this is associated with an increase in SMC growth.

Materials and Methods

PCR primers used in these studies were purchased from IDT. Small interfering RNAs were from Invitrogen Corp. C3 exotoxin was obtained from Cytoskeleton. Platelet-derived growth factor (PDGF) was from R&D, and S1P was purchased from Cayman Chemicals. The RhoA antibody was from Santa Cruz Biotechnology.

Original received August 29, 2006; resubmission received July 5, 2007; revised resubmission received August 22, 2007; accepted August 31, 2007. From the Department of Pathology (T.S., T.N., A.C., F.D., M.A.R.) and Surgery (D.S., G.D.) University of Washington, Seattle.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.107.159228
Animals and Surgical Procedure
SIP2−/− null mice and wild-type mice (C57BL/6×129) were kindly provided by Dr Richard L. Proia (NIH, Bethesda, Md).22 These mouse colonies were bred in our laboratory, and genotypes were verified by polymerase chain reaction analysis. Male mice between 7 to 8 weeks old (litter mates) were used for all the experiments. The left common carotid artery was dissected and ligated near the carotid bifurcation as previously described.24 All studies were performed within the guidelines for animal experimentation at the University of Washington.

Immunohistochemistry
Mice were injected intraperitoneally with bromodeoxyuridine (30 μg/g body weight), and carotid arteries were perfusion fixed with 4% paraformaldehyde in PBS for 3 minutes in situ.25 Hematoxylin-positive (total) and bromodeoxyuridine-positive (replicating) cells were counted on arterial sections (8 sections at 100 μm apart).

Mouse Arterial Smooth Muscle Cell Isolation
Arterial SMCs were isolated from adult male mouse carotid arteries by an enzyme dispersion approach using an enzyme mix (2 mg/mL BSA, 1 mg/mL collagenase, 0.375 mg/mL soybean trypsin inhibitor, and 0.125 mg/mL elastase type III in Hank’s balanced salt solution). After 10 minutes of incubation, the adventitial layer was removed and the remaining tissue was incubated at 37°C for a further 2 hours; cells were then collected. Ten carotids were usually pooled for this procedure, and cells were used up to the ninth passage.

Reverse Transcription–Polymerase Chain Reaction
Total RNA from 8 pooled carotid arteries was prepared using a kit from Qiagen. Following treatment with DNase I (Promega), RNA was reverse transcribed using Superscript reverse transcriptase (Promega). RNA was then PCR amplified for S1P1, S1P2, and S1P3 expression using a Perkin Elmer Gene Amp PCR system. Forward and reverse primers were CACCAGCTCTTCACTGCTGTC and CTCCCCAGTTGCCTCTTTCCTTCG for S1P1; GCCTCTACGGCAGTGACAAAAGC and GAGAGGCAGCCAGTGCCGAAGCAG for S1P2; and TTGGGAAGATCACCTTCCGGGAAA and TTGATCATGGTCAGGTGTCGCTCA for S1P3. The same RNA samples were PCR amplified for GAPDH.

In Vitro Migration
Migration was performed as described previously.25,26 Cells (1×10⁴) were placed in the upper chamber of a 24-well Costar Transwell precoated with 0.1% type I collagen. Medium containing PDGF (20 ng/mL) and S1P (1 μmol/L) was added to the lower chamber. After 7 hours, cells on the lower surface were fixed with methanol and stained with hematoxylin. The cells on the lower surface of membranes (9 fields/membrane) were then counted under a microscope at ×40 magnification. The data are expressed as the mean numbers of cells per field.

Measurement of SIP Levels
Plasma was deproteinated by addition of 80% acetonitrile. Extracts were cleared by centrifugation and subjected to reverse-phase chromatography on a Zorbax C-8 SB 2.1×50 mm SB column. SIP was eluted by a ballistic gradient (60% to 100% methanol) and measured by a Micromass Quattro Premier XE Tandem Quadrupole mass spectrometer (Waters) using a multiple reaction mode assay in positive ion (m/z) mass spectrometer (Waters) using a multiple reaction mode assay in positive ion. The S1P was detected in S1P2-null arteries with a peak in replication at days 7, 14, and 28 was detected in S1P2−/− arteries with a peak in replication at 14 days (Figure 4A). In a similar fashion, an increase in medial SMC replication was observed at all times after surgery (Figure 4B).

SIP2−/− SMCs Show an Increase in Migration
To measure migration, SMCs were isolated from carotid arteries of both SIP2−/− and wild-type mice, and migration was measured in vitro using a transwell chamber assay. A significant increase in migration of SIP2−/− SMCs occurred when stimulated with SIP alone and with a combination of SIP and PDGF as compared with wild-type SMCs. Both wild-type and SIP2−/− SMCs migrated equally well to PDGF (Figure 5).

Rho Activity
SMCs were seeded into 10-cm plates at 600 000 cells per plate and starved for 3 days. Cells were stimulated with 1 μmol/L SIP for 5 minutes, and Rho activity was measured in cell extracts with equal protein content using an ELISA technique following the instructions of the manufacturer (G-Elisa BK123, Cytoskeleton). Assays were performed in triplicate.

Inhibition of Rho Activity
Cell-permeable C3 Transferase (Cytoskeleton) was used to inhibit Rho proteins. SMCs were plated into 100-mm culture dishes at 700 000 cells per plate and allowed to recover overnight in 10% serum. The cells were rendered quiescent in serum-free DMEM for 32 hours before incubating with 2 μg/mL C3 Transferase in serum-free DMEM for 16 hours; they were then subjected to an in vitro migration assay.

Statistics
Differences between the wild-type group and the SIP2−/− group for morphometric data, bromodeoxyuridine index, and migration data were evaluated by unpaired Student’s t test. All data were considered significant at P<0.05.

Results
SIP Receptor Expression
As expected, SIP2−/− arteries expressed S1P1 and S1P3 but not S1P2. Wild-type arteries expressed S1P1 and S1P3, at similar levels as SIP2−/− arteries and also expressed S1P2 (Figure 1).

Carotid Artery Remodeling
The left common carotid artery of SIP2−/− null and wild-type mice was ligated with a 6/0 suture tied just proximal to the internal–external bifurcation. Fourteen days after surgery, a small neointimal lesion was present in SIP2−/− arteries, and by 28 days, the neointima was significantly larger (Figure 2 and 3). In wild-type arteries after 14 days, the neointimal lesions were comprised of 1 incomplete layer of SMCs (Figure 3A). Medial cell number remained constant throughout the experiment in both SIP2−/− null and wild-type arteries (Figure 3B).

In conjunction with the growth of the neointima, a significant increase in intimal SMC replication at days 7, 14, and 28 was detected in SIP2−/− arteries with a peak in replication at 14 days (Figure 4A). In a similar fashion, an increase in medial SMC replication was observed at all times after surgery (Figure 4B).

SIP2−/− SMCs Show an Increase in Migration
To measure migration, SMCs were isolated from carotid arteries of both SIP2−/− and wild-type mice, and migration was measured in vitro using a transwell chamber assay. A significant increase in migration of SIP2−/− SMCs occurred when stimulated with SIP alone and with a combination of SIP and PDGF as compared with wild-type SMCs. Both wild-type and SIP2−/− SMCs migrated equally well to PDGF (Figure 5).

Rho Activity
SIP1 is known to activate Rho via coupling to the G12/13, and in SMCs, this is associated with inhibition of cell movement.19 SIP activated Rho in wild-type but not in SIP2−/− SMCs under identical conditions (Figure 6). To determine that Rho activation was indeed important in regulating SMC migration, wild-type SMCs were preincu-
bated with 2 μg of the Rho inhibitor C3 exotoxin and the migration assay was repeated after 2 days. This concentration blocked S1P-induced Rho activity (data not shown). After treatment with C3, there was a significant increase in S1P-induced migration of SMCs (Figure 7) and in PDGF-induced migration (data not shown).

**Evaluation of S1P Levels**

As mentioned above, plasma S1P in humans correlates well with the reoccurrence of vascular events, and the differences in neointimal lesion size potentially could reflect variations in S1P levels in plasma of S1P2−/− mice.10 Plasma S1P levels in both sets of mice were similar before and at times after injury (Figure 8).

**Discussion**

The results of this study show that the deletion of S1P2 converts an unresponsive artery to an artery that develops large neointimal lesions. This highlights a role for S1P as a key regulator of vascular lesion growth, a fact that has previously been suggested but never proven. Furthermore, because loss of S1P2 permits arterial lesions to develop, this firmly establishes that this receptor functions as a potent suppressor of SMC growth. Indeed, an implication from this study is that downregulation or weak expression of S1P2 may predispose arteries to develop lesions after injury.

A key issue arising from this study is how the loss of S1P2 regulates the growth of arterial lesions. S1P is known to promote or inhibit cell migration depending on the S1P receptors expressed, and in general, activation of S1P1 and S1P3 have opposite effects.19,20,27 The predominant receptor in adult SMCs is S1P3, and migration is suppressed when cells are stimulated with S1P.20,28 This phenotype, however, can be changed readily by overexpressing S1P1 or by blocking expression of S1P3, and under such conditions S1P then stimulates SMC migration.20,27,28 In the
arteries of small mammals, migration of SMC into the intima is a critical step for lesion growth, and if this growth is blocked by proteases inhibitors or by genetic deletion of matrix metalloproteinases, then the growth of the neointimal lesion is blocked. The ligation of the carotid artery in mice stimulates the formation of a neointima comprised of SMCs, and it is generally assumed that migration of cells from the media is a necessary step. In the past few years, there are reports suggesting a role for circulating stem cells in arterial lesions. This possibility has been investigated by others, and it has been determined that very few bone marrow–derived cells are present in the lesions after carotid artery ligation. Furthermore, recent studies have cast major doubts on the likelihood of circulating stem cells contributing to arterial lesions.

It is difficult to measure SMC migration directly in arteries, especially when the endothelium is intact; however, our data would strongly suggest migration into the neointima must have occurred in the injured arteries. First, there are no intimal SMCs in mice carotid arteries before treatment, and SMCs must gain access to the intima before neointimal lesions can develop. Also, S1P is known to regulate cell movement, and our in vitro data directly show that S1P2−/− SMCs exhibit an enhanced migration. Furthermore, there is a marked peak in medial SMC replication at approximately day 14, reflecting an increase in the cell population yet the number of medial cells remains constant (Figure 2). One explanation for this is that cells have migrated into the neointima.

How activation of S1P receptors regulates migration is not completely understood, although this has been linked to...
to the activities of small GTPases of the Rho family. In SMCs, S1P binds to 3 G protein–coupled receptors, and differences in signaling depend on which G protein pathway is activated. The deletion of S1P2 did not influence expression of the other 2 receptors, as has previously been reported. S1P receptors couple to a variety of G protein–coupled receptors, and differences in signaling depend on which G protein pathway is activated. S1P couples only to Gi, and this results in the activation of Rac. In contrast, S1P1 couples to Gi, Gq, and G12/13, the latter resulting in Rho activation. Rho mediates formation of stress fiber and focal adhesion and importantly decreases SMC migration. In this study, S1P2−/− SMCs do not show any increase in Rho activation, and so the absence of this negative regulator may explain the enhanced migration of these cells. Supporting a role for Rho in downregulating SMC migration is our observation that the Rho inhibitor C3 exotoxin increased migration after stimulation with S1P. Collectively, these data support the hypothesis that differences in Rho activity attributable to variations in S1P1 expression influence SMC migration.

A surprising finding was that SMC replication was increased in S1P2−/− arteries, suggesting that activation of S1P1 normally functions as a suppressor of replication. There are many reports showing that S1P can act as a mitogen, but relatively few show that it inhibits replication, although it mainly acts through S1P1, which activates phospholipase C, Ras, and phosphatidylinositol 3-kinase. Stable transfection of S1P1 in SMCs enhances S1P-induced replication, and this correlated with an increase in p70S6k activity and expression of cyclin D1, both factors being important for cell growth. One possibility therefore is that in the absence of S1P2 expression, other S1P receptors such as S1P1 promote cell replication. Deletion of S1P1 is lethal, and so assessing the role of this receptor for neointimal growth will require a tissue specific knockout mouse.

Availability of S1P

Implicit in the results of this study is that S1P must be available to bind the S1P receptors of SMCs after arterial ligation. Mouse plasma has relatively high levels of S1P, approximately 0.2 to 0.9 μmol/L, although a large fraction (≈60%) is bound to lipoproteins and considered unavailable to interact with the S1P receptors. If plasma were the main source of S1P, then changes in plasma S1P levels could be important. Indeed, this is supported by a recent finding showing that high S1P levels are predictive of repeat cardiovascular events in man. In this study, however, the plasma S1P levels in S1P2−/− and wild-type mice were identical and did not change at times after surgery. One consideration, therefore, is that S1P is readily accessible to arterial SMCs and plasma levels are not rate limiting in this model.

A final issue of interest is that wild-type mice do not develop any significant neointimal lesions. There have been several reports that the arteries of different mouse strains and especially C57BL/6 do not develop arterial lesions after denuding injuries or after carotid ligation. This finding is somewhat controversial, but in this study, we show that C57BL/6×129 mice behave very much like C57BL/6 carotid arteries, ie, minimal neointimal lesions. Interestingly, we noted that C57BL/6 strongly express S1P2 and that FVB mice that develop large neointimal lesions show a lower expression of S1P2. One possibility, therefore, is that differences in the expression of arterial S1P receptors is a natural occurrence, at least in mice, and may be responsible for the marked variation in neointimal growth.

In summary, we show that the deletion of S1P2 promotes the rapid growth of neointimal lesions by increasing SMC replication and migration. One important inference is that arteries are normally subjected to inhibitory signals from S1P interacting with S1P2.

Acknowledgments

We thank Thomas F. Kalhorn at the Mass Spectrometry Center in the Department of Medicinal Chemistry at the University of Washington for S1P measurements.

Sources of Funding

This was funded by NIH grants HL70858, HL69907, and HL70850.
Disclosures

None.

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Circ Res. 2007;101:995-1000; originally published online September 13, 2007;
doi: 10.1161/CIRCRESAHA.107.159228

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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