

Review

Sphingosine 1-phosphate is a blood constituent released from activated platelets, possibly playing a variety of physiological and pathophysiological roles^{*⊙}

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We have found that sphingosine 1-phosphate (Sph-1-P) acts as an autocrine stimulator of platelets, being abundantly stored in platelets and released extracellularly, and that its exogenous addition induces platelet activation (Yatomi *et al.*, *Blood* 1995, 86, 193-202) through a specific receptor on the platelet surface (Yatomi *et al.*, *J. Biol. Chem.* 1997, 272, 5291-5297). Very recently, we identified Sph-1-P as a normal constituent of human plasma and serum. Sph-1-P levels in plasma and serum were 191 ± 79 and 484 ± 82 pmol/ml (mean \pm S.D., $n = 8$), respectively. Platelets are most likely the source of Sph-1-P discharged during blood clotting, since they abundantly store Sph-1-P as compared with other blood cells, and release considerable amounts of stored Sph-1-P extracellularly upon stimulation. The Sph-1-P released from activated platelets may be involved in a variety of physiological processes, including thrombosis, atherosclerosis, and wound healing. Moreover, we often observed that Sph-1-P injection into mice (iv., 10 mg/kg) caused immediate rigor and death. This may be related to the recent observations from an other laboratory that nanomolar concentrations of Sph-1-P affected atrial myocyte K^+ channel. These observations taken together strongly suggest pathophysiological roles of the released Sph-1-P in the blood.

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Abbreviations: C.C.I., corrected count increment of platelets; Cer, ceramide; IL1- β , interleukin 1- β ; IL-6, interleukin 6; PKC, protein kinase C; PDGF, platelet derived growth factor; Sph, sphingosine; Sph-1-P, sphingosine-1-phosphate; TLC, thin-layer chromatography.

As one example, we found that Sph-1-P content in the plasma of platelet concentrates correlated with poor platelet increments after transfusion and with the occurrence of transfusion reactions in patients.

Sphingosine-1-phosphate (Sph-1-P) is the initial product of the catabolism of Sph by Sph kinase and is then cleaved by Sph-1-P lyase to yield ethanolamine phosphate and a fatty aldehyde [1-3]. Recent studies showed that Sph-1-P exhibits several biological functions in addition to its role as an intermediate metabolite of Sph. It has been shown to be involved in a variety of cellular functions, including the stimulation of fibroblast growth [4, 5], regulation of the motility of cancer cells [6] and smooth muscle cells [7], activation of muscarinic K^+ currents in atrial myocytes [8, 9], Rho-dependent neurite retraction in neuronal cells [10], mediation of Fc ϵ RI antigen receptor signaling in mast cells [11], and suppression of ceramide-mediated apoptosis in HL-60 cells [12]. In human platelets, Sph-1-P induces a shape change and aggregation by itself, or synergistically elicits aggregation in combination with weak platelet agonists such as epinephrine and ADP [13].

Although physiological or pathophysiological roles of Sph-1-P have been strongly suggested, the current evidence for the involvement of Sph-1-P in signal transduction or cellular function(s) consists largely of data regarding the cellular and biochemical effects of exogenous Sph-1-P. Few studies have revealed the presence and quantitative changes of Sph-1-P in human tissues or body fluids under physiological conditions. To obtain clear evidence implicating endogenous Sph-1-P in cellular signal transduction, and to further assess its physiological and pathophysiological functions, we recently developed a method for quantifying the mass of Sph-1-P through its acylation into *N*-[3 H]acetylated Sph-1-P with [3 H]acetic anhydride, which made it possible to measure Sph-1-P in biological samples [14].

Using this newly-developed method, we measured Sph-1-P content in blood samples under normal or pathological conditions, and

found that 1) Sph-1-P is a normal constituent of plasma and serum, and platelets seem to release Sph-1-P into the serum during the blood clotting process, 2) Sph-1-P content in the plasma of platelet concentrates correlates with poor platelet increments after transfusion and with occurrence of transfusion reactions in patients.

Sph-1-P AS AN AUTOCRINE PLATELET ACTIVATING FACTOR AND AN INTERCELLULAR MESSENGER

We previously found functional roles for Sph-1-P in human platelets as an autocrine activator of platelets [13]. In brief, Sph-1-P induced platelet shape change and aggregation reactions, although it failed to elicit secretion. Sphingosine, ceramide, sphingomyelin, and *N,N*-dimethylsphingosine did not mimic the positive effects of Sph-1-P. Sph-1-P, together with weak platelet agonists such as ADP and epinephrine synergistically elicited aggregation, which may be important for efficient amplification of platelet activation. Sph-1-P induced intracellular Ca^{2+} mobilization, and the dose-response for Ca^{2+} release correlated closely with the concentration required for the induction of shape change. Interestingly, Sph-1-P accumulated in platelets was specifically released into the medium upon stimulation of platelets with physiological agonists through the activation of protein kinase (PKC). Furthermore, we recently reported that Sph-1-P activates platelets extracellularly through its binding to a putative receptor protein(s) on the cell surface, and that the Sph-1-P receptor might be shared with another bioactive lipid, lysophosphatidic acid [15]. Our findings are summarized in Fig. 1.

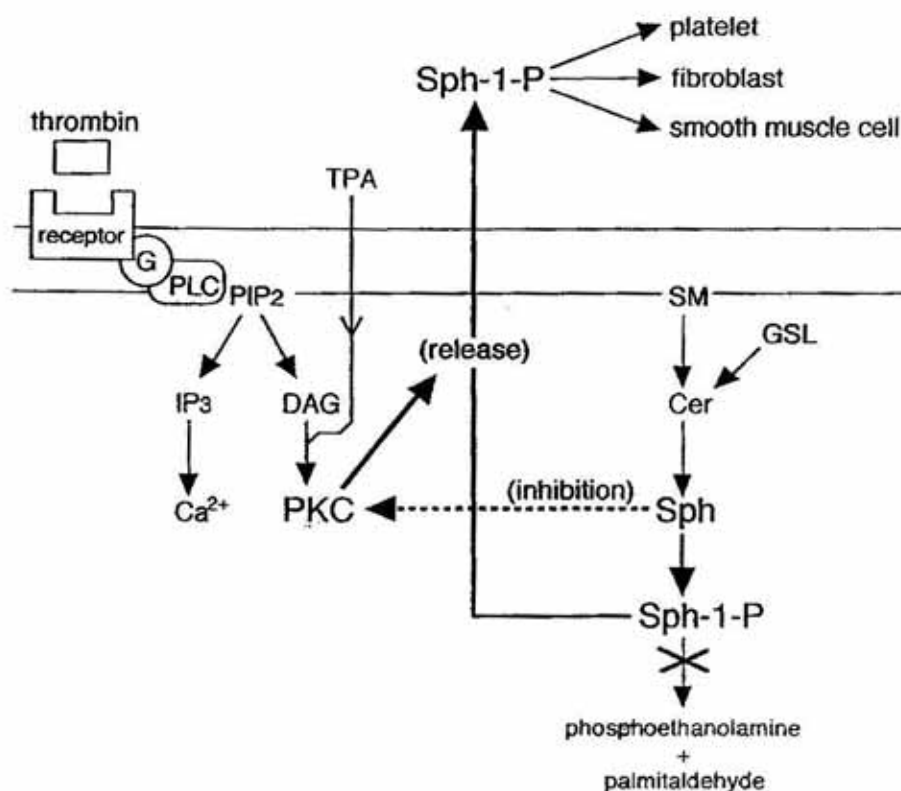


Figure 1. Sphingosine 1-phosphate as an intercellular messenger, released from platelets through mediation by PKC.

(See text for details).

ESTABLISHMENT OF A METHOD FOR QUANTIFICATION OF Sph-1-P

To examine Sph-1-P contents in various biological samples, we developed a facile, but very sensitive quantification method for this lipid by conjugating radioactive acetic anhydride to its free amino group [14]. In developing this assay, we utilized the unique solubility of this lipid. Namely, at neutral pH it is practically insoluble either in water or in any kind of organic solvents. Under alkaline conditions it shifts to the upper layer of the Folch's separation and under acidic conditions to lower organic layer. Sph-1-P thus extracted is N-acylated with [^3H]acetic anhydride into [^3H]C2-Cer-1-P (N-[^3H]acetylated Sph-1-P), and the radioactivity of this compound on the plates is measured after separation by thin-layer chromatography. Figure 2 depicts the essence of this method and the standard curve obtained for Sph-1-P. This as-

say allows quantification of Sph-1-P in the range of about 30 pmol to 10 nmol. The quantity of Sph-1-P extracted from biological samples was calculated by extrapolation of values of Sph-1-P standards subjected to the same procedures.

Sph-1-P LEVELS IN HUMAN BODY FLUIDS AND BLOOD CELLS

Using the above described method, we examined the Sph-1-P levels in blood samples. When plasma or serum extracts were N-acylated with [^3H]acetic anhydride for Sph-1-P measurements, one clear band, with a high degree of resolution, was detected on TLC (Fig. 3, left). The band (the acylated product from plasma and serum) coincided with FAB-MS identified C2-Cer-1-P in TLC mobility in three different solvent systems, i.e., butanol/acetic acid/water (3:1:1, by vol.) (Fig. 3,

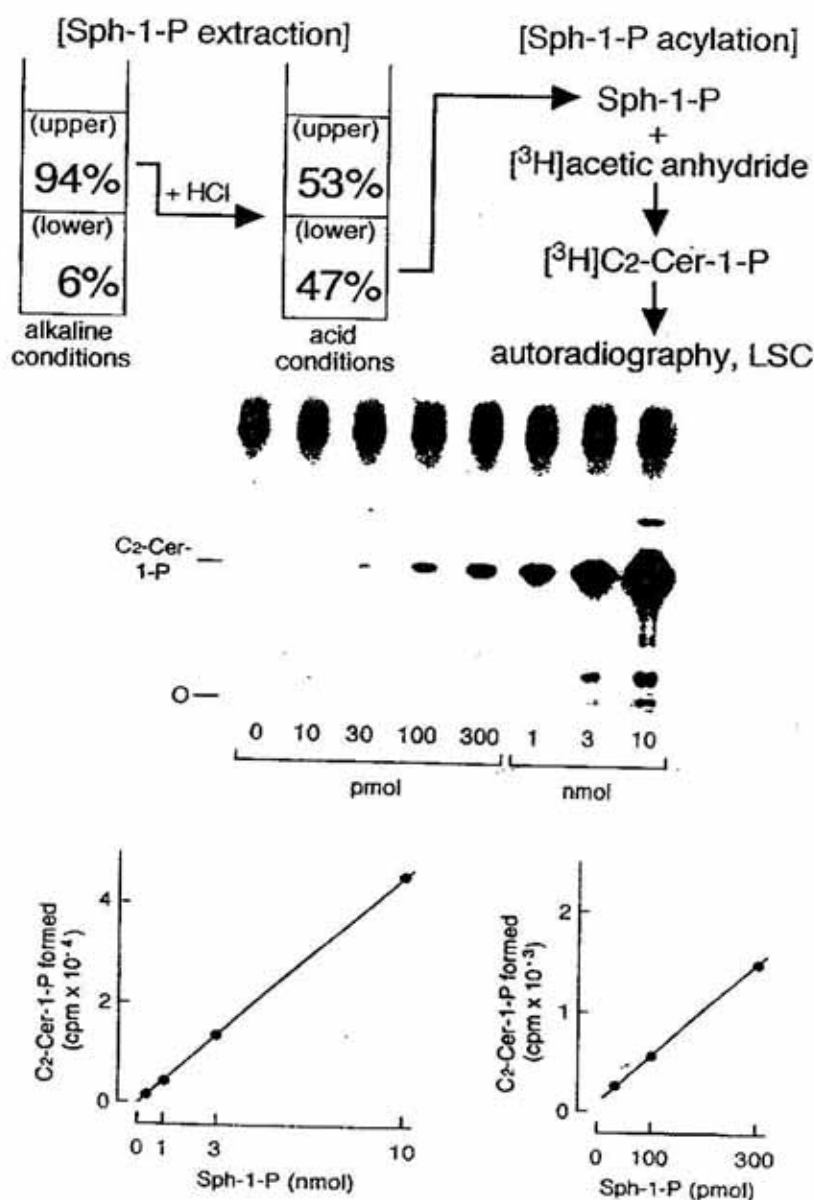


Figure 2. Quantification method of Sph-1-P

(See text for details).

left), chloroform/methanol/7 M NH₄OH/water (80:20:0.5:0.5, by vol.) (not shown) and chloroform/ methanol/acetic acid/water (65:43:1:3, by vol.) (not shown). These results indicate that Sph-1-P was clearly detected and identified by our procedures as a normal constituent of plasma and serum. Human plasma, which did not contain any platelet discharge (assuming no artificial activation of platelets occurs in the sampling process) contained about 190 pmol/ml of Sph-1-P, while clotted blood serum, into which the contents of plate-

lets should be released, contained about 480 pmol/ml of Sph-1-P (Table 1A). When the Sph-1-P levels in paired plasma and serum samples obtained from 6 healthy adults were measured, the serum Sph-1-P/plasma Sph-1-P ratio was found to range from 1.36 to 4.05, the average being 2.65 ± 1.26 (mean \pm S.D.) (Fig. 3, right). We also analyzed other body fluids, but Sph-1-P was not detectable in urine, ascites, pleural effusion or cerebrospinal fluid (Table 1A).

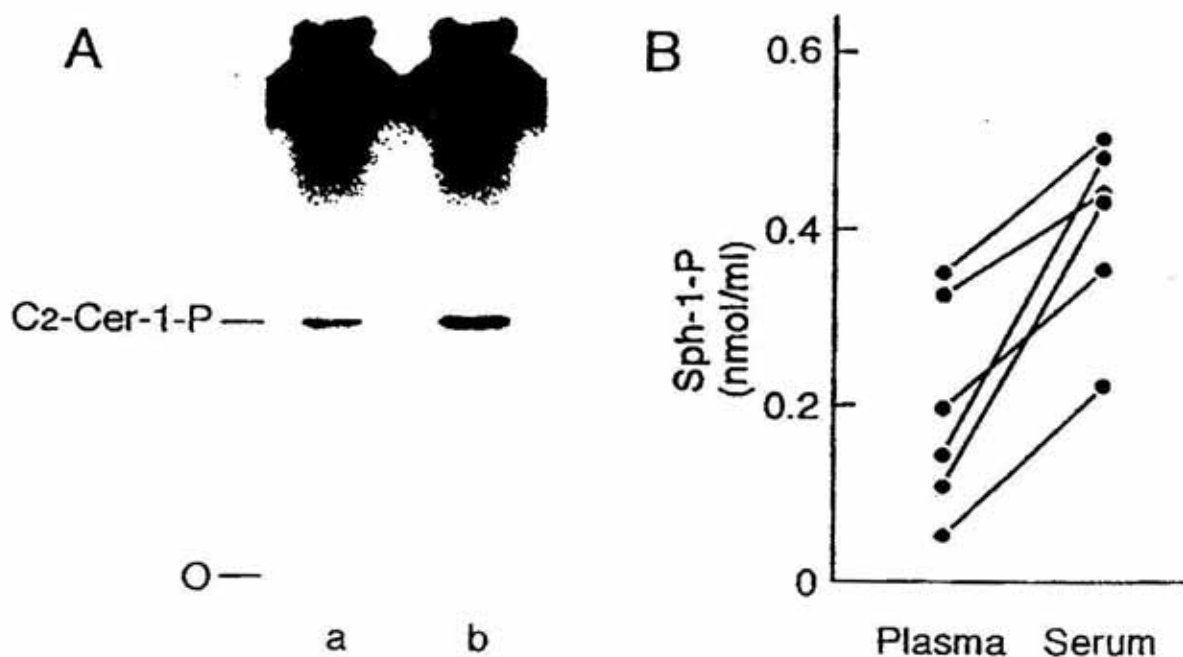


Figure 3. Sph-1-P contents in human plasma and serum.

(A) Sph-1-P extracted from human plasma (lane a) and serum (lane b) was N-acylated with [^3H]acetic anhydride into [^3H]C2-Cer-1-P. Radioactive spots corresponding to [^3H]C2-Cer-1-P on the TLC plate shown were scraped off and counted for quantification of Sph-1-P. A representative autoradiogram is shown. (B) Determination of the Sph-1-P levels in paired plasma and serum samples obtained from 6 healthy adults. The serum Sph-1-P/plasma Sph-1-P ratio was calculated to be 2.65 ± 1.26 (mean \pm S.D., $n = 6$).

Next, we analyzed blood cells for Sph-1-P. Platelets possess a very active Sph kinase but lack lyase activity for the degradation of Sph-1-P into a fatty aldehyde and ethanolamine phosphate [16-18]. In agreement with this, we previously reported that platelets contain as much as 1.4 nmol of Sph-1-P/ 10^9 platelets and that the amount of Sph-1-P present in platelets is much higher than that of Sph [15]. When a comparison was made using the mol % Sph-1-P/phospholipid value, the Sph-1-P level in platelets was found to be over 10 times higher than that in neutrophils or erythrocytes (Table 1B). Since Sph-1-P is most abundantly stored in platelets, compared with other blood cells, and can be released into the medium upon stimulation of platelets [13, 15], it is most likely that platelets are the source of discharged Sph-1-P during the clotting process.

METABOLISM OF [^3H]Sph-1-P IN PLASMA

Sph-1-P metabolism in plasma was next examined. Generally, Sph-1-P can be converted to Sph by phosphatase [19] or degraded to palmitaldehyde and ethanolamine phosphate by lyase [3]. However, [^3H]Sph-1-P, added to plasma, was metabolically stable and remained unchanged for at least 2 h (not shown). Thus, the Sph-1-P released from activated platelets may circulate in the body, at least for a certain period of time. Furthermore, when a mixture of albumin and Sph-1-P was applied to a gel-filtration column, Sph-1-P was co-eluted with albumin (not shown), suggesting that Sph-1-P released from platelets binds tightly to albumin or other lipophilic proteins in the blood (Fig. 4).

Table 1. Sphingosine 1-phosphate levels in human body fluids and blood cells

A. Body fluids		Sph-1-P content (pmol/ml)	
Plasma		191 ± 79 (n = 8)*	
Serum		484 ± 82 (n = 8)	
Urine		ND (n = 10)	
Ascites		ND (n = 6)	
Pleural effusion		ND (n = 2)	
Cerebrospinal fluid		ND (n = 6)	
B. Blood cells	Cell count	Sph-1-P (pmol)	mol% Sph-1-P/phospholipid
Platelets	10 ⁸	141 ± 4 (n = 3)	0.211 ± 0.014
Neutrophils	10 ⁷	15.2 ± 2.8 (n = 3)	0.018 ± 0.003
Erythrocytes	10 ⁸	7.17 ± 1.66 (n = 3)	0.019 ± 0.002

* Values are means ± S.D., and the numbers of experiments are shown in parentheses. ND, not detectable.

POSSIBLE PHYSIOLOGICAL ROLES OF Sph-1-P RELEASED FROM PLATELETS INTO BLOOD

Based upon the findings from our or other laboratories, we may speculate on several physiological roles for Sph-1-P released from platelets into blood.

Acceleration of hemostasis or thrombosis

As mentioned above, Sph-1-P can synergistically elicit platelet aggregation with weak but physiological agonists such as ADP or epinephrine [13]. Thus, Sph-1-P released from activated platelets may further enhance the platelet aggregation and accelerate the hemostatic or thrombotic process, in an autocrine or paracrine manner.

Involvement in wound healing processes

Activated platelets release a certain amount of accumulated Sph-1-P [13, 15] and similar levels of Sph-1-P (0.5–1 μM) added exogenously are known to stimulate proliferation of fibroblastic cells [4, 5]. These findings taken together strongly suggest that Sph-1-P, released from platelets activated through the attachment to collagen fibers around wounded

vessels, may serve as a growth factor for wounded tissues, in collaboration with other factors such as platelet derived growth factor (PDGF) and lysophosphatidic acid which are also released from activated platelets.

Inhibition of motility of cancer cells and smooth muscle cells

We found that exogenously added Sph-1-P can inhibit cell motility of various cancer cells as well as aortic smooth muscle cells at very low (nM) concentrations [6, 7]. Abnormal motility enhancement of cancer cells is regarded as a major factor in cancer metastasis through blood vessels. Likewise, continuous stimulation of motility of smooth muscle cells by PDGF or other cytokines in injured vessels has been regarded as a dangerous factor favoring the development of atherosclerosis [20]. Therefore, one cannot deny the possibility that Sph-1-P may function as a factor preventing cancer metastasis or atherogenesis, although it seems rather paradoxical since activated platelets are believed to play negative roles in these pathophysiological processes [20–22].

The possible physiological or pathophysiological roles of Sph-1-P released from platelets into the blood as well as Sph-1-P-related me-

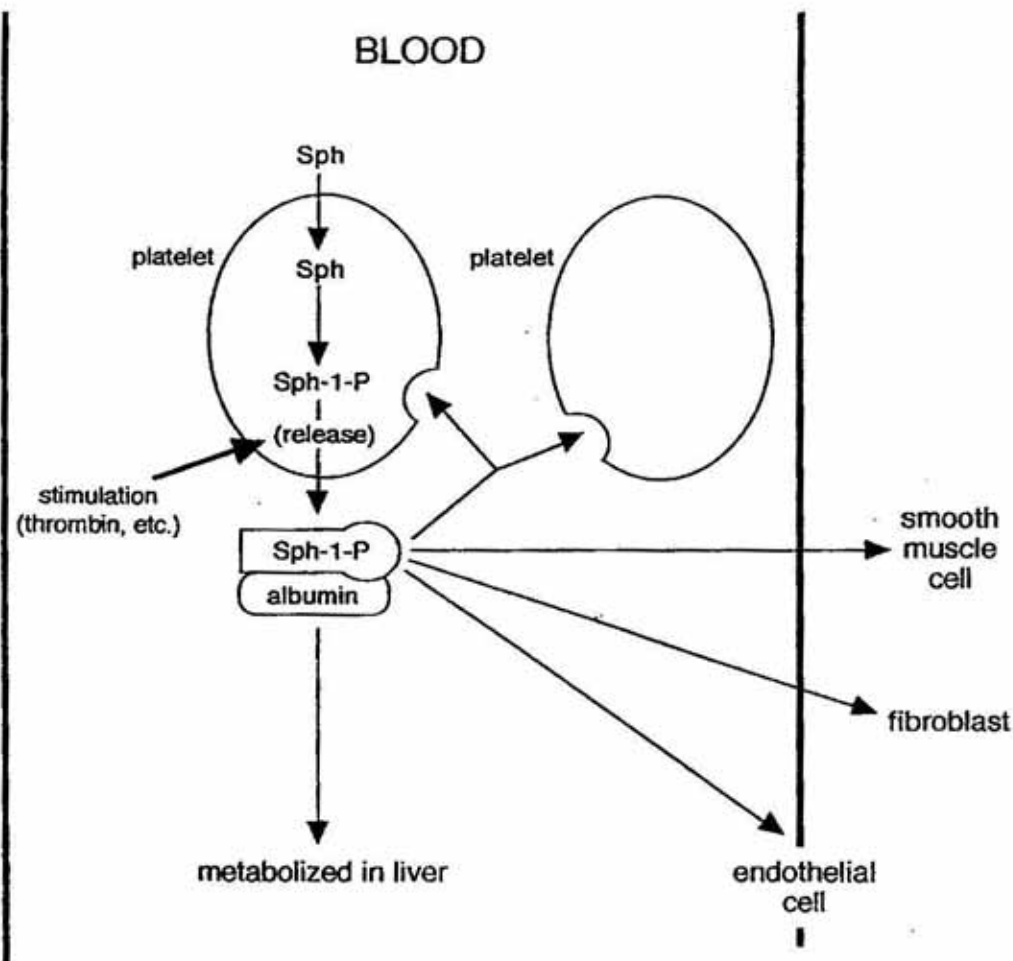


Figure 4. Metabolism of Sph-1-P in blood and possible physiological or pathophysiological roles of Sph-1-P released from platelets into blood.

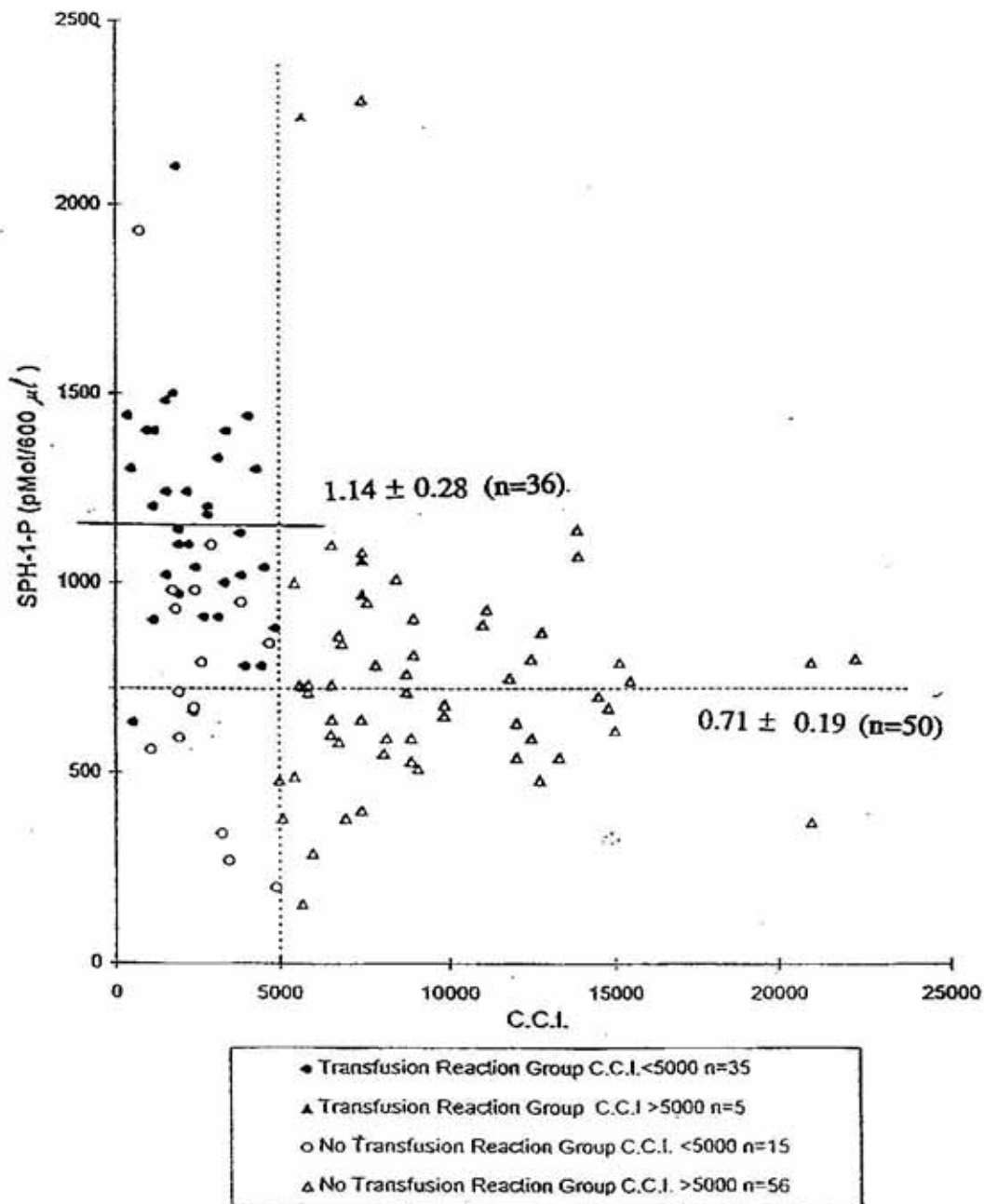
Platelet incorporates sphingosine from plasma and rapidly converts it into Sph-1-P, which is then released extracellularly upon stimulation. Released Sph-1-P, existing as a complex with albumin and being metabolically inactive in plasma, may interact with other platelets (autocrine acceleration of thrombosis) and with the components of the vessel wall such as endothelial cells and fibroblasts (wound healing) or smooth muscle cells (motility inhibition (see ref. [6] and [7])), possibly involved in prevention of atherosclerosis), finally being metabolized in liver where lyase activity, the degrading enzyme of Sph-1-P, is abundant.

metabolism in the blood are depicted and summarized in Fig. 4.

SIGNIFICANCE OF MEASUREMENT OF Sph-1-P CONTENT IN PLASMA

The plasma level of Sph-1-P may be an index of *in vivo* platelet activation in patients with thrombotic disorders, as is the case with β -thromboglobulin [23, 24] and platelet factor 4 [24]. Recently, some of the important bioac-

tivities reported for Sph-1-P, such as Ach-sensitive K^+ channel activation in myocytes [8, 9] and retraction of neurite outgrowth [10], have been observed at concentrations below 100 nM. In our previous studies, we often found that Sph-1-P injection in mice (iv., 10 mg/kg mouse) caused immediate rigors and death [25]. Another study also reported that, together with sphingosine, Sph-1-P plays mediatory roles in the immediate negative inotropic effects of $TNF\alpha$ on cardiac myocytes [26]. Since the plasma concentration of Sph-1-



C.C.I. = (pretransfusion platelet count - post transfusion count) x surface area of the patient in square meters divided by the number of platelets transfused.

Figure 5. Spingosine 1-phosphate content in platelet plasma samples correlates with poor platelet increments after transfusion and with occurrence of transfusion reactions in patients.

(See text for details).

P ranges from 50 pmol/ml (50 nM) to 400 pmol/ml (400 nM), it is possible that variations in the plasma level of Sph-1-P may be related to some clinical disorders, and measure-

ment of the plasma Sph-1-P level may provide information on the pathophysiological implications of Sph-1-P.

CORRELATION OF Sph-1-P CONTENT WITH COMPLICATIONS AFTER PLATELET TRANSFUSION

As an example for the possible pathophysiological roles of Sph-1-P, we recently examined the relationship between Sph-1-P content in the plasma of platelet concentrates and transfusion reactions in patients. Clinical studies suggest that complications after platelet transfusion are often caused by bioactive substances formed in the platelet concentrates during storage. Likely candidates considered for causing detrimental reactions have been cytokines such as IL-1 β , IL-6, or TNF α , released from contaminating leukocytes during storage [27], although other studies do not fully support this theory [28].

To clarify the relationships between plasma Sph-1-P contents in platelet concentrates and occurrence of complications as well as with corrected count increments (C.C.I.; an index for the successful transfusion) after transfusion, we measured Sph-1-P contents in the plasma from platelet samples given for transfusions to patients (John Hopkins Hospital, Baltimore) with or without subsequent transfusion reactions (fever, vomiting etc.) and with differing C.C.I. values. We have arrived at two important findings (Fig. 5): 1) Poor platelet C.C.I. after transfusion correlates strongly with high Sph-1-P content in transfused platelet plasma. 2) A significant difference in Sph-1-P content prior to transfusion between the non-reaction and the transfusion reaction groups is apparent, namely 0.71 ± 0.19 nmol/600 μ l plasma ($n = 50$) and 1.1 ± 0.28 nmol/600 μ l plasma ($n = 36$), respectively (P -value of Student's t -test < 0.001). We have also observed a 2-3 fold increase of Sph-1-P in plasma during 5 days storage. Aged platelet concentrates are often claimed to cause more complications and poor C.C.I. These results taken together strongly suggest that Sph-1-P released from stored platelets is a reasonable candidate substance, in addition to various cytokines, for causing platelet transfusion reac-

tions and poor C.C.I. in the patients. They also imply that inhibitors of Sph-1-P synthesis in, or its release from, activated platelets, such as methylsphingosines [29], would be possible candidates for efficient drugs to mitigate platelet transfusion reactions and to improve poor C.C.I. after transfusion.

CONCLUSION

Since Sph-1-P is highly abundant in platelets and is released from platelets upon stimulation, the plasma Sph-1-P level may be elevated as a result of platelet activation or thrombus formation. Accordingly, the plasma level of Sph-1-P, like β -thromboglobulin and platelet factor 4, may be an index of *in vivo* platelet activation in patients with thrombotic disorders. Furthermore, it is conceivable that variations in the plasma level of Sph-1-P may be related to some clinical disorders, and that measurement of the plasma Sph-1-P level may provide information on the pathophysiological implications of Sph-1-P. As an example for the possible pathophysiological roles of Sph-1-P, we report here our findings that high Sph-1-P content in the plasma of platelet concentrates correlates with poor platelet increments after transfusion and with occurrence of transfusion reactions in patients.

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