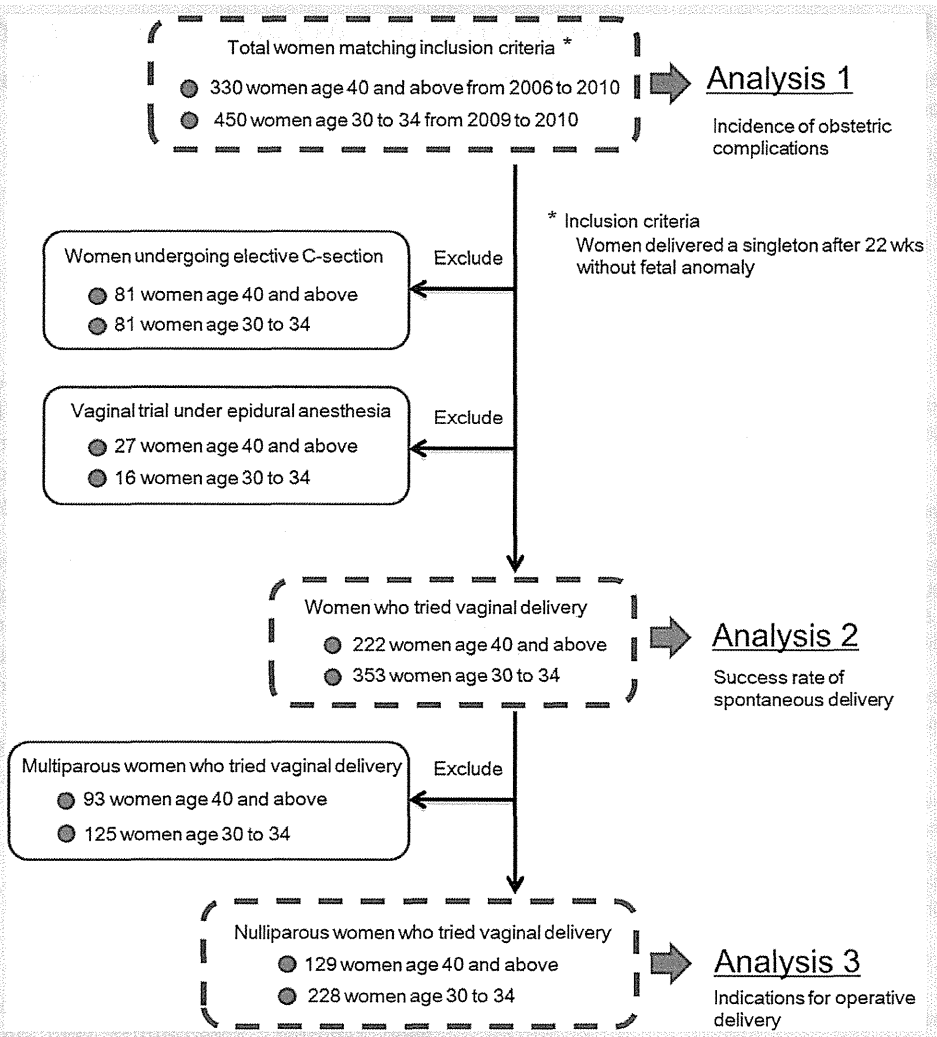
SUPPLEMENTAL FIGURE 1



Flow chart of study design. The number of women in each group is indicated, and the target populations of the three analyses are highlighted in dotted boxes.

Toshimitsu. IVF pregnancy outcomes in women over 40. Fertil Steril 2014.

Emerging Roles for Lysophospholipid Mediators in Pregnancy

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Recent progress in lipid research has unveiled new biologic roles for lysophospholipids as mediators of intercellular signaling. Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are representative lysophospholipids. Accumulating evidence suggests that, acting as intercellular mediators, these and other lysophospholipids may play important roles in physiological and pathological situations. This review discusses the possible involvement of LPA and S1P in reproductive processes, with a focus on the regulatory mechanisms of pregnancy maintenance. As LPA promotes prostaglandin synthesis, mediators in the LPA pathway may also play a significant role in implantation and parturition. S1P signaling is thought to be essential in vascular formation within the uteroplacental unit and in fetomaternal immunologic interactions. Derangements in either one of these lysophospholipid signaling pathways could result in pregnancy complications that may include implantation failure, preeclampsia, and preterm labor.

Introduction

With recent rapid advances in lipid research, numerous biologic functions have been elucidated for a new generation of intercellular lipid mediators. These newly recognized mediators transmit cell-to-cell signals, in direct contrast to the better defined group of intracellular lipid mediators, such as diacylglycerol and phosphatidylinositol trisphosphate, that act as second messengers.

Lysophospholipids are structurally characterized by a phosphate head linked to a single fatty acid backbone molecule such as monoacylglyceride or sphingosine. Lysophospholipids were previously recognized only as intermediate and transient metabolites that were generated during the process of phospholipid production and degradation. In the past several decades, their physiological roles in intercellular signal transduction have been unveiled.[1] Although signaling through nuclear receptors has been reported,[2] intercellular lipid signaling molecules exert biologic activity mainly through binding to cell membrane G-protein-coupled receptors (GPCR).

The availability of substrates and the expression level and activity of rate-limiting enzymes control the production of these lipid mediators and consequently determine their local concentration. Lipid components within the plasma membrane, so-called structural lipids, are the primary source of lysophospholipid mediators. The biologic activities of lipid mediators cover a wide spectrum of physiological processes, including tissue formation and immune regulation. Additionally, impaired regulation of lipid-mediated signaling systems has been linked to a variety of pathologic situations. Important roles for intercellular lipid mediators have been suggested in a broad range of reproductive processes, including

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spermatogenesis, ovarian function, implantation, pregnancy maintenance, and parturition. This review addresses the emerging roles of intercellular lysophospholipid mediators in reproduction, with a particular focus on pregnancy.

Molecular structure, metabolism, and signal transduction

Lysophospholipid mediators are classified into two groups, the lysoglycerophospholipids and the lysosphingolipids. Categorization is based mainly on the characteristics of the molecular backbone (e.g. glycerol versus sphingosine). These backbones are linked to a hydrophilic phosphate head and a hydrophobic carbon chain as shown in Fig. 1. Lysophosphatidic acid (LPA) is a representative mediator in the lysoglycerophospholipid group; sphingosine 1-phosphate (S1P) is an important representative of the lysosphingolipid group.[1,3] Generally, lysophospholipid mediators are synthesized from phospholipids, a major component of cell membranes. Although the inciting stimuli are varied, activation leads to the conversion of cell membrane phospholipid to lysophospholipid via the induction of a series of intracellular or extracellular enzymes.

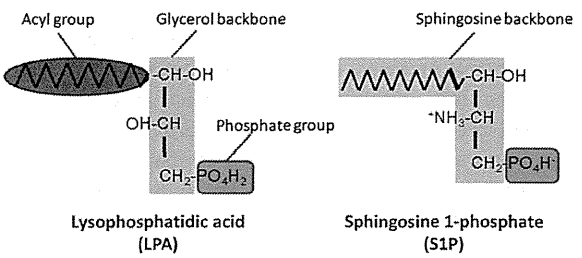

Fig. 1 The molecular structures of LPA and S1P. An acyl group and a phosphate group are connected to a glycerol backbone in LPA. S1P is composed of a sphingosine backbone linked to a phosphate group.

Phospholipid degradation following local tissue activation generates LPA. Currently, two metabolic pathways to LPA production have been described in living organisms (Fig. 2). In one route, lysoglycerophospholipid is generated following the removal of one of two fatty acid chains residing at the sn-1 and sn-2 positions of a glycerophospholipid. This degradation is catalyzed by phospholipase A1/A2. Next, the phosphate head of lysoglycerophospholipid is removed by lysophospholipase D. In the other route, these steps occur in a reverse order; that is, phosphatidic acid without phosphate is produced primarily and phosphatidic acid is subsequently converted into LPA through the action of phospholipase A1/A2. Autotaxin (ATX), a secretory peptide enzyme with lysophospholipase D bioactivity, is central to the second step in the first pathway mentioned above,[4,5] whereas phosphatidic acid-selective phospholipase A1 (PA-PLA1) is central to the second step of the latter pathway.[6,7] The first, ATX-mediated, pathway is thought to be dominant in the regulation of general LPA production. This is consistent with the finding that plasma LPA levels are reduced by 50% in heterozygous ATX-deleted mice.[8]

Although peroxisome proliferation-activating receptor gamma (PPARγ) has been described as an intracellular LPA receptor,[9] the biologic activities of

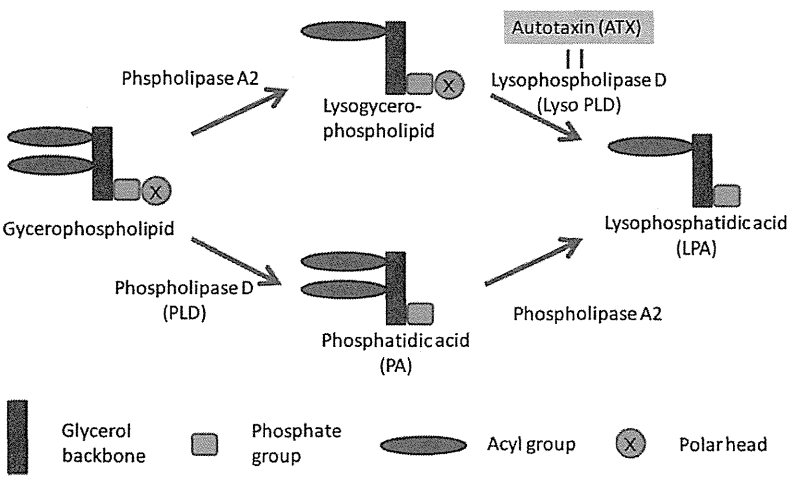

Fig. 2 Pathways for LPA synthesis. Two pathways for LPA synthesis have been identified. The molecular intermediates and metabolic enzymes involved in each pathway are shown.

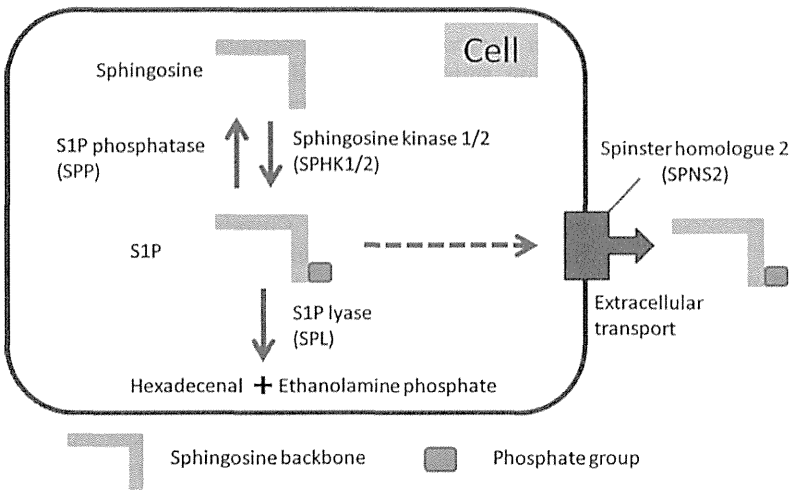

Fig. 3 S1P synthesis, degradation, and export. Known enzymes and intermediates in S1P metabolism and export are demonstrated. The dotted arrow indicates transport across the cell membrane via *spinster homologue 2*.

LPA are mainly transmitted by the several types of GPCRs that can be present on the cell surface. So far, six GPCRs, designated LPA1-6, are proven LPA receptors. Three of these LPA receptors, LPA1, LPA2, and LPA3, are categorized within the endothelial differentiation gene (Edg) family and display very high amino acid homology. LPA2-6 expression is fairly tissue specific, while LPA1 is ubiquitously expressed. The types of G proteins coupled to each LPA receptor and their major expression sites in reproductive organs have been summarized in a review by Choi et al.[10]

S1P, a well-studied lysosphingolipid mediator, elicits pleiotropic biologic effects within the immune, cardiovascular, and central nervous systems. S1P is the product of sphingosine phosphorylation, which is catalyzed by sphingosine kinase (SPHK). There are two SPHK isozymes, termed SPHK1 and SPHK2.[11] Degradation of S1P occurs via two distinct pathways, one reversible and the other irreversible. In the first, dephosphorylation of S1P is mediated by S1P phosphatases (SPP) to generate sphingosine, a reversal of the S1P production process. In the second, S1P lyase (SPL) irreversibly disassembles S1P into hexadecenal and ethanolamine phosphate. Production and degradation of S1P occurs intracellularly. Although the mechanisms for exporting S1P from the cell are not fully described, spinster homologue 2 (SPNS2) and members of the ATP-binding cassette (ABC) family have been implicated (Fig. 3).[12–14]

Five GPCRs belonging to the Edg family of receptors, designated S1P1-5, have been confirmed to bind to S1P at the cell surface and interactions with these receptors can explain most of diverse biologic effects of S1P.[15] S1P1-3 are broadly distributed on the cells within cardiovascular and lymphatic organs, whereas the expression of S1P4 and S1P5 is relatively restricted to cells associated with the immune and central nervous systems, respectively.[15,16] The identities and activities of the particular G-protein-coupled receptor and its corresponding downstream signaling pathway in a given cell or tissue determine the consequent cellular response to S1P. Signaling via S1P1 mainly activates Gi-dependent pathways, promoting lymphocyte emigration from lymphatic organs and stabilization of the angiogenic process. S1P2 signaling opposes S1P1-derived biologic effects by activating G12/13-linked pathways and thereby suppressing the Gi-linked downstream signaling induced by S1P1.[15,17]

Ovarian function

Data from several animal models suggest that S1P-mediated signaling supports ovarian function. Progesterone produced from the corpus luteum, the temporal ovarian endocrine structure formed after ovulation, is indispensable for the maintenance of pregnancy. Prostaglandin F2α (PGF2α) participates in the termination of corpus luteum function, an event that, in rodents, will initiate delivery. S1P suppresses the luteolytic effect of PGF2α in pregnant rats. S1P prevents caspase-mediated apoptosis of luteal cells and maintains blood vessel density in the corpus luteum.[18] In mouse and primate models of radiation and chemotherapy-induced ovarian damage, the

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administration of S1P and its metabolic precursors protects ovarian reserve.[19–21]

There is increasing evidence for an important role for the LPA and S1P pathways in human ovarian function and fertility. LPA and S1P are present in human follicular fluid.[22,23] Concentrations of S1P and LPA in follicular fluid from women undergoing pharmacologic ovarian stimulation are higher than those found in women during natural ovulatory cycles.[23,24] S1P signaling through S1P1 and S1P3 receptors protects granulosa cells from apoptosis via activation of the PI3K/Akt pathway.[25] S1P promotes granulosa cell migration and stimulates endothelial cell growth.[26] LPA increases the production of the proangiogenic cytokines IL-6 and IL-8 in primary human granulosa cell cultures.[24] Taken together, these observations indicate that lysophospholipid mediators contribute to the development of the corpus luteum by protecting granulosa cells from apoptosis and by promoting local microvasculature formation.

Endometrial receptivity and implantation

LPA and S1P are critical in the functional maturation of the uterine endometrium to support embryonic growth, also known as decidualization. Sphingolipid pathway molecules, including S1P, affect uterine endothelial and smooth muscle cell function.[27] The local expression patterns of S1P metabolic enzymes and S1P receptors within the uterine endometrium are temporospatially controlled during the progress of pregnancy in sheep. Further, sphingolipid expression patterns can be correlated with those of several proangiogenic factors, implying a role for S1P in vascular development at the fetomaternal interface.[28] Prominent endometrial expression of S1P receptors and of key enzymes for S1P synthesis and degradation during pregnancy has been also confirmed in mice and humans.[29,30] Both SPHK1-null and SPHK2-null mice are fertile, but double knockout of SPHK1 and SPHK2 is embryonic lethal secondary to impairments in neurogenesis and vasculogenesis.[31] Interestingly, SPHK1−/−SPHK2−/+ mice are infertile. Their reproductive tract phenotype is characterized by neutrophil infiltration and hemorrhage in the endometrium and disturbed endometrial decidualization.[32] Appropriate S1P formation and recognition may be essential for integrated endometrial function.

S1P upregulates the expression of cyclooxygenase (COX) 2 but not prostacyclin synthase in decidual stromal cells.[30] As pharmacological prostaglandin (PG) blockade and transgenic deletion of the rate-limiting enzyme in PG synthesis (COX2) cause impaired fertility, prostanoids appear to be indispensable for successful decidualization and implantation in mice.[33] It is hypothesized that S1P supports decidualization by promoting local vascularization and enhancing PG production.

A critical role for LPA signaling in normal implantation can be gleaned from a targeted deletion study in mice by Ye et al.[34] Delayed implantation and altered implant positioning within the uterine horns, as well as prolonged pregnancy duration and reduced litter size, were detected in LPA3-deleted females. No obvious changes were observed in ovulatory status, egg transport or blastocyst development. The authors of this study also reported reduced endometrial expression of COX2 during the implantation period in LPA3-deleted mice. Moreover, administration of the COX2 products, PGE2 and PGI2, rescued the implantation delay but did not improve embryo spacing. The authors concluded that LPA3-dependent PGs biosynthesis, mediated by COX2, plays a key role in implantation. In contrast, alterations in other LPA and S1P receptors do not appear to affect implantation in mice.

Studies performed in several other animal species support the importance of LPA signaling in implantation. In pigs, LPA equipped with a variety of fatty acid chains was detected in uterine luminal fluid and the biologic activity of the LPA-producing enzyme, ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2; synonym for ATX), was higher in early pregnancy than during estrous cycles.[35,36] A dramatic increase in mRNA for the LPA3 receptors, but not the LPA1 and LPA2 receptors, was also reported in these animals.[35] Woclawec-Potocka et al.[37] confirmed ample production of LPA and increased LPA receptor expression in the endometrium of pregnant cows when compared to non-pregnant controls. In this bovine study, intravaginal infusion of LPA elicited a luteotrophic effect by promoting progesterone and PGE2 synthesis, whereas pharmacologic blockade of LPA1 significantly reduced pregnancy rates.[38] In rats, LPA3-mediated signals increase the uterine expression of fatty acid amide hydrolase (FAAH) during the implantation window.[39] Enhanced FAAH activity might decrease the local concentration of endocannabinoids, which are reported to have negative effects on implantation.[40,41] Supporting the importance of the

LPA-LAP3 axis in animal implantation is the finding that local expression of LPA3, cytosolic phospholipase A2α, and COX2 is decreased in the endometria of patients with repeated implantation failure.[42] Collectively, the existing data support a role for LPA-, particularly LPA3-, mediated modulation of PGE2 synthesis and endocannabinoid activity in the regulation of animal and human implantation.

Endometriosis impairs female fecundity, although the precise mechanisms for this finding are not completely elucidated. Previous reports have suggested that deregulation of phospholipid mediator signaling may be involved. The expression profiles of sphingosine metabolic enzymes and S1P receptors in ectopic and eutopic endometria from endometriotic women differ from those in healthy women.[43] Immunohistochemical analysis of tissues from patients with endometriosis exhibits reduced expression of the implantation-related biomarkers LPA3 and HOXA10 in mid- to late secretory endometria when compared to tissues from normal patients.[44]

Fetomaternal immune regulation

S1P signaling via S1P1 is essential for the egress of lymphocytes from the thymus and lymphoid organs; T cells are absent from peripheral blood and lymph nodes in S1P1 gene-deleted mice.[45] Pharmacological S1P signal blockade using a S1P mimetic, FYT720, causes S1P receptor internalization.[45] Such downregulation of S1P signaling using antagonists has been regarded as a promising therapeutic approach to autoimmune disorders such as multiple sclerosis and autoimmune diabetes.[46,47] These and other known immunologic activities of the lysophospholipid mediators allow for a potential role for these substances in modulating maternal immune responsivity during pregnancy.

A tight control of the balance between maternal tolerance to the fetal semiallograft and maternal defense against exogenous pathogens is the key to the continued health of the pregnant mother and her developing gestation. Regulatory T cells have been shown to be protolerogenic at the fetomaternal interface and their presence appears to be essential for pregnancy maintenance.[48] Components of the S1P-S1P1 pathway negatively impact the generation of regulatory T cells and hamper the development of general immune tolerance in non-pregnant animals.[49] The link between the S1P system and fetomaternal immunology was first proposed in a study using an abortion-prone, non-obese diabetic (NOD) mouse model. In these animals, the combination of TGF-β and S1P signal blockade using FTY720 decreased embryo resorption rates. Mechanistically, this is likely explained by the finding that FYT720 induces the generation of Foxp3+ regulatory T cells.[50]

In cows, it has been shown that components of the LPA pathway affect implantation by modulating the typically tight balance in prostaglandin (PG), prostacyclin, and thromboxane synthesis. In these animals, LPA caused an elevation in PGE2 synthesis along with an inhibition of PGF2α production, leading to a PGE2-dominant environment in uterine endometrium.[38] Through the augmentation of PGE2 production, LPA might indirectly affect fetomaternal immunologic interactions during early pregnancy. Decidual PGE2 supports fetomaternal immune tolerance by suppressing harmful lymphocyte activation and by diminishing the antigen-presenting abilities of decidual macrophages.[51,52] Several lines of evidences have suggested a direct impact of LPA signaling on immunocompetent cells. Various types of immune cells express LPA receptors, and activation of these receptors by LPA alters the chemotactic capacity and chemokine productivity in these cells.[53]

Trophoblast function and placentation

Biologic regulation of trophoblast cell proliferation and functional differentiation are crucial to the development of a healthy placenta. Although the biophysiological function of lysophospholipid signaling in the placenta is incompletely understood, several pioneering studies suggest important roles for S1P and LPA in trophoblast cell physiology. The placenta is a robust source of lysophospholipid metabolites. Normal pregnancy is characterized by progressive elevations in maternal peripheral LPA concentrations; these elevations rapidly return to non-pregnant levels after delivery.[54] The LPA increases in pregnancy are paralleled by increases in serum ATX activity.[55] The source for this enhanced activity appears to be placental ATX production as continued increases in placental ATX activity over the course of gestation fully account for the drastic changes in peripheral blood LPA levels that can be measured in pregnant women.[56] LPA receptors have been detected on trophoblast cells in humans and in several animal species.[57,58] LPA stimulates the production of several chemokines, including IL-8,

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monocyte chemo-attractant protein-1, and growth-regulated oncogene-α, in human cytotrophoblasts in a manner dependent on nuclear factor-kappa B (NF-κB). LPA-induced chemokines from invading trophoblasts may control the migration of lymphocytes and macrophages within the uterine wall. This may, in turn, alter local immunity and angiogenesis.[58]

The S1P pathway has also been implicated in placental function and placental immunology. The S1P receptors, S1P1, S1P2, and S1P3, are expressed in human placenta.[59,60] An investigation using primary human cytotrophoblast cells reported that S1P reduced intracellular cAMP and thereby inhibited cytotrophoblast differentiation into syncytiotrophoblast.[61] Signaling via S1P2 stimulates IL-6 gene expression and IL-6 secretion in the trophoblast cell line, BeWo. This response required a functional Rho-PI3K intracellular pathway.[60] $CD56^{bright}CD16^{-}$ large granular lymphocytes, so-called decidual NK cells, are the dominant decidual immune cells during early pregnancy. Angiogenic and chemotactic factors produced by this unique cell population contribute to local vascular remodeling by supporting cytotrophoblast invasion.[62,63] Zhang et al.[64] demonstrated that S1P signaling regulates decidual NK cell angiogenic activities. In their study in humans, VEGF production by decidual NK cells expressing S1P5 was inhibited upon exposure to the immunosuppressive sphingosine analogue, FTY720, which exerted its immune effects through blockade of S1P1- and S1P5-mediated signaling.

Over the last decade, several studies have indicated that deterioration in the lysophospholipid system can lead to adverse pregnancy outcomes. In one report, serum ATX levels in the third trimester were markedly decreased in pregnant women with PIH when compared to healthy pregnant controls.[54] Others used immunohistochemistry to demonstrate that this same pregnancy disorder is associated with lower expression of the LPA receptors, LPA2(Edg4) and LPA3(Edg7).[65] Arteries dissected from the chorionic plate of normal pregnancies will constrict in response to S1P and this effect is enhanced in the presence of L-NAME, a inhibitor of nitric oxide (NO) synthesis.[59] Vasoconstriction induced by L-NAME treatment causes symptoms similar to preeclampsia in pregnant rats.[66] Taken together, it seems likely that components of the S1P pathway combine with NO to balance vascular tone and appropriate blood supply in the placental bed and that derangement in this balance may foster the development of preeclampsia.

Delivery

Uterine contractility in labor requires the integration of mechanical, hormonal, and immunologic inciters whose consequences are mediated by a multitude of molecular signaling pathways. Several past studies have proposed a regulatory role for the lysophospholipid mediators in this complex process. LPA can alter muscle cell function. An increase in the cytosolic concentration of Ca^{2+} is an indispensable part

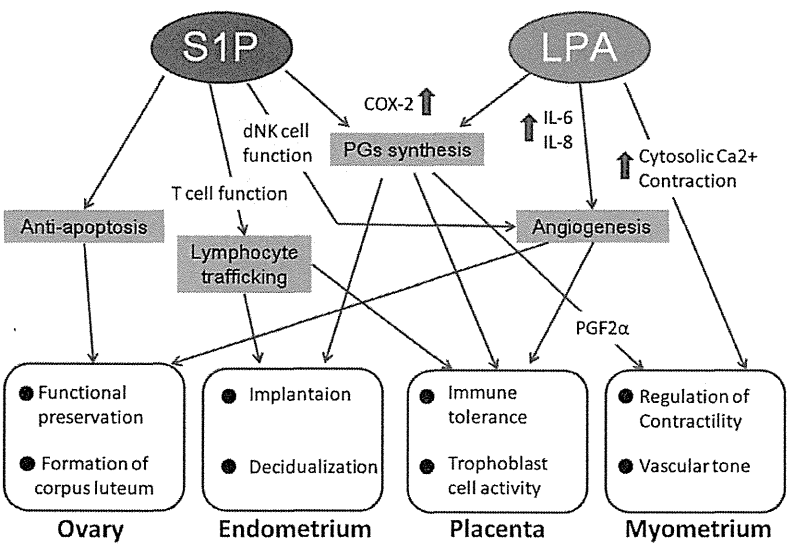

Fig. 4 Proposed roles for S1P and LPA in pregnancy.

of muscle contraction. LPA enhances the elevations in cytosolic free Ca^{2+} that occur in response to mechanical stress in smooth muscle cells isolated from guinea pig ileum.[67] In vitro oxytocin and LPA exposures promote Rho-kinase intracellular signaling and stress fiber formation in human uterine myometrial cells.[68] In pigs, LPA receptor agonists increase uterine myometrial contractile tension.[69] The amplitude and the frequency of this effect vary according to the types of expressed LPA receptors and depend on the presence or absence of embryos.[69] In a classic work by Tokumura et al.,[70] intravenously administered LPA increased intrauterine pressure in rats, a biologic effect similar to that of PGF2α.

The potential involvement of S1P signaling in labor has been studied in human amnion. The enzymatic activity of SPHK1 generates S1P and this activity is higher in post-labor amnion than in pre-labor amnion. The same was not true for SPL, an enzyme that inactivates S1P.[71] PGs and endothelin-1 produced from amnion are known enhancers of uterine contractility.[72,73] S1P increases the expression of prostaglandin endoperoxide synthase 2 (COX2) as well as endothelin-1 in amnion cells.[74,75] This is consistent with the hypothesis that the promotion of S1P production in the amnion at term may be one of the triggers for labor onset.

Evidence directly linking lysophospholipid pathways and mediators to pathologic labor is limited. In endometrial cells, bacterial cell wall components stimulate phospholipase A2 (PLA2)-mediated degradation of phospholipids into lysophospholipids and fatty acids.[76,77] In addition, studies on chronic inflammatory disorders demonstrate enhanced activity of several phospholipid metabolizing enzymes, including LPA2 and ATX.[1] Intrauterine infection is a major cause of preterm labor. The mechanisms for this may be several and involvement of LPA mediators is enticing. Preterm intrauterine infection may amplify local phospholipid degradation and thereby increase the local supply of the prostaglandin precursor, arachidonic acid. It may simultaneously augment LPA production and stimulate COX2 expression as described above. Together, this would facilitate local inflammatory PG synthesis, which would predictably result in preterm uterine contractions.

Summary

Evidence supporting the participation of the LPA and S1P systems in a wide spectrum of reproductive processes is rapidly accumulating (Fig. 4) and it is becoming increasingly clear that these and other lysophospholipid mediators exert important and elaborate effects on uteroplacental function. In fact, appropriate activity of these novel intercellular signaling molecules may be indispensable for healthy fetal growth and delivery. The biologic outcomes of lysophospholipid signaling are determined by tissue-specific receptor expression and the local availability of lysophospholipid-metabolizing enzymes. The actions of LPA signaling pathways and molecules during implantation and parturition are closely associated with prostaglandin synthesis. Although not fully elucidated, S1P-mediated regulation of vascular function and immune cell trafficking may be essential to establish an adequate uteroplacental microvasculature and to develop essential immune tolerance at the fetomaternal interface. Future work in this field should clarify the involvement of the lysophospholipid system in the pathogenesis of pregnancy disorders and aid in the discovery of novel therapeutic approaches for these disorders.

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Safety and efficacy of preoperative autologous blood donation for high-risk pregnant women: Experience of a large university hospital in Japan

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Abstract

Aim: Preoperative autologous blood donation (PAD) has the advantages over allogeneic blood transfusion of theoretically no risk of viral infection and alloimmunization. However, there are some concerns regarding PAD in pregnant women, as they sometimes become anemic and adverse effects such as low blood pressure could be harmful to fetuses. In our hospital, the PAD program was implemented in 2006 and has been used in pregnant women at high risk of massive hemorrhage. In this study, the safety of PAD in pregnant women and its efficacy for avoiding allogeneic blood transfusion were investigated.

Methods: The hospital records of pregnant women who delivered at our hospital from January 2009 to June 2012 were reviewed and those who were enrolled in the PAD program for predicted massive hemorrhage were analyzed.

Results: Among the total of 3095 deliveries, 69 cases enrolled in the PAD program were analyzed. Blood donation was performed 189 times for the 69 cases. The median donated blood volume was 1200 mL (range, 400–2000). The mean blood loss during delivery was 1976 ± 1654 mL. Autologous blood was transfused in 64 cases. Allogeneic blood transfusion was required in five cases of massive blood loss exceeding 5000 mL. In the other 64 cases, no additional allogeneic blood transfusion was required. No adverse events were observed in either the pregnant women or fetuses.

Conclusion: For pregnant women at a high risk of massive hemorrhage, our PAD program was safe and effective for avoiding allogeneic blood transfusion.

Key words: allogeneic, autologous, hemorrhage, pregnancy, transfusion.

Introduction

In many countries, maternal death rates have been dramatically reduced by some reasons such as increasing education level and improvement of the health-care environment.[1–4] Maternal hemorrhage remains a major concern for maternal health, as hemorrhage during delivery can be unpredictable and uncontrollable.

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In pregnancy with placenta previa, life-threatening massive hemorrhage occasionally occurs during cesarean section. Although transfusion is an important treatment modality for massive hemorrhage, allogeneic blood transfusion is associated with the risk of viral infection transmission, alloimmunization and non-hemolytic transfusion reactions dependent on the infusion of allogeneic antigens. Although the risk of blood-borne viral infection, particularly hepatitis B virus, hepatitis C virus and HIV, has significantly decreased in recent years following the implementation of the nucleic acid test by the Japanese Red Cross Blood Center (JRCBC), the risk of transmission of emergent or new viruses, which are not routinely tested, remains. In addition, while the JRCBC has implemented universal leukocyte reduction of all blood products, it is impossible to completely eliminate the risk of alloimmunization against red cells, platelets or leukocyte antigens due to allogeneic transfusion. Alloimmunization may induce hemolytic disease of the newborn, neonatal alloimmune thrombocytopenia or neonatal alloimmune neutropenia. On the other hand, the risk of graft-versus-host disease, which has a higher incidence in Japan compared to other countries, decreased tremendously after the JRCBC started the provision of irradiated blood in 1999.[5]

In addition to these concerns regarding the safety of allogeneic blood transfusion, it is predicted that a blood shortage will occur in the near future in Japan, owing to the declining birth rate, aging population and consequent decrease in the number of potential blood donors. Therefore, the Ministry of Health, Labor and Welfare of Japan adopted a plan to encourage the use of autologous blood. Through the specific Law and Guidelines, the Ministry created rules to provide a strong incentive by payment of autologous blood collection and transfusion, which has been effective. In our hospital, preoperative autologous blood donation (PAD) was introduced approximately 20 years ago, mainly in the field of orthopedics, cardiovascular surgery, and gynecology. We previously reported on modified PAD for malignant gynecologic surgery, in which autologous plasma was doubly collected.[6]

To date, a series of reports have demonstrated the safety and efficacy of PAD during pregnancy.[7–12] In our hospital, we have been using PAD for pregnant women at high risk of massive hemorrhage, such as those with placenta previa, since the mid-1990s. However, PAD in pregnant women requires a guarantee of safety for the mother and fetus. Thus, the establishment of an adequate PAD program is essential. The following topics should be carefully considered: (i) the minimal hemoglobin level necessary for PAD; (ii) the appropriate volume and interval of blood collection; (iii) the ideal gestational age to start the collection; (iv) the method of preservation of autologous blood; and (v) the system for the 24-h provision of autologous blood. In our hospital, the PAD program was fully implemented in 2006, in which the specialists in blood transfusion, specialized nurses and medical technologists perform all of the steps of PAD, including patient consultation, blood collection, labeling, control and provision of autologous blood.[13]

However, in recent years, the use of PAD in pregnant women has been re-evaluated because of some weak points. Blood collection is started weeks before delivery, but emergent events such as bleeding often break the schedule. The optimal volume of autologous blood-stock has not been established for pregnant women at high risk of massive hemorrhage. Blood collection from pregnant women may cause hypotension and anemia, which may result in placental insufficiency and could harm the fetus. In fact, some authors consider it inappropriate to use PAD in pregnant women.[14,15]

In the present study, we aimed to validate our present PAD protocol for pregnant women at risk of massive hemorrhage and to propose an ideal PAD program, focusing particularly on the safety of PAD in pregnant women and its efficacy in avoiding allogeneic blood transfusion.

Methods

Patients

The hospital records of pregnant women who had delivered at our hospital from January 2009 to June 2012 were reviewed, and those enrolled in the PAD program were selected. Among these patients, those at high risk of massive hemorrhage during pregnancy and/or delivery, including those with placenta previa, placenta accreta and uterine fibroma, were analyzed. The cases enrolled in the PAD program because of rare blood group type or the presence of irregular antibodies were excluded from this study, as they were not at high risk of massive hemorrhage.

Autologous blood collection

Autologous blood was collected in polyvinyl chloride bags plasticized with bis(2-ethylhexyl) phthalate, containing the preservative citrate phosphate dextrose adenine, which allowed for the preservation of blood

for 35 days at 4°C. The collected blood was separated into red cell concentrate (RCC) and fresh frozen plasma (FFP), and the RCC was preserved refrigerated (4–6°C) for a maximum of 35 days. If the cesarean section was not planned before expiration of RCC for medical reasons, the RCC was converted into frozen preservation.

In the PAD program of our hospital, all the steps of PAD were performed by experienced doctors in the blood transfusion department, monitored by specialized nurses and medical technologists. For pregnant women, the obstetrician continuously monitored the fetus by cardiotocogram. To avoid abrupt circulatory volume changes, blood withdrawal was performed slowly, without a tourniquet, taking more than 10 min for each collection. The minimum interval of each blood collection was 1 week to allow for partial recovery of the anemic state, and the cesarean section was planned within 5 weeks from the first blood collection, but not within 1 week from the last collection.

For the control of anemia, p.o. iron administration was initiated prior to the first blood collection, and i.v. iron administration was added just after each blood collection. In case the blood withdrawal-dependent anemia did not recover to meet the prerequisites for the next blood collection, the use of erythropoietin was considered.

PAD program for pregnant women

In cases other than pregnant women, the maximum volume of blood collection per time is 400 mL (2 units) when the following prerequisites are fulfilled: hemoglobin (Hb) concentration of 11.0 g/dL or more and bodyweight of 50 kg or more. In cases of bodyweight of less than 50 kg, the total circulatory volume is estimated from the patient's height and bodyweight, and a maximum of 13% of this volume is allowed per time. For pregnant women, we established a minimal Hb level of 10.0 g/dL as the allowable value, because pregnant women tend to present slight anemia and sometimes do not achieve a Hb level of 11.0 g/dL. Patients with bodyweight of less than 50 kg required more frequent visits to the hospital for PAD.

The collection schedule was generally planned to start at 32–33 weeks of gestation and the cesarean section was planned at 37–38 weeks, before the expiration date of the collected autologous RCC. For cases with total/partial previa, low-lying placenta and suspected placenta accreta, the total volume of autologous blood scheduled to be collected was 1200 mL. In cases in which the risk of emergent hemorrhage during pregnancy was considered to be high and emergent termination of pregnancy was predicted, autologous blood collection was started at 30–31 weeks of gestation. If 1200 mL of blood was collected and emergent events had not occurred, an additional 400 mL or 800 mL of autologous blood collection was considered if the patient's general condition met the aforementioned prerequisites. For those cases at risk of moderate hemorrhage during delivery, a maximum of 800 mL of autologous blood was scheduled.

Results

From January 2009 to June 2012, 3095 deliveries occurred at the University of Tokyo Hospital. Among these cases, PAD was applied in 70 (2.3%), consisting of 30 total previa, seven partial previa, 19 low-lying placenta, two suspected placenta accreta, one vasa previa, six uterine fibromas, one adenomyosis, two cervical carcinoma, one intrapelvic arteriovenous aneurysm in Klippel–Trenaunay–Weber syndrome and one patient who was positive for high-titer anti-Jra antibody. Excluding the case with the irregular antibody, the 69 cases of predicted massive hemorrhage during delivery were analyzed retrospectively (Table 1).

The mean age (±standard deviation [SD]) of the 69 cases was 35.1 ± 4.1 years. Sixty cases (87.0%) were delivered by cesarean section and nine (13.0%) vaginally (Table 1). One patient with partial previa and seven with low-lying placenta tried and succeeded in vaginal delivery. In another case of low-lying placenta, vaginal delivery was attempted, but vaginal bleeding

Table 1 Mode of delivery of 69 pregnant women enrolled in the preoperative autologous blood donation program

Diagnosis	No. of cases ($n = 69$)	Cesarean section ($n = 60$)	Vaginal delivery ($n = 9$)
Total previa	30	30	0
Partial previa	7	6	1
Low-lying placenta	19	12	7
Suspected placenta accreta	2	2	0
Vasa previa	1	1	0
Uterine fibroma	6	6	0
Adenomyosis	1	0	1
Cervical carcinoma	2	2	0
Intrapelvic arteriovenous aneurysm	1	1	0

exceeded 500 g during delivery and emergent cesarean section was performed. Vaginal delivery was also tried in one case of uterine fibroma, but the progression was insufficient because of weak uterine contraction and cesarean section was performed. In cases of low-lying placenta and partial previa, the mode of delivery was re-evaluated during pregnancy progression and vaginal delivery was considered feasible if the distance between the internal cervical os and the lower end of the placenta was more than 2 cm. In cases of uterine fibroma and adenomyosis, the mode of delivery was discussed and decided in individual cases, considering the size and the position of the fibroma nodules and past history of myomectomy.

A total of 189 blood donations were performed in the 69 cases (mean, 2.7 times/patient). The mean gestational age (±SD) at delivery of the total 69 cases was 37.4 ± 2.0 weeks and that of the 60 cesarean section delivery cases was 37.0 ± 1.8 weeks (Table 2). In some cases, cesarean section was performed at 35–37 weeks of gestation because of bleeding during pregnancy ($n = 10$), threatened premature labor ($n = 5$), non-reassuring fetal status ($n = 2$) and premature rupture of membrane ($n = 2$). In two cases of cervical cancer, cesarean section was performed at 28 and 29 weeks, respectively. The median donated blood volume was 1200 mL (range, 400–2000). In 12 cases, the donated blood volume exceeded the usual 1200 mL: 1400 mL in one case, 1600 mL in eight cases and 2000 mL in three cases. The mean gestational age (± SD) at blood collection initiation, the number of blood collections, and the mean Hb concentration (±SD) at blood collection initiation were 33.2 ± 2.0 weeks, 3 (range, 1–5) and 11.1 ± 0.7 g/dL, respectively (Table 3). The mean Hb concentration (±SD) at delivery was 10.8 ± 0.9 g/dL in 68 cases, which were checked 3.3 ± 3.2 days before delivery. One patient with low-lying placenta delivered vaginally 33 days after the second blood collection without having the Hb recovery assessed. In five cases, 24 000 IU of erythropoietin was administrated s.c. 1–4 times along with an iron prescription.

The mean blood loss (±SD) during delivery among the 69 total cases was 1976 ± 1654 mL, and that of the 37 total or partial previa cases was 2328 ± 1940 mL (Table 2). In this study, blood loss was calculated by adding aspirated blood with a suction device and gain in gauze weight. Therefore, the count included the amniotic fluid volume. The patient distribution is shown in Figure 1. Autologous blood was transfused in 64 cases (92.8%). Among the pre-deposited autologous blood, 75.1% of the RCC and 54.5% of the FFP were transfused. In the five cases with blood losses below 1000 mL (110, 370, 430, 562 and 920 mL), transfusion of autologous blood was not required. In the other 14 cases with blood losses below 1000 mL, autologous blood was transfused according to the patient's general status and Hb levels, intra- or postoperatively. In these 14 cases, 69.4% of the RCC and 33.3% of the FFP were transfused. In our hospital, we usually consider allogeneic blood transfusion when the blood loss exceeds

Table 2 Delivery outcomes

Mode of delivery		
Cesarean section	60 (87.0%)	
Vaginal delivery	9 (13.0%)	
Gestational age at delivery (weeks)	37.4 ± 2.0	(mean ± SD)
Blood loss during delivery (mL)	1976 ± 1654	(mean ± SD)
Total/partial previa ($n = 37$)	2328 ± 1940	(mean ± SD)

SD, standard deviation.

Table 3 Data of autologous blood donation during pregnancy ($n = 69$)

Gestational age at blood donation initiation (weeks)	33.2 ± 2.0	(mean ± SD)
Volume of donated blood (mL)	1200, 400–2000	(median, range)
No. of blood donations (times)	3, 1–5	(median, range)
Hemoglobin concentration (g/dL)		
at the beginning of donation ($n = 69$)	11.1 ± 0.7	(mean ± SD)
at delivery ($n = 68$)	10.8 ± 0.9	(mean ± SD)
after transfusion of autologous blood only ($n = 59$)	10.1 ± 1.4	(mean ± SD)
after delivery of cases that did not receive autologous blood ($n = 5$)	10.1, 9.0–12.5	(mean, range)
Vasovagal reflex	0/189 donations	
Hypotension during blood donation	0/189 donations	
Non-reassuring fetal heart rate during blood donation	0/189 donations	
Aggregate formation in the bag	0/189 donations	

SD, standard deviation.

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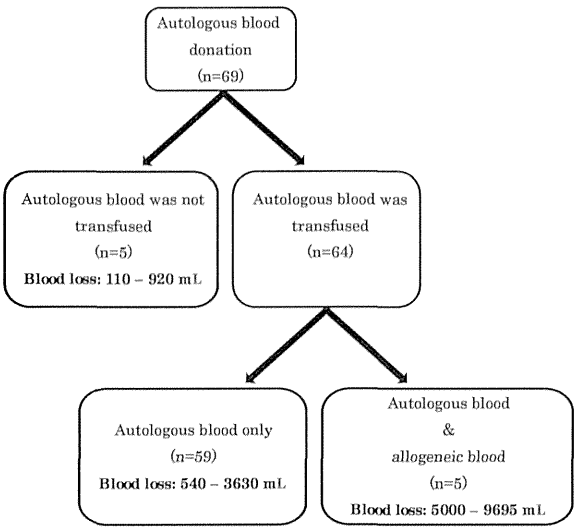

Figure 1 Patient distribution during the study period.

2000 mL or the shock index (pulse rate/systolic blood pressure) exceeds 1.0. Final decisions regarding transfusing allogeneic blood were made after taking into account the situation of the operation, Hb concentrations before and during the operation, and the general status of the patients. When autologous blood was available, we did not hesitate to transfuse it when the operators observed any signs of severe bleeding, even when the patient's general condition did not meet the aforementioned transfusion criteria.

All five cases with blood loss exceeding 5000 mL received allogeneic blood in addition to autologous transfusion, but no other cases required allogeneic blood (Table 4). Allogeneic blood transfusion was avoided in 92.2% (59/64) of patients that received transfusion. Among the five patients that received allogeneic blood, four had total previa, all of whom required hysterectomy and were pathologically diagnosed with placenta increta or accreta. Placenta accreta was suspected by ultrasound and magnetic resonance imaging in the case with a blood loss of 5400 mL, but hysterectomy was not performed because the active bleeding from the endometrium was finally controlled by suture of the uterine myometrium and intrauterine gauze tamponade. Pathological examination of the placenta suggested placenta accreta. Allogeneic blood transfusions were not required in the three cases with blood losses between 3000 and 3999 mL, as well as the 16 cases with blood losses between 2000 and 2999 mL (Table 5).

Table 4 Characteristics of the five cases requiring allogeneic blood in addition to autologous blood transfusion

Blood loss (mL)	Diagnosis	Pathological diagnosis	Procedure	Autologous blood transfusion (mL)	Red cells (units†)	Fresh frozen plasma (units†)	Platelet concentrate (units†)
9695	Total previa	Total previa, placenta increta	Hysterectomy	2000	24	16	30
8750	Total previa	Total previa, placenta increta	Hysterectomy	1600	18	10	20
5400	Suspected placenta accreta	Placenta accreta	Cesarean section	1200	4	4	20
5195	Total previa, twice of previous C/S	Total previa, placenta accreta, twice of previous C/S	Hysterectomy, perioperative balloon occlusion of the internal iliac artery	1200	4	4	0
5000	Total previa	Total previa, placenta increta	Hysterectomy	400	4	4	0

†One unit of red cells/fresh frozen plasma originates from 200 mL of whole blood. C/S, cesarean section.

Table 5 Characteristics of the 19 cases with blood loss ranging between 2000 and 3999 mL

Blood loss (mL)	Diagnosis	Donated volume of autologous blood (mL)	Autologous RCC transfusion (mL)	Autologous FFP transfusion (mL)	Hb after transfusion of autologous blood (g/dL)
3630	Uterine fibroma	800	800	800	8.6
3350	Cervical carcinoma	800	800	800	7.9
3050	Total previa	1200	1200	1200	8.2
2900	Total previa	1600	1600	1600	9.9
2900	Low-lying placenta	1600	1600	1600	8.7
2770	Total previa	1200	1200	800	9.5
2660	Uterine fibroma	800	800	800	5.9
2650	Total previa	1600	1600	800	10.5
2600	Uterine fibroma	800	800	800	10.1
2520	Low-lying placenta	1200	1200	1200	11.2
2470	Total previa	1600	1200	800	11
2450	Total previa	1200	1200	400	8.3
2380	Total previa	800	800	800	8.3
2370	Total previa	1200	1200	0	8.9
2230	Partial previa	1200	800	400	10.9
2200	Low-lying placenta	800	800	800	10.7
2110	Total previa	1400	1050	1050	8.4
2060	Total previa	1600	1200	1600	8.3
2004	Low-lying placenta	800	400	400	10

All 19 cases with blood loss between 2000 and 3999 mL could be managed only with autologous blood. Without preoperative autologous blood donation, many of them might have received allogeneic blood. FFP, fresh frozen plasma; Hb, hemoglobin; RCC, red cell concentrate.

No adverse events, such as vasovagal reflex, hypotension and non-reassuring fetal heart rate on cardiotocogram, were observed during the 189 blood donation sessions. Neither bacterial contamination nor aggregate formation in the bag, which renders the transfusion unfeasible, was observed by visual examination in this study. Neonatal outcomes were uneventful in all cases.

Discussion

Autologous blood transfusion is an ideal alternative to overcome the infectious and immunological adverse events associated with allogeneic blood transfusion. It can be performed perioperatively using different methods, including acute normovolemic hemodilution (ANH), intraoperative blood salvage (IBS) and PAD. ANH consists of autologous blood withdrawal just before surgery, usually in the operating room, followed by administration of crystalloid and colloid fluids. Thus, the maximum collectable blood volume is limited, and is therefore inadequate for emergent surgeries such as sudden bleeding in pregnant women with placenta previa. The IBS technique, in which shed blood is retrieved from the surgical field, washed and returned to the patient, is applicable for emergent operations. There have been some concerns regarding contamination of fetal cells and amniotic fluid when using IBS for pregnant women. Thus, leukocyte-reduction filters are used during reinfusion and anti-D immunoglobulin is administrated post-partum to Rh(D)-negative women.[16] To reduce the risk of contaminating fetal components, some technical procedures have been applied in IBS.[17] Salvage begins after the removal of placenta and amniotic fluid, and salvaged blood cells are washed thoroughly. In recent years, the safety and efficacy of IBS have been reported and accepted in the field of obstetrics. In Japan, however, IBS has not been widely adopted by obstetricians, possibly owing to the lack of specialized equipment and trained medical engineering technicians for IBS in Japan.

In fact, PAD has been widely introduced in the obstetric field in Japan. However, in recent years, the efficacy of PAD in avoiding allogeneic blood usage, the cost/benefit of reducing the allogeneic blood supply, the wastage of autologous blood and its potential side-effects have been re-evaluated, and in some Western countries, there is a tendency to abandon its routine use.[14,15] The American College of Obstetricians and Gynecologists noted that the role of PAD during pregnancy is limited to cases of rare antibodies because it is often difficult to anticipate the need for transfusion in pregnant women at risk of massive hemorrhage, and

pregnant women are often too anemic for autologous blood to be collected.[18] The Royal College of Obstetricians and Gynecologists (RCOG) does not recommend PAD during pregnancy because of concerns regarding placental insufficiency.[19] Instead, the RCOG recommends cell salvage. Thus, a converse phenomenon is observed in Western countries and Japan. In Japan, there is a strong tendency to promote the use of autologous blood transfusion, even in obstetrics, for societal, political and economic reasons. The costs of PAD and autologous blood transfusion are now covered by the Japanese national insurance system, and the government strongly advocates its application, which is also stipulated in the Blood Law (the law for the guarantee of stable provision of safe blood products) enacted in 2003 and in the guidelines released by the government.

While PAD is widely accepted by obstetricians in Japan, there is no standardized protocol for PAD in pregnant women, which we considered essential. Thus, we founded the PAD program in 2006 in our hospital. In 2011, Watanabe *et al.* reported the feasibility and safety of PAD in pregnant women[12] and proposed that placenta previa may be a good indication for PAD, as the autologous blood re-transfusion rate was the highest (42.4%) in this group of patients in their study. In our study, we aimed to elucidate whether PAD is effective in avoiding allogeneic blood transfusion by focusing on the amount of blood loss. We also tried to confirm the safety of PAD for both pregnant women and the fetuses. We retrospectively analyzed the obstetric patients at risk of massive hemorrhage who received PAD under our PAD program, in an attempt to validate the program and, if possible, to propose an ideal protocol for the obstetric field.

Watanabe *et al.* stored collected autologous blood as whole blood.[12] In our PAD program, RCC and FFP were preserved separately, because frozen plasma contains active coagulation factors that would be of great use in obstetric massive hemorrhage. Our present PAD protocol for obstetric patients is as follows: (i) start PAD at 32–33 weeks of gestation; (ii) perform cesarean section (or vaginal delivery, when feasible) at 37–38 weeks of gestation; (iii) Hb level of 10.0 g/dL or more, a sine qua non condition for blood collection; (iv) the amount of blood collection per time decided according to the patient's bodyweight (bodyweight 50 kg or more enables collection of 400 mL of blood per time); (v) a total of 800 mL of blood collected for those at moderate risk and 1200 mL for those at high risk of massive bleeding; (vi) preservation of RCC and FFP separately; (vii) starting p.o. administration of iron prior to blood collection and administrating iron i.v. just after blood collection; and (viii) administrating erythropoiesis-stimulating agents only when strictly necessary. This protocol allows for the preservation of autologous RCC refrigerated (4°C) for up to 35 days, thus autologous RCC can be provided 24 h a day, even in an emergent situation. If the storage period exceeds 35 days because of medical reasons, autologous RCC is converted to frozen preservation. In such cases, the department of transfusion medicine provides an on-call system for the thawing of frozen-preserved blood. In Watanabe *et al.*'s protocol, pregnant women who were scheduled to deliver by spontaneous onset of labor began donating blood at 36 weeks of gestation.[12] However, in cases of partial previa and low-lying placenta, the mode of delivery was finally decided at approximately 36 weeks, depending on the distance between the placental edge and internal cervical os. In this study, 18 of 26 cases finally delivered by cesarean section. If autologous blood collection was started after cesarean section was selected in these 18 cases, the optimum blood volume may not be achieved.

By analyzing the 69 cases enrolled in this study, we demonstrated that autologous blood collection could be safely performed in pregnant women without any adverse events. Anemia tends to be severe in pregnant women after blood collection, and the next collection was sometimes postponed for 1 or 2 weeks. However, we believe that the anemia was controlled by p.o. or parenteral iron administration, because the scheduled autologous blood volume was achieved eventually in all cases. Other risks of PAD were not observed in this study, such as a drop in blood pressure, vasovagal reflex and non-reassuring fetal status during the procedure.

Autologous blood was transfused in 64 cases (92.8%, 64/69). From another point of view, 75.1% of the RCC and 54.5% of the FFP were transfused, showing a wastage rate of approximately 25% for RCC and 45% for FFP. Nineteen cases (27.5%, 19/69) had blood loss below 1000 mL, of which five cases (26.3%, 5/19) did not require blood transfusion, whereas all five cases (7.2%, 5/69) with blood loss exceeding 5000 mL required additional allogeneic blood. For the five cases of massive hemorrhage, we consider that transfusion of allogeneic blood was unavoidable regardless of autologous blood storage. Nineteen cases (27.5%, 19/69) with blood loss between 2000 and 3999 mL received only autologous blood and did not require additional allogeneic blood. In Watanabe *et al.*'s article, only 17.8% of the autologous blood was transfused.[12]

Their policy for transfusing autologous blood was the same as that for allogeneic blood, as they stated that autologous blood transfusion should not be performed when the vital signs were stable for the risk of side-effects such as bacterial contamination. However, in this study, we did not hesitate to use autologous blood if the operators observed any signs of severe bleeding, because of the safety of viral transfection and alloimmunization. We have never experienced bacterial contamination. The aim of this study was to evaluate the efficacy of PAD in avoiding allogeneic blood transfusion. The strict criteria for using autologous blood did not affect our results.

The most important observation in this study is that the 19 cases of blood loss between 2000 and 3999 mL could be managed only with autologous blood, as many of these cases might have received allogeneic blood if autologous blood had not been prepared. From a different point of view, our PAD protocol was effective for avoiding allogeneic blood transfusion when the blood loss did not exceed 4000 mL. In the remaining 45 cases with blood losses below 2000 mL, bleeding could be adequately controlled, but we believe that PAD was useful also for this group because 40 cases received all or part of the pre-deposited autologous blood. If the autologous blood had not been available, most of these 40 cases might have gone through post-partum without transfusion; however, the transfusions of autologous blood certainly promoted their recovery.

In addition to the efficacy for avoidance of allogeneic blood transfusion, the wastage rate of pre-deposited autologous blood also shows the effectiveness of the PAD program. Our data revealed a relatively high wastage rate, especially for FFP. Considering that the indication of transfusing autologous blood usually is not as strict as transfusing allogeneic blood, the wastage rate of both RCC and FFP would be higher, which demonstrates that an excessive volume of autologous blood is being pre-deposited in our PAD program and suggests the need for re-evaluation of an appropriate collection volume. However, it is sometimes difficult to predict the amount of bleeding and thereby the necessary autologous blood volume to be pre-deposited. When the equipment and personnel needed for cell salvage are available, smaller volumes of pre-deposited autologous blood may be sufficient in combination with the salvaged blood.

In conclusion, PAD during pregnancy is safe for both pregnant women and fetuses, if it is performed in an institution with a fully implemented PAD program. In cases of high-risk pregnancy, including placenta previa, our PAD program was effective in avoiding allogeneic blood transfusion when the blood loss did not exceed 4000 mL. Thus, our protocol is effective in the present form, but there is need to carefully analyze the individual cases in an attempt to select those who will not require blood transfusion and to reduce autologous blood wastage.

Disclosure

The authors report no conflict of interest.

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