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Involvement of Sphingosine 1-Phosphate, a Platelet-Derived Bioactive Lipid, in Contraction of Mesangium Cells

Makoto Osada[1], Yutaka Yatomi[2,*], Tsukasa Ohmori[1], Shinya Aoki[2], Shigemi Hosogaya[1] and Yukio Ozaki[1]

[1]Department of Clinical and Laboratory Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi; and [2]Department of Clinical Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

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Platelet-derived mediators may play an important role in the development of renal diseases through interaction with glomerular mesangial cells (MCs), and we, in this study, examined the effect of sphingosine 1-phosphate (Sph-1-P), a bioactive lipid released from activated platelets, on the contraction of MCs. Sph-1-P was found to induce MC contraction through mediation of Rho kinase both in cell shape change and collagen gel contraction assays. The specific antagonist of the Sph-1-P receptor $S1P_2$ inhibited the response. Similar results were obtained when the supernatant from activated platelet suspensions were used instead of Sph-1-P. Our findings suggest that platelet-derived Sph-1-P may be involved in MC contraction *via* $S1P_2$ and that regulation of this receptor might be useful therapeutically.

Key words: contraction, mesangial cell, platelet, Rho kinase, $S1P_2$, sphingosine 1-phosphate.

Abbreviations: MC, mesangial cell; Sph-1-P, sphingosine 1-phosphate.

Glomerular mesangial cells (MCs) are smooth muscle-like pericytes and play an important role in renal (patho)physiology (*1*). MCs surround the filtration capillaries within the glomerulus, and their excitability regulates the filtration rate *in vivo* (*1*). It is well established that MCs contract and relax in response to a number of vasoactive mediators to regulate renal blood flow and glomerular filtration rate (*1–3*). In this context, it is worthy of notice that a lot of circulating and locally produced cellular products can directly influence MC function because of the proximity of MCs to the glomerular microcirculation. Determining the factors controlling the responses of glomerular MCs is important for understanding how glomerular filtration rate is regulated in health and disease states.

Increasing evidence has accumulated to show an involvement of platelets in inflammatory and thrombotic kidney disorders (*4–7*). The activation of platelets and the resultant extracellular release of bioactive substances are believed to induce important functional responses in glomerular MCs, including contraction, proliferation and cytokine production (*4–7*). In fact, platelet-derived mediators including but not limited to platelet-derived growth factor, 5-hydroxytryptamine, and lysophosphatidic acid have been shown to affect MC responses. Also *in vivo*, platelets were found together with neutrophils in damaged or inflamed glomeruli (*4*). In anti-Thy-1 nephritis, an experimental model of mesangioproliferative glomerulonephritis, platelet depletion by anti-platelet antibody decreased the extent of MC proliferation (*5*).

*To whom correspondence should be addressed. Tel: +81-3-5800-8721, Fax: +81-3-5689-0495, E-mail: yatoyuta-tky@umin.ac.jp

Sphingosine 1-phosphate (Sph-1-P) has recently been added to the list of bioactive lipids acting intercellularly (*8*, *9*). This phosphorylated sphingoid base exerts a variety of biological responses in diverse cell types mainly through the interaction with the cell surface receptors S1Ps (*8*, *9*). Since blood platelets abundantly store Sph-1-P and release it upon activation (*10*, *11*), it is important to examine the effects of this bioactive lipid on MC functions from the viewpoint of platelet–MC interactions. In this study, we examined the effect of Sph-1-P on MC contraction. We also analysed the relative involvement of this bioactive lipid in platelet–MC interactions with the use of the supernatant prepared from activated platelets and a specific S1P antagonist.

MATERIALS AND METHODS

Materials—The pyrazolopyridine derivative JTE-013, a specific $S1P_2$ antagonist (*12*, *13*), was obtained from the Central Pharmaceutical Research Institute, Japan Tobacco Incorporation, Osaka, Japan and Calbiochem (San Diego, CA); the effects of this compound from these two sources were similar. The following materials were obtained from the indicated suppliers: Sph-1-P (Biomol, Plymouth Meeting, PA); Y-27632 and Ro-318220 (Calbiochem, San Diego, CA); HA1077 (Upstate Biotechnology, Lake Placid, NY); pertussis toxin (Sigma, St. Louis, MO); VPC23019 (Avanti Polar Lipids, Alabaster, AL).

Cell Culture—Normal human MCs were purchased from BioWhittaker, Inc. (Walkersville, MD), and maintained in RPMI medium 1640 (Gibco, NY, USA) with 5% fetal bovine serum (ICN Biomedicals, Aurora, OH), penicillin G (100 U/ml) and streptomycin sulphate

(100 μg/ml) at 37°C under an atmosphere of 5% CO2 and 95% room air. The cells were not used after the seventh passage.

Preparation of Platelet Supernatant—Human washed platelets (at a cell density of 5×10^8/ml) were prepared as previously described (*10*), and stimulated with 20 μg/ml of type I collagen (Hormon-Chemie, Munich, Germany) for 15 min under continuous stirring at 1,000 r.p.m. The samples were then centrifuged at 15,000*g* for 1 min, and the resultant supernatants were used to stimulate MCs. It was confirmed that collagen, by itself, failed to affect the response of MCs in the present study (data not shown). Furthermore, collagen was shown to induce Sph-1-P release from human platelets (*10*).

Cell Shape Change Assay—In order to visualize stimulus-dependent contractility, suspensions of MCs were seeded onto dishes coated with 0.2% gelatin; the cells were allowed to adhere to and spread on the substrate for 24 h. The cells were serum-starved for 3 h, and then challenged with the indicated stimulus for 30 min unless otherwise stated. The cells were fixed with 3% paraformaldehyde for 40 min, and then permeabilized with 0.2% Triton X-100 for 10 min. Actin filaments were detected with 0.1 μg/ml of tetramethyl rhodamine isothiocyanate-phalloidin (Sigma Chemical Co., St. Louis, MO). Cell morphology was observed with a confocal microscope, and the images were digitalized with a digital scanner (Canoscan D2400U, Canon Inc., Tokyo, Japan). For quantitative evaluation, the maximal cord length of the cells was measured and was defined as the cell length.

Collagen Gel Contraction Assay—Collagen lattices were prepared using type I collagen (Cellmatrix Type I-A, Nitta Gelatin Inc., Osaka, Japan) according to the manufacturer's instructions. Five hundred microlitres of collagen solution in RPMI1640 medium (with a final concentration of collagen being 2.1 mg/ml) was aliquoted into 12-well plates. Collagen lattices were polymerized for 10 min in a humidified 5% CO_2 atmosphere at 37°C. After the preparation of this collagen base layers, MCs (5×10^5 cells) in 500 μl of collagen solution were seeded and further incubated for 10 min. Five hundred microlitres of RPMI 1640 medium containing 5% fetal calf serum was then added. The samples were incubated for 24–36 h, followed by 5 h incubation in serum-free medium. To initiate collagen gel contraction, polymerized gels were gently released from the underlying culture dish and cells were immediately stimulated as indicated. The degree of collagen gel contraction was determined after 30 min. The diameter of the gels were measured in millimetre and recorded as the average values of the major and minor axes.

Statistics—When indicated, statistical analysis was performed by Student's *t*-test, and $P < 0.05$ was considered significant.

RESULTS

MCs modulate intraglomerular haemodynamics through contraction, and we first evaluated this response by examining the cell shape change. As shown in Fig. 1, Sph-1-P induced MC contraction. The onset of contraction was observed as early as 3 min after the addition of Sph-1-P (data not shown), and the response was concentration-dependent (Fig. 2A). We then characterized the determinants of this contractility. We examined the effect of JTE-013, a specific and potent $S1P_2$ antagonist (*12*, *13*), to check $S1P_2$ involvement in the Sph-1-P-induced MC contraction since Sph-1-P-induced and $S1P_2$-mediated contraction was observed in vascular smooth muscle cells (*14*) and since MCs do express this Sph-1-P receptor (*15–17*). This pyrazolopyridine derivative significantly reversed Sph-1-P-induced MC contraction (Figs 1 and 2B), indicating involvement of $S1P_2$.

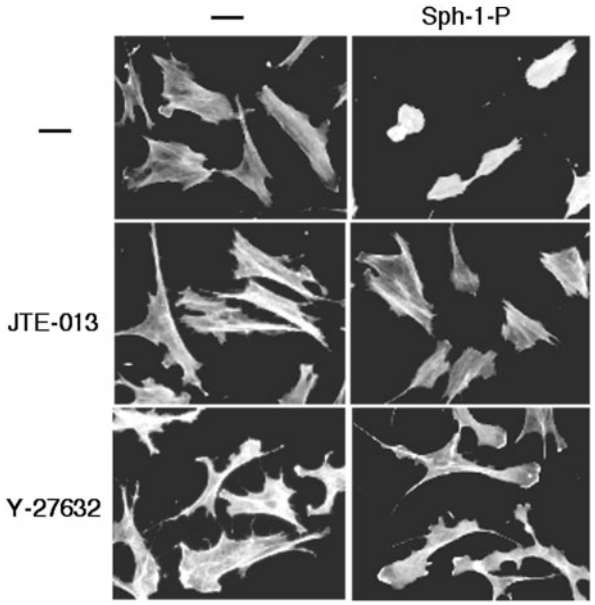

Fig. 1. **Sph-1-P-induced MC shape change and its inhibition by JTE-013 or Y-27632.** MCs pre-treated without (Upper Panels) or with 10 μM JTE-013 for 10 min (Middle Panels) or 20 μM Y-27632 for 30 min (Lower Panels) were challenged without (Left Panels) or with (Right Panels) 1 μM Sph-1-P. The cells were fixed, permeabilized and then stained with tetramethyl rhodamine isothiocyanate-phalloidin for evaluation of their contraction. The results are representative of three independent experiments.

A guanine nucleotide exchange factor for Rho, p115RhoGEF, has been identified as a target for the α subunit of the $G_{12/13}$ family, with which $S1P_2$ communicates (*9*, *18*). Furthermore, the small GTPase Rho and its downstream targets Rho kinase (and myosin light chain phosphatase) play an important role in phosphorylation of myosin light chain and thereby induce actomyosin contractile force (*19*, *20*). Accordingly, the involvement of this signalling pathway was examined. As expected, pre-treatment of MCs with the specific Rho kinase inhibitors Y-27632 (*21*) and HA1077 (*22*) reduced the MC contraction induced by Sph-1-P (Figs 1 and 2B). In contrast, pertussis toxin, the protein kinase C inhibitor Ro-318220 (*23*) and the $S1P_1$ and $S1P_3$ antagonist VPC23019 (*24*) failed to inhibit the response. These results indicate that Sph-1-P-induced MC

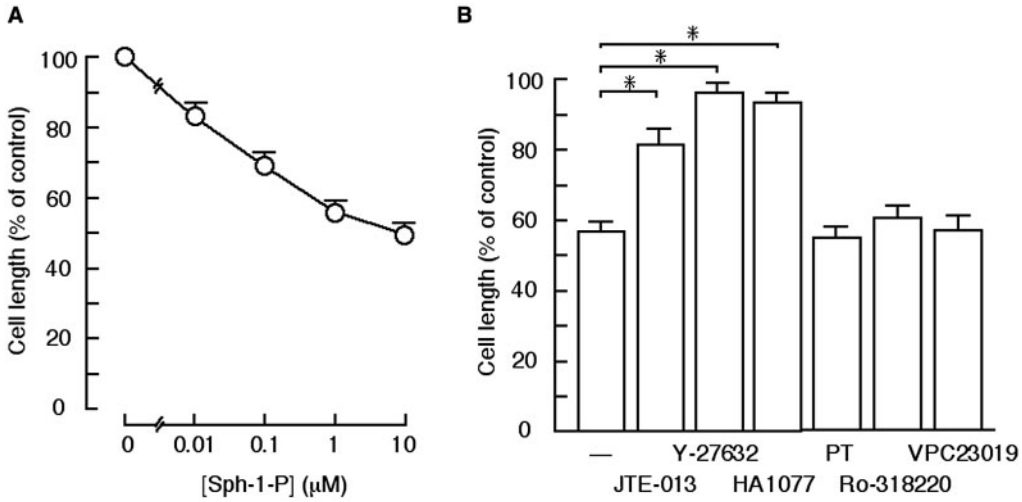

Fig. 2. **Sph-1-P-induced MC contraction, assessed by shape change studies.** (A) MCs were stimulated with various concentrations of Sph-1-P. The average cell length from 100 cells is shown as a percentage of the control cells (without Sph-1-P). Data represent the mean ± SD ($n = 3$). (B) MCs were pre-treated without (−) or with 10 μM JTE-013 for 10 min, 20 μM Y-27632 for 30 min, 10 μM HA1077 for 10 min, 500 ng/ml of pertussis toxin (PT) for 2 h, 10 μM Ro-318220 for 10 min or 2 μM VPC23019 for 10 min and then challenged with 1 μM Sph-1-P or not. The results are shown as a percentage of the control cells (without any treatment). Columns and error bars represent the mean ± SD ($n = 3$). *Statistically significant compared with the Sph-1-P-treated control cells (without pre-treatment).

contraction is mediated by the Rho/Rho kinase pathway signalled *via* $S1P_2$.

We next examined the MC contraction by collagen gel contraction assay, and confirmed that Sph-1-P induced the contraction in a concentration-dependent manner (Figs 3A and 4A) and that the response was again blocked by JTE-013 or Y-27632 (Fig. 3B). We further examined involvement of this bioactive lysophospholipid in platelet-mediated MC contraction. For this purpose, the supernatant from platelet suspensions was prepared; Sph-1-P is a bioactive lysophospholipid released from activated platelets. As expected, the supernatant of activated platelets induced strong contraction of MCs (Fig. 3B). The response was mimicked by the boiled supernatant (data not shown), confirming that the ingredients released from activated platelets and responsible for MC contraction are not peptide or protein mediators. Importantly, the response induced by the platelet supernatant (as well as Sph-1-P) was inhibited by JTE-013 and Y-27632 (Fig. 4). This indicates that platelets seem to contract MCs by releasing Sph-1-P at least under the conditions employed.

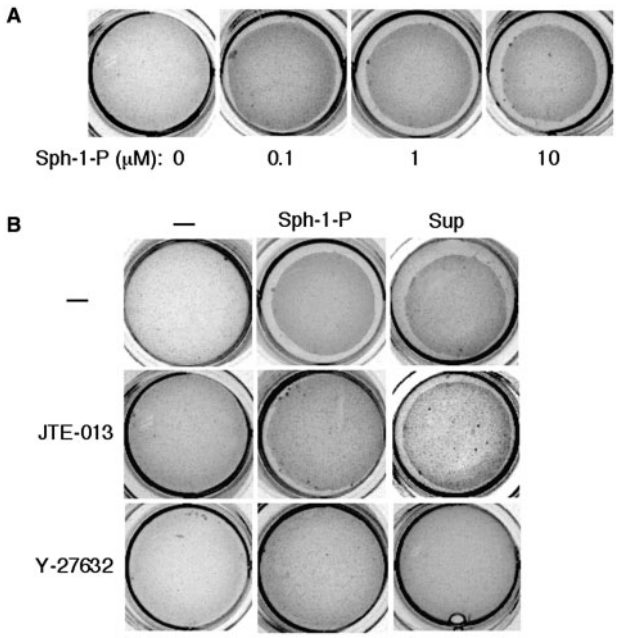

Fig. 3. **Sph-1-P-induced contraction of MCs embedded into type I collagen lattices.** (A) MCs were stimulated with the indicated concentrations of Sph-1-P. (B) MCs were pre-treated without (−) or with 10 μM JTE-013 for 10 min or 20 μM Y-27632 for 30 min and then challenged with 1 μM Sph-1-P or the supernatant from activated platelets (Sup). The results are representative of three independent experiments.

DISCUSSION

In this study, we found that MC contraction was induced by Sph-1-P and platelet supernatants, in which Sph-1-P is included. Importantly, JTE-013, a specific $S1P_2$ antagonist (*12*, *13*), inhibited not only the response induced by Sph-1-P but also that induced by the platelet supernatant. This indicates that platelets seem to contract MCs by releasing Sph-1-P. Given that 140 pmol Sph-1-P is stored in 10^8 platelets (*25*) and that 40% of it is released upon activation (*10*, *11*), the concentration of Sph-1-P in the supernatant prepared from activated platelets (5×10^8/ml) is calculated as 280 nM. In fact, we confirmed that the similar levels of Sph-1-P did exist in the supernatant, by conversion of extracted Sph-1-P to the fluorescent isoindol derivative by ortho-phthaldialdehyde, followed by HPLC separation

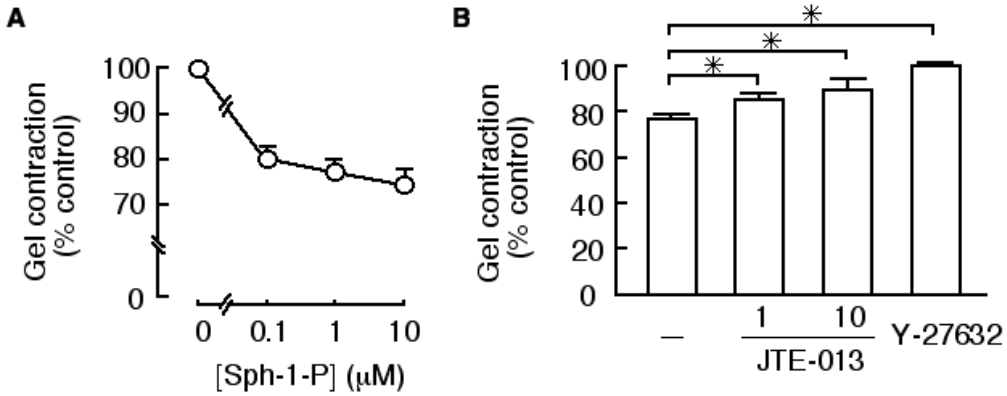

Fig. 4. **Sph-1-P-induced MC contraction, assessed by collagen gel contraction studies.** (A) MCs were stimulated with various concentrations of Sph-1-P. The contraction of collagen gel was measured in millimetre and recorded as the average value of the major and minor axes; the reduction in the diameter of the gels was expressed as a percentage of the diameter of the control preparation (without Sph-1-P treatment). Data represent the mean ± SD ($n = 3$). (B) MCs were pre-treated without (−) or with 1 or 10 μM JTE-013 for 10 min or 20 μM Y-27632 for 30 min and then challenged with the supernatant from activated platelets or not. The contraction of collagen gel was measured, and the reduction in the diameter of the gels was expressed as a percentage of the diameter of the control preparation (without any treatment). Data represent the mean ± SD ($n = 3$). *Statistically significant compared with the Sph-1-P-treated control cells (without pre-treatment).

and fluorescence monitoring (data not shown). Based on our results on the concentration-dependent effect of Sph-1-P, this concentration of Sph-1-P is expected to contract MCs. This is consistent with the fact that MCs do express $S1P_2$ and that the similar findings were obtained with the use of vascular smooth muscle cells (*14*); MCs are considered to be specialized smooth muscle cells.

This is not the first report for Sph-1-P-elicited responses in MCs. Sph-1-P has been shown to induce proliferation of MCs, in a pertussis toxin-sensitive manner (*15*, *17*). Furthermore, when a pattern of gene expression associated with immunoglobulin A nephropathy, the most common primary renal glomerular disease worldwide, was elucidated by analysing the diseased kidneys on cDNA microarrays, an enhanced expression of $S1P_2$ (formerly named EDG-5) was observed (*16*) Furthermore, platelet-derived growth factor (PDGF), a potent mesangial mitogen released from activated platelets, reportedly induces a marked up-regulation of $S1P_2$ in proliferative MCs and promotes cell proliferation synergistically with Sph-1-P (*16*). Hence, it has been suggested that enhanced PDGF-$S1P_2$ signalling plays an important role in the progression of glomerular diseases such as immunoglobulin A nephropathy (*16*). These findings all support the importance of $S1P_2$ in the pathophysiology of MCs although not only $S1P_2$ but also $S1P_3$ (formerly named as EDG-3) is reportedly involved in MC proliferation and survival (*17*).

Our present demonstration that platelet-derived Sph-1-P induces contraction of MCs may have important clinical implications in relation to the pathogenesis of diverse renal disorders. MCs are separated from the glomerular capillary lumen by the fenestrated endothelium, and the entry of Sph-1-P, presumably released from activated platelets at the site of injury, may be facilitated and can thereby modulate MC function and possibly contribute to the pathophysiology of the glomerular disorders. Sph-1-P may contribute in a paracrine manner in the process of amplifying and propagating glomerular injury. Sph-1-P is attracting much attention as a bioactive sphingolipid released from platelets, and the development of its receptor agonists/antagonists of the S1P family is expected to lead to a new therapeutic approach to regulate various diseases (*26*, *27*); the targets may include glomerulonephritis and renal dysfunction.

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