Review

Lysophospholipids in laboratory medicine

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Abstract: Lysophospholipids (LPLs), such as lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and lysophosphatidylserine (LysoPS), are attracting attention as second-generation lipid mediators. In our laboratory, the functional roles of these lipid mediators and the mechanisms by which the levels of these mediators are regulated *in vivo* have been studied. Based on these studies, the clinical introduction of assays for LPLs and related proteins has been pursued and will be described in this review. Although assays of these lipids themselves are possible, autotaxin (ATX), apolipoprotein M (ApoM), and phosphatidylserine-specific phospholipase A_1 (PS-PLA_1) are more promising as alternate biomarkers for LPA, S1P, and LysoPS, respectively. Presently, ATX, which produces LPA through its lysophospholipase D activity, has been shown to be a useful laboratory test for the diagnosis and staging of liver fibrosis, whereas PS-PLA_1 and ApoM are considered to be promising clinical markers reflecting the *in vivo* actions induced by LysoPS and S1P.

Keywords: lysophospholipids, lysophosphatidic acid, sphingosine 1-phosphate, lysophosphatidylserine, autotaxin, apolipoprotein M

Introduction

The (patho)physiological significance of eicosanoid mediators, namely first-generation bioactive lipids such as prostaglandins and leukotrienes, is well known and can be easily understood by considering the established importance of aspirin, which is used for the treatment of various diseases by irreversibly inhibiting cyclooxygenases. In addition to eicosanoid mediators, lysophospholipids (LPLs), which are second-generation bioactive lipids, have now been shown to play very important (patho)physiological roles in the body. LPLs have a common structure consisting of a hydrophilic head portion of a phosphate group and a hydrophobic tail portion, and they are involved in a variety of (patho)physiological responses mainly as intercellular signaling molecules. In particular, considerable knowledge has been accumulated regarding the glycerophospholipid mediator lysophosphatidic acid (LPA)[1)–3)] and the sphingolipid mediator sphingosine 1-phosphate (S1P).[4),5)] The importance of these LPLs has been shown at an individual level using genetically modified mice, and the S1P receptor modulator fingolimod (FTY720) has been introduced into actual clinical settings as a therapeutic agent for multiple sclerosis.[6),7)] Furthermore, the drug applications of LPL receptor agonists/antagonists and inhibitors

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Abbreviations: ACS: acute coronary syndrome; ApoM: apolipoprotein M; ATX: autotaxin; CETP: cholesteryl ester transfer protein; CSF: cerebrospinal fluid; GPCR: G protein-coupled receptor; HCC: hepatocellular carcinoma; HEV: high endothelial venule; LC-MS/MS: liquid chromatography–tandem mass spectrometry; LPA: lysophosphatidic acid; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; LPI: lysophosphatidylinositol; LPL: lysophospholipid; LPP: lipid phosphate phosphatase; lysoPLD: lysophospholipase D; LysoPS: lysophosphatidylserine; MAG: monoacylglycerol; PA: phosphatidic acid; PIH: pregnancy-induced hypertension; PLA_1: phospholipase A_1; PLA_2: phospholipase A_2; PS: phosphatidylserine; PS-PLA_1: phosphatidylserine-specific phospholipase A_1; RA: rheumatoid arthritis; RBC: red blood cell; SF: synovial fluid; SK: sphingosine kinase; S1P: sphingosine 1-phosphate.

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of LPL production have been studied extensively.[8] To elucidate the functional roles of LPLs *in vivo*, especially in clinical settings, we have tried to clarify the (patho)physiological roles of LPLs and the mechanisms by which the metabolism of this new class of bioactive lipids are controlled; our final aim is the inclusion of assays for these molecules in laboratory testing.

Eicosanoid mediators are derived from arachidonic acid liberated from the cell membrane by phospholipase A_2s (PLA_2s) in association with cell activation; they are the products of a series of sequential reactions in which unstable mediators are produced and released at the same time as cell activation. In contrast, the regulation of LPL metabolism varies greatly. For example, LPA is continuously produced in plasma, because both the substrate and enzyme responsible for the production of LPA co-exist in this blood component; at the same time, LPA undergoes degradation through the action of lipid phosphate phosphatase (LPP), an ectoenzyme expressed on the cell surface (such as vascular endothelial cells) that is in contact with blood.[9),10] On the other hand, S1P is produced by intracellular sphingosine kinases (SKs), and is released extracellularly.[11),12] Blood cells, particularly platelets, already store S1P abundantly in the resting state; upon activation, the cells release this preformed S1P, which is then metabolized by LPP.[13)–15] As described above, LPLs are formed continuously and metabolized dynamically in body fluids, especially blood plasma. Understanding the regulatory mechanisms responsible for this dynamism is important for understanding the *in vivo* significance of cellular responses resulting from specific cell-surface receptors for each LPL. This dynamism also implies the importance of the suitable sampling of plasma and other clinical samples to minimize undesired artifacts arising from the *in vitro* metabolism of LPLs after venipuncture and/or sample preparation, which are both essential for the application of LPL assays for clinical diagnosis, (*i.e.*, laboratory medicine applications).

This review will explore the metabolic control mechanism of LPLs in body fluids, especially in the plasma and the application of assays for LPLs and related proteins in laboratory medicine.

1. Production and action of LPLs present in body fluids

The LPLs referred to in this review are LPA, S1P, and lysophosphatidylserine (LysoPS), the production and action of which are summarized in Fig. 1. LPA is mainly produced extracellularly (i) from substrate LPLs (such as lysophosphatidylcholine [LPC]) through the activity of lysophospholipase D (lysoPLD) of autotaxin (ATX) and (ii) from phosphatidic acid (PA) through the action of PA-specific phospholipase A_1 (PA-PLA$_1\alpha$/LIPH).[2),3] On the other hand, S1P is produced from sphingosine through the action of two SKs, *i.e.*, SK1 and SK2, present intracellularly; S1P thus produced is then transported out of the cell by specific transporters such as Spns2[16] and Mfsd2b,[17] and by rather non-specific ones such as ABC (ATP binding cassette) transporter[18] and an anion exchanger.[19]

On the other hand, LysoPS has been shown recently to induce a variety of cellular responses via its interactions with specific receptors,[20),21] and phosphatidylserine-specific PLA_1 (PS-PLA_1) has attracted attention as a possible enzyme involved in the production of ligand LysoPS.[22] PS-PLA_1 is highly homologous with PA-PLA$_1\alpha$/LIPH, as mentioned above, and specifically hydrolyzes the *sn*-1 fatty acid of phosphatidylserine (PS). PS-PLA_1 is an enzyme that is secreted extracellularly, and utilizes PS exposed on the cell membrane as a substrate to produce LysoPS,[20),22] although enzymes other than PS-PLA_1 may produce LysoPS; at least intracellularly, LysoPS is reportedly regulated by the ABHD16A-ABHD12 axis.[23]

As described above, compared with the eicosanoid mediators that are produced and released from activated cells and then act as autacoids, the regulation of the metabolism of LPL mediators is complicated; LPLs can both circulate in the blood like hormones and function as local mediators produced locally. In any case, LPA (produced extracellularly), S1P (produced intracellularly and released extracellularly), and LysoPS (possibly produced extracellularly), function through specific G protein-coupled receptors (GPCRs) expressed on the surfaces of target cells. Six, five, and three kinds of specific receptors have been identified for LPA, S1P, and LysoPS, respectively, and the elucidation of their expression patterns *in vivo* is important for discussions of the (patho)physiological roles of these LPL mediators.[3),4),8),20] Revealing the mechanism by which the levels of these LPLs are regulated and measuring their concentrations in body fluids (that are in contact with the cell surfaces where GPCRs are expressed) are important. In fact, relatively high concentrations of LPLs are known to exist in body fluids such as blood (plasma, serum), which can be

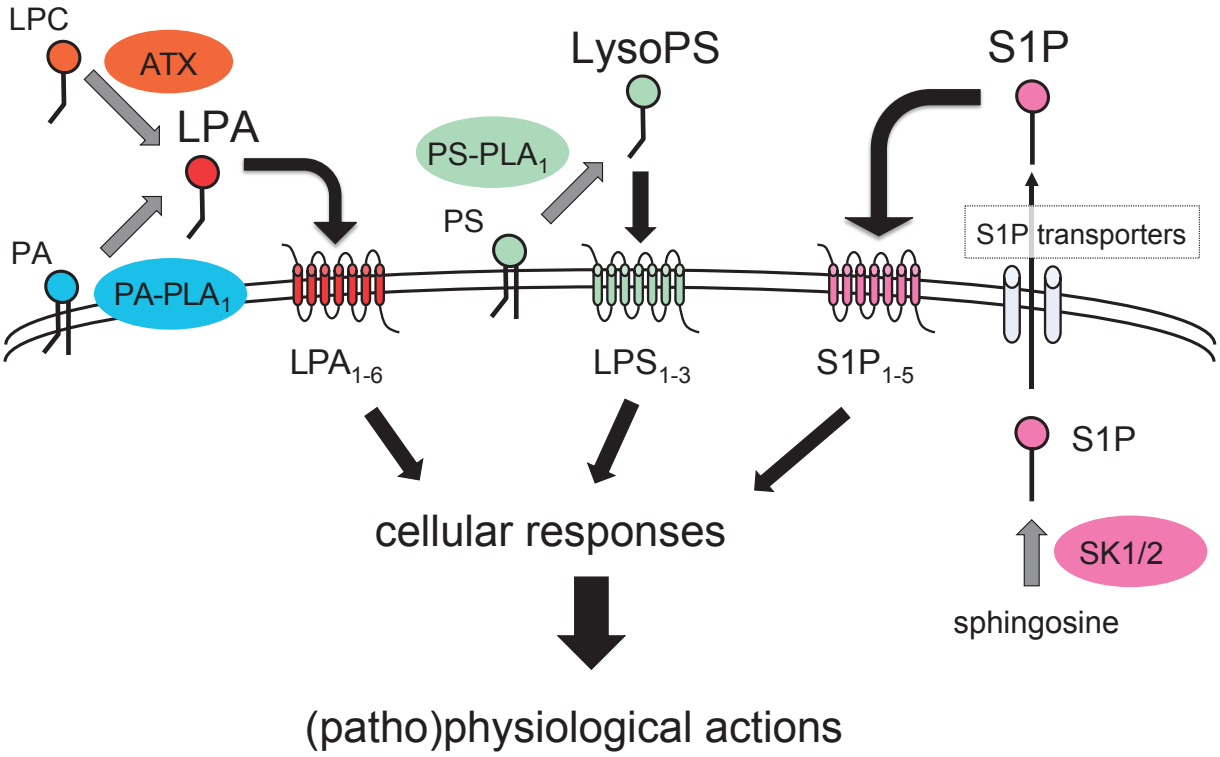

Fig. 1. The three major LPLs: LPA, S1P, and LysoPS. The production and actions of these lipid mediators are illustrated. Although the mechanisms by which these bioactive lipids are formed differ, they act on specific GPCRs expressed on the cell surface, resulting in a variety of cellular responses. See the text for details.

obtained easily as human clinical samples; future clinical applications are thus promising if clinically meaningful findings can be obtained. Urine is another promising sample for LPL assays that requires no burden to the subject, although our preliminary study suggested that procedures to concentrate the sample are needed. Of note, the enzymes producing these LPLs and their carriers also exist in body fluids, and this observation is important from the viewpoint of clinical applications, as described later.

2. LPA

2.1. Overview. In plasma, LPA is mainly produced by ATX from LPLs such as LPC, and this ATX/LPA system is essential for the stabilization of blood vessels and for the embryonic vasculature.[24] Although platelets were once reported as a source of blood or serum LPA, their contribution is now thought to be relatively small.[25),26] As mentioned above, LPA is actively produced and simultaneously subjected to degradation by LPP, as shown by *in vivo* experiments using ATX inhibitors; the concentration of plasma LPA decreases rapidly when the production pathway of blood LPA is suppressed by the injection of ATX inhibitors (Fig. 2).[9] Furthermore, accumulation of the atherosclerotic mediator LPA by the reduction of LPP activity is reportedly involved in the onset and progression of atherosclerotic diseases.[10] On the other hand, PA-PLA$_1\alpha$/LIPH is abundantly expressed in the outer sheath of the hair root and hydrolyzes the substrate PA to produce LPA locally, which plays an important role in hair follicle development through the action of LPA$_6$.[27] In addition, at sites where tissue damage and inflammation occur, various types of PLA$_2$ and PLA$_1$ are somehow activated and produce LPLs, which can serve as the substrate of ATX and hence the precursor of LPA. Thus, LPA can be produced by a variety of mechanisms and can serve as both a blood-borne lipid mediator and a local mediator.

LPA is produced not only extracellularly but also intracellularly by fatty acid esterification of glycerol 3-phosphate, a metabolic intermediate in the glycolysis reaction. Because this pathway is believed to play an important role in the *de novo* synthesis of

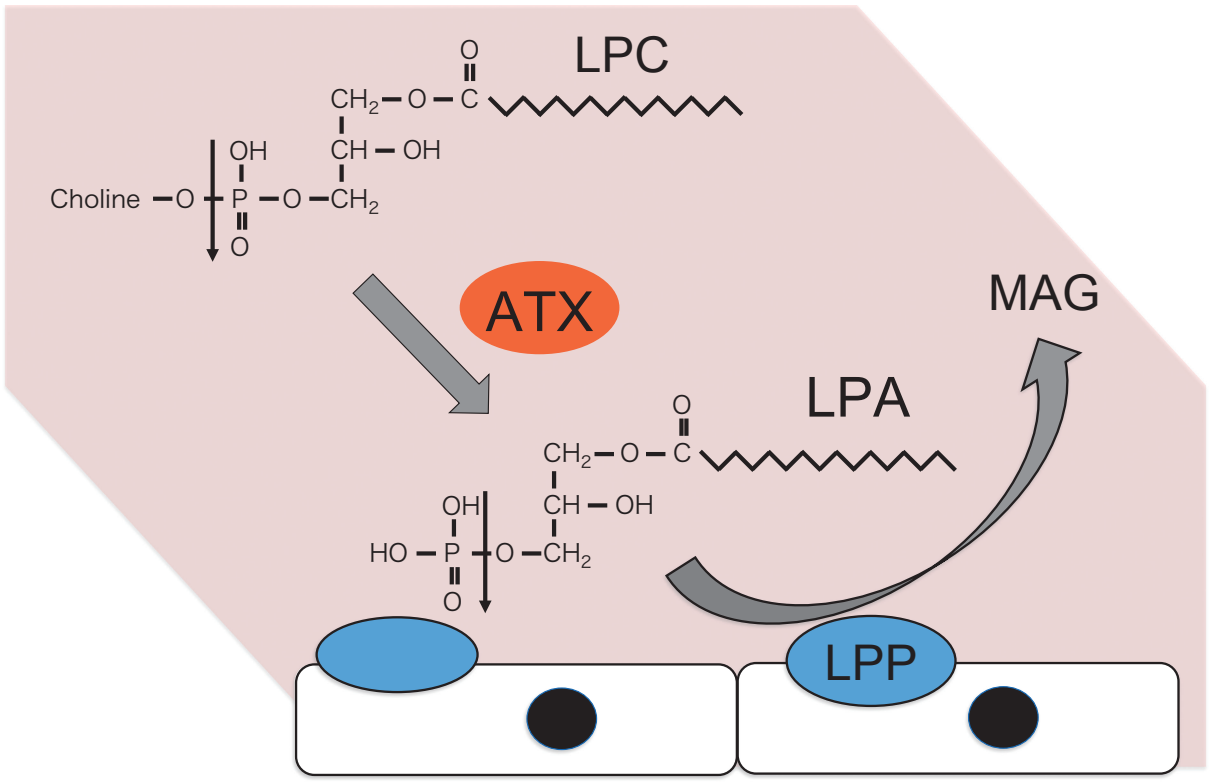

Fig. 2. LPA metabolism in the blood. In plasma, LPA is produced by the lysoPLD activity of ATX from substrate LPLs. LPC is the most important substrate; *sn*-1 acyl LPC is shown in the figure. The product LPA undergoes dephosphorylation by the action of the ectoenzyme LPP, resulting in the formation of monoacylglycerol (MAG).

phospholipids, this is not referred to in the present review, which focuses on the clinical applications of LPA assays.

Although numerous phospholipases are capable of producing LPA from their corresponding precursors *in vitro*, only ATX and PA-PLA$_1\alpha$/LIPH are known to produce and provide LPA, functioning as a ligand for its GPCR *in vivo*. Of note, these two enzymes possess a signal sequence at the N-terminus, and are both secreted.[28] PA-PLA$_1\alpha$/LIPH, however, is subjected to binding to the cell membrane after secretion, and hence is known to be hardly detectable in the culture medium and in body fluids. On the other hand, high concentrations of ATX are present in body fluids such as plasma or serum, as can be easily understood from the fact that the purification of ATX was initially achieved using serum as a starting material,[29),30)] and this point is relevant to the clinical applications of an ATX assay, referred to later.

2.2. LPA assays. Several methods can be used to determine LPA concentrations *in vitro*. For the purpose of assaying clinical samples, we have used an enzymatic cycling method, which can be performed with an automatic analyzer,[31)] and mass spectrometry, which enables each LPA species to be assayed.[32),33)] In this manner, we confirmed that the total LPA concentration measured using the former method was nearly the same as the sum total LPA of each LPA molecular species measured using the latter method. The most important sample for clinical purposes is plasma, but its suitable preparation is a difficult task because the blood levels of LPA can easily be increased by *in vitro* manipulation after venipuncture due to the co-existence of the enzyme ATX and the substrate LPC, and possibly due to the absence of endothelial LPP in test tubes (not *in vivo*).[34)] We previously examined the optimal conditions for the preparation of plasma samples for the measurement of LPA and found that the undesired and artificial formation of LPA after blood sampling became negligible when whole blood samples were mixed with EDTA plus an anti-platelet cocktail and all of the procedures, including the plasma preparation and preservation until measurement, were performed at 4 °C.[35)] Using these strict

sampling conditions, we reported that the plasma LPA concentration was significantly higher in women (0.103 ± 0.032 µmol/L; n = 47) than in men (0.077 ± 0.026 µmol/L; n = 99).[36] Unfortunately, erroneously high concentrations of plasma LPA have likely been reported in the past due to inadequate sampling procedures.

A multiple regression analysis showed a strong positive correlation between plasma LPA concentration and serum lysoPLD activity,[36] which is consistent with the fact that production by ATX/lysoPLD is a major route for plasma LPA. We further confirmed correlations between plasma LPA and serum ATX levels in disease states.[37),38] We think that the higher plasma LPA level in women than in men, as described above, can be explained by the fact that the serum ATX level is higher in women.[39]

Although the plasma levels of LPA can be determined using these methods, the laboratory testing for this important bioactive lipid for clinical purposes seems unpractical because the conditions required for suitable sample preparation, which are essential for suppressing artifact LPA production after blood sampling, are too stringent. We then established an immunoassay for the quantitative determination of ATX,[39] which enabled us to handle numerous serum samples as part of routine clinical practice. Based on the correlation between plasma LPA and serum ATX, the latter measurement may be both useful and practical as a surrogate marker for the former. In fact, the serum ATX antigen concentration in healthy subjects was strongly correlated with serum lysoPLD activity.[39] Consequently, we have mainly examined serum ATX to investigate the clinical significance of LPA and to establish laboratory medicine for LPA/ATX.

2.3. Clinical significance of serum ATX assay (Fig. 3). ATX is expressed in a variety of cells and tissues, and relatively high concentrations of ATX exist in human serum. The central 95th percentile reference interval for the serum ATX antigen concentration in healthy subjects was reported as 0.468–1.134 mg/L (n = 120); the concentration of ATX was significantly ($P < 0.001$) higher in women (0.625–1.323 mg/L) than in men (0.438–0.914 mg/L).[39] The main source of ATX in the bloodstream is adipose tissue,[40),41] and the oral administration of prednisolone decreases circulating ATX through the regulation of adipose tissue ATX expression.[42] Other cell types also express ATX. For example, high endothelial venule (HEV) reportedly expresses ATX, and HEV-associated ATX generates LPA locally, which, in turn, acts on HEV endothelial cells for constitutive lymphocyte transmigration across the basal lamina of HEVs.[43] The contribution of HEV to the ATX level *in vivo*, however, remains to be resolved.

Our earlier studies revealed that serum ATX activity and plasma LPA levels are increased in patients with chronic hepatitis C and liver fibrosis,[38] as supported by results obtained in a rat model;[44] therefore, we measured the serum ATX antigen level as a marker of liver fibrosis in two cohorts of patients with chronic liver disease caused by hepatitis C virus.[45] In the first cohort, the serum ATX level was significantly correlated with the liver fibrosis stage and was the best parameter for the prediction of cirrhosis, with an area under the receiver operating characteristic curve (AUROC) of 0.756 in men and 0.760 in women, compared with the serum hyaluronic acid and aminotransferase-to-platelet ratio index, which are established markers of liver fibrosis. In another cohort, the serum ATX level was significantly correlated with liver stiffness, a novel reliable marker of liver fibrosis, and was the second-best parameter in men (AUROC, 0.799) and women (AUROC, 0.876) for the prediction of significant fibrosis, and the best parameter in men (AUROC, 0.863) and the third-best parameter in women (AUROC, 0.872) for the prediction of cirrhosis, both of which were evaluated based on liver stiffness. Considering that the increase in ATX in patients with liver fibrosis is more specific than that of conventional markers, the serum ATX level has now been established as a novel and reliable marker of liver fibrosis,[46),47] and these results have been reproduced in other studies.[48)–50] Recently, the serum ATX level was also found to be a useful disease progression marker in patients with primary biliary cholangitis.[51] The increase in serum ATX in chronic liver disease is possibly due to the reduction in ATX clearance from the circulation by the scavenger receptors of liver sinusoidal endothelial cells.[44),52]

LPA involvement in the female reproductive system is well-known,[53] and the serum ATX antigen levels were reported to be significantly higher in normal pregnant women than in non-pregnant women.[29] The serum ATX antigen levels in normal pregnant women were also significantly and positively correlated with the gestational week ($r = 0.809$, $P < 0.001$), and the serum ATX antigen levels of patients with pregnancy-induced hypertension (PIH) (3.299 ± 1.720 mg/L) were significantly lower than those of normal pregnant women (4.915 ± 2.323 mg/L) during the third trimester, indicating

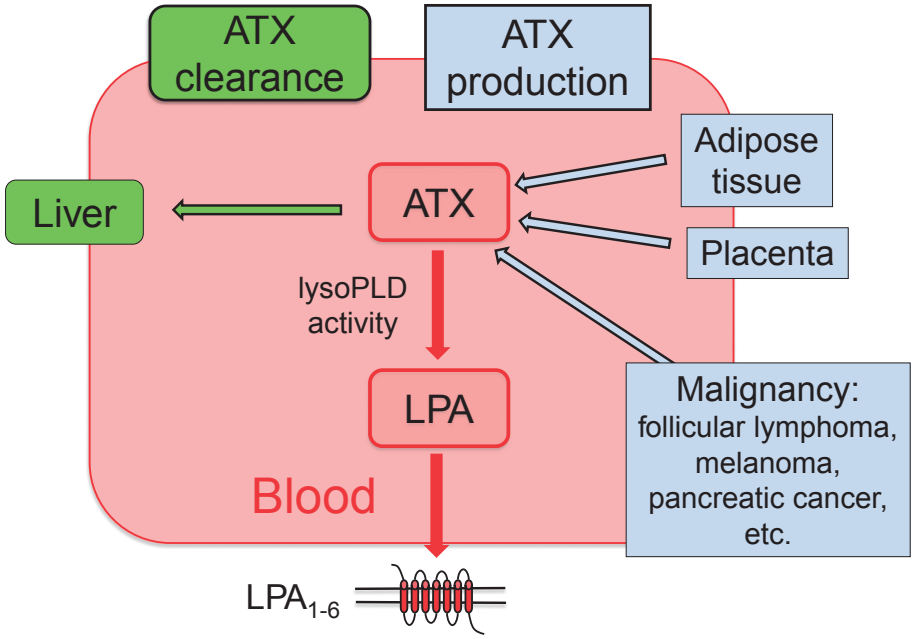

Fig. 3. Regulation of the ATX level and resultant LPA production in the blood. Although ATX is expressed in a variety of cells and tissues, the main source of ATX in the bloodstream is considered to be adipose tissue. The placenta produces ATX, and the serum ATX level in normal pregnant women is positively correlated with the gestational week; the serum ATX antigen level of patients with PIH is lower than that of normal pregnant women. As for malignancy, the serum ATX level is increased in follicular lymphoma and may be a promising marker for this B-cell lymphoma. ATX was cloned from the supernatant of melanoma cells, and serum ATX is reportedly elevated in patients with melanoma, but only in progressive cases. Serum ATX activity may be useful for identifying pancreatic cancer when used together with other serum markers of pancreatic cancer. The liver seems to be important for the regulation of blood ATX by its clearance from the circulation by scavenger receptors on liver sinusoidal endothelial cells; serum ATX is increased in chronic liver disease, possibly because of the reduction of ATX clearance by the liver. In most cases, changes in serum ATX are accompanied by parallel variations in plasma LPA. The only exception seems to be ACS; only the plasma LPA level, and not the serum ATX level, increases in patients with ACS. See the text for details.

that the serum ATX level may be a serological marker for the prediction of PIH,[54] as supported by the concurrent expression of ATX in the placenta.[55),56]

Although the involvement of LPA/ATX in cancer initiation and progression has been reported frequently, the role of ATX as a biomarker for cancer has not received much attention. ATX was cloned from the supernatant of melanoma cells, and an elevation in serum ATX in patients with melanoma has been described, but only in progressive cases.[57] When we measured the serum ATX antigen levels in patients with hematological malignancies, we found that the serum ATX antigen level may be a promising and novel marker for follicular lymphoma.[37] When the potential clinical significance of the serum ATX level in patients with cancers of the digestive system was examined, we found that the serum ATX activity level may be useful for identifying pancreatic cancer when used together with other serum markers for pancreatic cancer.[58]

Furthermore, ATX has been shown to be involved in pruritis in cholestatic liver diseases and atopic dermatitis,[59)–61] which may be a promising and important finding from the viewpoint of therapeutic approaches.

2.4. ATX isoform. To date, five alternative splicing isoforms of ATX have been reported. Three alternative splicing isoforms, *i.e.*, classical ATX including ATXα, ATXβ, and ATXγ, were reported initially, and two novel alternative splicing ATX isoforms, *i.e.*, novel ATX including ATXδ and ATXε, were identified later. ATXδ and ATXε possess a 4-amino acid deletion in the L2 linker region of ATXβ and ATXα, respectively. ATXβ and ATXδ have similar divalent cation sensitivities and cell motility-stimulating activities and are present as the major isoforms in a wide range of organisms from fish to mammals.[62),63] We developed enzyme immunoassays to measure the serum concentrations of "classical ATX" and "novel ATX" antigens and evaluated the usefulness of these assays using human

serum samples; these assays may be helpful for elucidating the distinct functional roles of each ATX isoform. However, while the concentrations of both classical ATX and novel ATX in normal pregnant subjects and patients with chronic liver diseases or follicular lymphoma were significantly higher than those in healthy subjects, the ratio of both ATX isoforms to total ATX did not vary in these groups. The clinical significance of assaying each ATX isoform remains to be clarified.

2.5. ATX-independent increase in plasma LPA level in acute coronary syndrome (ACS). As described above, the serum ATX level varies in several (patho)physiological conditions, accompanied by parallel variations in plasma LPA. As an exception, however, we found that only the plasma LPA levels, but not the serum ATX levels, were elevated in patients with ACS.[64),65)] Using liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis, notable increases in the levels of long-chain unsaturated LPAs (such as 22:6 LPA, 18:2 LPA, and 20:4 LPA), but not saturated LPA, were observed in patients with ACS.[33)] Although a possible correlation between the plasma 18:2 LPA and serum ATX levels were observed, consistent with *in vitro* experiments revealing that serum incubation mainly increases the 18:2 LPA level possibly because of the lysoPLD activity of ATX, the level of 22:6 LPA, which showed the greatest increase in ACS, was not correlated with the ATX level. Furthermore, a multiple regression analysis also revealed that lysophosphatidylinositol (LPI), LPC, and lysophosphatidylethanolamine (LPE) were found to be independent explanatory variables for several LPA species, and 22:6 LPA, in particular, was strongly correlated with corresponding LPE, LPI, and LPC. Thus, the increases in long-chain unsaturated LPA, such as 22:6 LPA, may be determined by precursor LPLs including but not limited to LPC, but not by ATX. Although the details of this ATX-independent LPA formation remain to be clarified, it may be noteworthy that unsaturated fatty acids such as docosahexaenoic acid are abundant in the heart;[66)] unsaturated LPLs may be released from damaged heart tissue and become a substrate for ATX, which may be supported by the fact that the culprit coronary arteries in ACS contain significantly more LPA than the systemic arterial circulation.[65),67)] These findings are important because LPA is deeply involved in atherosclerosis and ischemic heart disease and differences exist in the abilities of LPA species to activate the LPA receptor; in fact, 22:6 LPA may be unique in its exertion of a cardioprotective effect (Kano, *et al.* unpublished data). Furthermore, not only LPA, but also other LPLs may be involved in these pathological states.

2.6. Clinical significance of ATX assays using samples other than serum. ATX exists not only in the bloodstream, but also in other body fluids. For example, ATX has been found in synovial fluid (SF). ATX was highly and specifically expressed in rheumatoid arthritis (RA) synovium, and the level of ATX was high in RA SF. Using LPA_1-deficient mice, LPA/LPA_1 signaling was found to contribute to the development of arthritis via cellular infiltration, Th17 differentiation, and osteoclastogenesis, indicating that LPA/LPA_1 may be a promising target for RA therapy.[68)] The increase in ATX in the SF of patients with RA seems to be due to the enhanced expression of ATX by TNF in synovial fibroblasts.

ATX has been detected in abundance in cerebrospinal fluid (CSF). The ATX concentration of the normal CSF (1.320 ± 0.196 mg/L, $n = 46$) was higher than that in normal serum (see above), and no significant difference in the CSF ATX concentration was seen between men and women.[69)] On the other hand, in the case of CSF, although the enzyme ATX exists, the substrate LPC does not; we failed to detect LPC in normal CSF using our enzymatic colorimetric assay (unpublished data). Consistent with this finding, no formation of LPA was observed in CSF even after 6 h of incubation at 37 °C, probably because of the absence of substrate. When CSF was mixed with LPC or serum, followed by incubation at 37 °C, the formation of LPA was observed. Accordingly, an increase in LPA must occur under pathological conditions where CSF LPC increases, such as bloody CSF. CSF may be promising as a clinical sample with which LPA and its precursor LPLs can be used as potential disease biomarkers. Furthermore, previous results using CSF samples have revealed that ATX activity is increased in patients with multiple sclerosis,[70)] and ATX in the elderly was correlated with greater odds of having Alzheimer's disease-related markers and poor executive function and memory scores, indicating that ATX may be a useful dysmetabolism biomarker for examining the outcomes and risk of this disease.[71)] These studies indicate that LPA/ATX may be important biomarkers in CSF, reflecting various neurological disorders.

Of note, an increase in ATX in SF and CSF is not accompanied by an increase in serum; no significant correlation in ATX concentration was

seen between CSF and serum.[69] These results indicated that the mechanism by which the ATX concentration in local body fluids changes is independent of that in the blood. Accordingly, even if a significant change in serum ATX levels is not seen in some disorders, the involvement of ATX cannot be ruled out. Finally, it is noteworthy that ATX/LPA/LPC is present in aqueous humor, and higher ATX and LPA concentrations are significantly correlated with intraocular pressure, suggesting that the ATX-LPA pathway might be involved in glaucoma.[72),73]

3. S1P

3.1. Overview. S1P acts on each of five specific receptors on cell surfaces, and is involved in a variety of (patho)physiological responses in areas including but not limited to vascular biology, tumor biology, hematology, and immunology. S1P has also attracted attention as a drug target, and the best example is definitely fingolimod (FTY720), which has been shown to be effective for the treatment of multiple sclerosis.[6),7] FTY720 is phosphorylated to FTY720 phosphate by SK2 *in vivo*, and the resulting FTY720 phosphate acts as a functional antagonist for $S1P_1$, inhibiting lymphocyte egress from the thymus and secondary lymphoid organs.[74]

S1P acts via cell surface GPCR, and S1P present extracellularly (or in the cell membrane outer layer) is believed to function as a ligand. Hence, the concentrations of S1P and the mechanisms by which S1P is regulated in body fluids are important, as is the case with LPA. On the other hand, S1P also plays an important role intracellularly.[75),76] This, however, will not be referred to in the present review because assays of clinical samples are the main topic.

3.2. Assay of plasma S1P. The production of S1P is catalyzed by intracellular enzymes called SK1 and SK2, and SK activity in blood cells is generally high; the main source of plasma S1P is blood cells.[77] Blood platelets are unique in that they store S1P abundantly (possibly because of the existence of highly active SK2 and the lack of S1P lyase), and they release this bioactive lipid extracellularly upon stimulation. On the other hand, red blood cells (RBCs) possess SK1 and produce S1P, which is discharged constantly.

When adjusted according to phospholipids, platelets have a much greater abundance of S1P than RBCs. In *in vivo* plasma, however, RBCs have been shown to contribute to the regulation of S1P, rather than platelets.[74] This is probably due to the fact that the volume of RBCs greatly surpasses that of platelets, and RBCs, but not platelets, leak S1P constantly, *i.e.*, without stimulation.[78] In fact, strong positive correlations were found between the plasma S1P concentration and RBCs, supporting the involvement of RBCs in the regulation of plasma S1P concentrations.[79] This involvement can also explain the fact that plasma S1P concentrations are significantly higher in men (413.1 ± 52.0 nmol/L; mean $\pm$ SD) than in women (352.4 ± 39.7 nmol/L).[79]

Although the contribution of platelets may not be important in physiological states, high concentrations of S1P must be released from platelets where platelets are activated, *i.e.*, at sites of thrombus formation or atheroma.[25] In fact, close correlations between the serum S1P level and platelet count (but not RBC count) have been observed, suggesting that S1P may be released from platelets and may mediate crosstalk between platelet activation and the formation of atherosclerotic lesions.[80),81]

We attempted to test the possibility of the clinical introduction of plasma S1P measurement and found that great attention should be paid to the preparation of suitable plasma samples, *i.e.* plasma without platelet releasates, because platelets are easily activated during handling or the centrifugation of blood specimens.[12),79] It seems likely that erroneously high concentrations of S1P have been reported in the past because of inadequate sampling, as is the case with LPA.

S1P is present at a concentration sufficient to activate the S1P receptor in plasma. Accordingly, S1P is thought to control adhesion between vascular endothelial cells in a positive manner and to play an important role in the maintenance of vessel integrity.[77),82),83] On the other hand, S1P is produced in lymphatic endothelial cells in lymph nodes, and Spns2 is needed in endothelial cells to supply lymph S1P and support lymphocyte circulation.[84] In addition, the liver, through the production of apolipoprotein M (ApoM),[85),86] seems to contribute to the regulation of blood S1P.

3.3. ApoM as the carrier of S1P. Most LPLs, such as LPA, are believed to bind to albumin in the circulation. In contrast, two-thirds of plasma S1P is known to be associated with HDL, which is very important when considering the *in vivo* action of S1P.[87] The main role of HDL is its involvement in the reverse cholesterol transfer system. In other words, HDL exerts an anti-atherosclerotic effect by extracting cholesterol from peripheral tissues, includ-

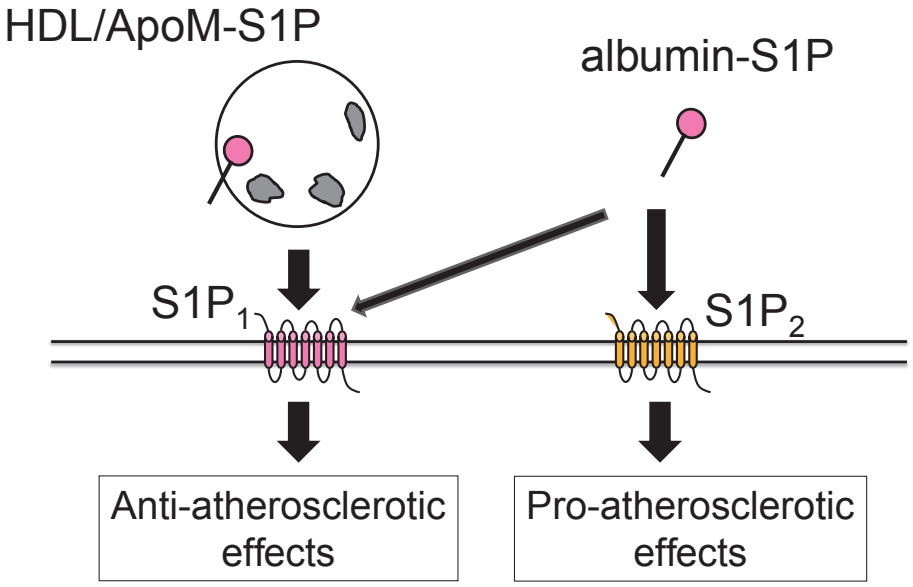

Fig. 4. Carrier-dependent effects of S1P in atherosclerosis. At least part of the HDL pleiotropic effects are thought to be due to S1P present on HDL, and HDL-associated S1P is bound specifically to ApoM. ApoM is not just a carrier of S1P, but is also an important modifier that increases the total amount of S1P in the body by protecting S1P from degradation. Furthermore, ApoM strengthens the agonist properties of S1P toward $S1P_1$, which is anti-atherosclerotic, while it weakens the agonist properties for $S1P_2$, which is pro-atherosclerotic. On the other hand, S1P bound to albumin preferably interacts with $S1P_2$.

ing blood vessel walls. However, a number of studies have suggested that in addition to this reverse cholesterol transport effect, HDL also has pleiotropic effects such as anti-oxidant and anti-apoptotic activities, a cell growth-promoting action, and a blood vessel relaxation action. S1P, which is present primarily on HDL, has the same effects and is considered to play at least a part in the pleiotropic effects of HDL.[88),89)] How S1P resides on HDL was clarified by Christoffersen *et al.*, who reported that HDL-associated S1P is bound specifically to ApoM and that ApoM, by delivering S1P to the $S1P_1$ receptor on endothelial cells, acts as a vasculoprotective component of HDL.[90)] We further reported that ApoM is not just a carrier of S1P, but also an important modifier that increases the total amount of S1P in the body by protecting S1P from degradation.[85),89)] Furthermore, moderate concentrations of resveratrol, which possesses anti-oxidant, anti-inflammatory, and anti-angiogenic properties, increased the intra- and extracellular levels of ApoM, along with intracellular S1P levels, suggesting that the beneficial effects of resveratrol may be mediated by the effects on the metabolic kinetics of S1P/ApoM, at least in part.[91)] Furthermore, ApoM was shown to possess protective properties against LPS-induced organ injuries and to have potential as a novel therapy for severe conditions that are consequent to sepsis.[92)]

Importantly, the biological activities of S1P attached to ApoM (ApoM-S1P) are different from those of S1P attached to albumin (Alb-S1P)[83),93)] (Fig. 4). In this sense, the contrasting responses elicited through $S1P_1$ and $S1P_2$ seem to be very important. $S1P_1$, which is mainly expressed on endothelial cells, mediates embryonic vascular maturation and maintains vascular integrity by contributing to eNOS activation, inhibiting vascular permeability and inducing endothelial cell chemotaxis via G_i-coupled mechanisms, thus playing an anti-atherogenic role. In contrast, $S1P_2$, which is mainly expressed in vascular smooth muscle cells, inhibits Rac and cell migration via a $G_{12/13}$- and Rho-dependent mechanism, thus playing a pro-atherogenic role. It seems likely that ApoM-S1P exerts a strong action toward $S1P_1$, which, among S1P receptors, is thought to be responsible for the beneficial effects that protect vascular remodeling, whereas the opposite is true for Alb-S1P, which preferably interacts with $S1P_2$.[83),89),94)] Accordingly, not only the concentration of S1P, but also its association with carriers is important (Fig. 4). This is true not only in the area of vascular biology, but also for carbohydrate metabolism. ApoM may aug-

ment insulin secretion by maintaining the S1P concentration both *in vivo* and *in vitro* in a manner mediated by $S1P_1$.[95] Furthermore, plasma ApoM has been shown to be negatively correlated with acute myocardial infarction, endotoxemia, diabetes, metabolic syndrome, and body mass index.[96),97] The plasma ApoM level in hepatocellular carcinoma (HCC) patients is reportedly higher than that in normal subjects but lower than those in patients with chronic hepatitis or cirrhosis,[98] possibly because ApoM is mainly expressed in liver parenchymal cells. Finally, the serum ApoM level decreased in patients with primary nephrotic syndrome.[99]

Recently, new aspects of ApoM/S1P metabolism have been found. LDL cholesterol and ApoB, but not HDL cholesterol or ApoA-I, exhibited a significant positive correlation with the plasma S1P level,[79] suggesting that the metabolic pathway for LDL might play some role in the metabolism of S1P. In LDL receptor-overexpressing mice, both the plasma S1P levels and ApoM levels were decreased, and exogenously administered C_{17}S1P bound to ApoM-containing lipoproteins was cleared more rapidly. Unlike the situation in wild-type mice, LDL receptor overexpression in ApoE-deficient mice did not reduce plasma S1P or ApoM levels, suggesting that ApoE might be a ligand for the LDL receptor during the clearance of these factors. Based on these findings, the involvement of the LDL receptor and ApoE in the clearance of S1P can be postulated.[100] Statins, which lower LDL-cholesterol, may also lower ApoM/S1P. In fact, ApoM is reportedly decreased by 7% in response to simvastatin.[101] Furthermore, when we studied the involvement of cholesteryl ester transfer protein (CETP) in S1P metabolism, CETP, which greatly influences HDL quantities, was shown to modulate the distribution of S1P among lipoproteins, which affects the bioactivities of S1P.[102]

In summary, serum ApoM may reflect the beneficial effects of S1P in the bloodstream and hence be a promising clinical biomarker. In fact, plasma ApoM has been shown to be reduced in clinical disorders such as acute myocardial infarction, endotoxemia, and diabetes.[96),97] Therapeutically, ApoM-Fc has been shown to be a viable therapeutic strategy in vascular diseases due to its selective and sustained targeting of endothelial S1P receptors.[103]

4. LysoPS

Recently, 3 LysoPS GPCRs have been identified, and their involvement in immunomodulation is receiving attention.[21),23),104)–106] For example, the LysoPS receptor GPR174 constrains regulatory T cell development and function.[107] Accordingly, a LysoPS assay for body fluids is important from a clinical viewpoint. In the case of rat platelets, the cells secrete two types of PLAs. One is secreted PLA_2 group IIA, which is responsible for the production of saturated LysoPS and other LPLs, whereas another PLA is PS-PLA_1, which specifically hydrolyzes fatty acid at the *sn*-1 position of PS and produces unsaturated LysoPS.[108] Although the mechanisms by which extracellular LysoPS is produced *in vivo* in humans remain to be identified, PS-PLA_1 is believed to be an important candidate for involvement. PS-PLA_1 is a PLA_1 that acts specifically on PS as its substrate and belongs to a secreted lipase family.[20] In fact, PS-PLA_1 can be detected in various body fluids including plasma/serum, unlike PA-$PLA_1\alpha$/LIPH despite the latter being homologous to PS-PLA_1. In this context, we developed an easy immunoassay method for PS-PLA_1. The mean ± SD of the serum PS-PLA_1 concentration in 191 healthy subjects was 33.8 ± 16.6 µg/L, and the central 95th percentile reference interval for the serum PS-PLA_1 antigen concentration was 13.8–74.1 µg/L. The concentration was significantly ($P < 0.001$) higher among men (13.8–80.6 µg/L) than among women (12.1–68.8 µg/L).[109] This PS-PLA_1 assay method can be applied to clinical laboratory testing, as is the case with ATX. In fact, we have found that the serum PS-PLA_1 level is altered in several immunological disorders (data not shown).

Because platelets are involved in the formation of LPLs other than S1P and LPA, we investigated the correlations between the blood levels of each glycero-LPL and serotonin, a biomarker of platelet activation in human subjects, to elucidate the involvement of platelet activation in glycero-LPLs *in vivo*.[110] We found a specific positive correlation between the blood levels of serotonin and LysoPS. Thus, LysoPS might be specifically involved in platelet activation, which is associated with the release of serotonin. Upon activation, platelets expose PS to the outer surface, which may act as the substrate of PS-PLA_1 in plasma, although further studies are necessary. In fact, a positive correlation between LysoPS and PS-PLA_1 was observed in ACS. Together with our observation that LysoPS possesses bilateral effects on macrophages in the pathogenesis of atherosclerosis[111] and that platelets abundantly express the LysoPS re-

ceptor P2Y10,[112] it seems likely that LysoPS may be involved in the pathogenesis of atherosclerotic diseases, in addition to its established involvement in immunomodulation.

Recently, we used ascites samples obtained from patients with gastric cancer and from those with cirrhosis (as a control) in assays for LysoPS and PS-PLA_1. The LysoPS level was shown to be significantly higher in ascites from patients with gastric cancer. We also found a significant positive correlation between LysoPS and PS-PLA_1 only in the gastric cancer group.[113] Although further studies are necessary, it seems likely that the PS-PLA_1-mediated pathway may be involved in the production of LysoPS in gastric cancer and that body fluids other than serum may be useful as clinically important samples for PS-PLA_1 assays.

5. Investigations using surgical pathological tissues

Although the description in this review focuses mainly on the measurement of LPLs in body fluids, especially plasma and serum, and their applications for clinical laboratory testing, important clinical information can be obtained from the examination of pathological tissues, such as surgical samples; for example, the quantification of LPLs in small quantities of liver is possible using LC-MS/MS.[114]

We showed that the expression levels of SK1, Spns2, and $S1P_2$ increased during the progression of liver fibrosis using human liver tissues,[115] suggesting that S1P released from hepatocytes may interact with cell-surface $S1P_2$ and be involved in the resultant fibrosis. This is consistent with our previous results using $S1P_2$-deficient mice.[116),117] Furthermore, by comparing HCC tissues and surrounding non-malignant tissues, a high expression of SK was observed and was shown to be correlated with poorer differentiation and earlier recurrence; however, this was not accompanied by an increase in S1P, possibly because of the elevated expression of S1P lyase.[118] Recently, we similarly reported an elevated expression of S1P lyase in human colon cancer tissues. S1P lyase may be important for the progression of cancer, as suggested by *in vitro* data showing that a reduction in S1P lyase expression through silencing led to reduced proliferation and invasion, whereas the overexpression of S1P lyase enhanced proliferation in colon cancer cell lines.[119] In breast cancer tissues, associations with an elevated expression of SK1, higher levels of S1P,[120] and lymphatic metastasis were reported.[121]

As for LPA, higher LPA_2 mRNA levels were associated with poorer differentiation, and higher LPA_6 levels were associated with microvascular invasion in HCC; both of these factors are risk factors for recurrence after surgical treatment when combined with increased serum ATX levels.[122] On the other hand, increased LPA_1 and LPA_3 expression localized to the HCC- the non-tumor liver margin was reported.[123] As for LysoPS, PS-PLA_1 expression was found to be correlated with tumor invasion and hematogenous metastasis using colorectal cancer tissues.[124]

We believe that the analysis of human tissue samples can provide information that cannot be obtained *in vitro* or using genetically modified animals and insights into the pathophysiology of LPLs. Of note, however, is that the LPL amounts measured using these samples reflect those of both intracellular and extracellular spaces and that cellular components are not homogeneous, including the vasculature. Furthermore, tissue analysis may be difficult to be introduced into clinical laboratory testing because of difficulty in standardization.

6. Toward the establishment of laboratory medicine for LPLs

Therapeutic applications for eicosanoid mediators are much more advanced than those for LPLs. In contrast, the diagnostic applications of assays are more promising for LPLs than for eicosanoids. Eicosanoid mediators are typically autacoids and, upon cell activation, are produced in and released from the cell's interior, then rapidly metabolized. In contrast, LPLs are basically more stable, and the metabolism of extracellular LPLs, which act on cell surface GPCRs, occurs in extracellular body fluids, such as plasma, CSF, and ascites.

Taking these characteristics of LPLs into consideration, assays for LPLs and/or related molecules in extracellular body fluids are promising clinically. In other words, the inclusion of LPL assays in laboratory medicine is feasible, as is the case for peptide mediators, which are important components of ordinary clinical laboratory testing using automated immunological devices.

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Profile

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