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ORIGINAL ARTICLE

Lysophosphatidylcholines: Bioactive Lipids Generated During Storage of Blood Components

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Abstract Transfusion-related acute lung injury (TRALI) is suggested to be a "two hit" event, resulting from priming and activation of pulmonary neutrophils. It is known that neutrophil activation may result from infusion of lysophosphatidylcholines (LysoPCs) accumulated during storage of blood components. The aim of our study was to verify whether the LysoPCs are released into the storage medium of blood components. We measured the LysoPCs concentration in the supernatants from stored apheresis platelet concentrates (PLTs), packed non-leukoreduced red blood cell concentrates (RBCs), leukoreduced red blood cell concentrates (L-RBCs), fresh frozen plasma (FFP) and donor plasma (control). Lipids were separated on high-performance thin-layer chromatography, detected by primulin spray and quantified by photodensitometric scanning. The LysoPCs concentration in donor plasma was similar to that in FFP. During storage the LysoPCs content in PLTs increased almost two-fold as compared to the fresh isolated platelets. In RBCs and L-RBCs the LysoPC level was very low or below detection limit and did not increase throughout the storage period. According to our observations bioactive LysoPCs may be considered a neutrophil-activating factor only following PLT transfusions but not RBCs transfusions.

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Abbreviations

CPDA-1	Anticoagulant (citrate, phosphate, dextrose, adenine)
FFP	Fresh frozen plasma
GMP	Good manufacturing practice
HLA	Human leukocyte antigen
HNA	Human neutrophil antigen
HPTLC	High-performance thin-layer chromatography
LysoPCs	Lysophosphatidylcholines
L-RBCs	Leukoreduced red blood cell concentrates
PLTs	Platelet concentrates
RBCs	Red blood cell concentrates
SAGM	Additive solution (sodium chloride, adenine, glucose, mannitol)
TRALI	Transfusion-related acute lung injury

Introduction

Transfusion-related acute lung injury (TRALI) is the most common cause of transfusion-related morbidity and mortality (Bux 2005; Goldman et al. 2005; Holness et al. 2004; Silliman and McLaughlin 2006; Silliman et al. 2009; Stainsby et al. 2006; Van Stein et al. 2010). The exact mechanism of TRALI is not yet completely understood but in most TRALI cases an immune antibody-mediated mechanism has been implicated. It was first postulated in 1970's by Ward (1970) and by Thompson et al. (1971) but the first extended clinical studies were reported by Popovsky et al. (1983; Popovsky and Moore 1985). Numerous reports have documented the presence of human leukocyte antigen (HLA)-specific or granulocyte antibodies in plasma of

donors of the implicated blood components (Kopko et al. 2002; Maślanka et al. 2007; Reil et al. 2008; Żupańska et al. 2007). In some reported cases however no antibody was identified therefore an alternative "two hit" mechanism was postulated. The "first hit" is provoked by a pro-inflammatory condition, e.g. pneumonia, sepsis and causes activation of lung endothelium with subsequent sequestration and priming of neutrophils. The "second hit" is provided by transfusion of a blood component that contains either antibodies (anti-HLA, anti-HNA) or biologically active lipids (lysophosphatidylcholines: LysoPCs) and cytokines. The age of blood components has also been found significant for TRALI pathogenesis; first confirmed in a clinical cohort of 90 TRALI cases by Silliman et al. (2003b). The "two hit" model is supported by experimental studies with bioactive lipids as well as outdated blood products to cause TRALI after a "first hit" (Silliman et al. 1994, 1996, 1998).

Discrepant results however have been reported in a recent study (Sachs et al. 2010; Vlaar et al. 2010b, 2011b) of the effect of LysoPCs in stored blood products on the activity of lung neutrophils. In our study therefore we compared the LysoPCs content in supernatants from stored platelet and packed red blood cell concentrates (RBCs) with the LysoPCs in plasma of random donors.

Materials and Methods

The LysoPCs content was measured in the supernatant samples from storage bags containing: apheresis platelet concentrates (PLTs), 39 samples; packed (non-leukoreduced) RBCs from whole blood collected onto CPDA-1, 62 samples, leukoreduced RBCs (L-RBCs) ($<1 \times 10^6$ white bloods cells) collected in Atreus System suspended in SAGM additive solution, 40 samples; fresh frozen plasma (FFP) derived from single-donor apheresis donations, 10 units and in plasma samples from 40 random donors. Blood was collected from donors and subjected to quality control according to Polish "Medical Standards for collection, preparation and distribution of blood and blood components" based on the recommendations of the Council of Europe (2010). RBC concentrates were stored at 2–6°C, PLTs at 20–22°C on horizontal flatbed shaker (30 cycles/min; Helmer; USA). The cell-free supernatant samples were obtained by centrifugation of 2 ml of blood components, for 20 min at 1,550 g then analyzed at various storage periods.

Lipids extraction was performed with modified Bligh and Dyer procedure, under acidic conditions for effective LysoPCs recovery (Weerheim et al. 2002, Vlaar et al. 2010a, b, 2011a, b). In short, 100 µl of supernatant from stored blood components or 100 µl of plasma were extracted. The separated chloroform layers were combined and dried. Samples were dissolved in 500 µl of methanol: chloroform mixtures (2:1 v/v) for further analysis. Lipids were separated on one-dimensional high-performance thin-layer chromatography (HPTLC) using silica gel G coated plates (10 × 20 cm) without fluorescent indicator (Merck, Ref. 5721). Lipid samples (of 100 µl each; i.e. equivalent of lipid extracted from 20 µl of plasma or supernatant from blood components) were deposited on plates as 5 mm streaks. The 12 lipid samples were applied on one plate: 8 spots of analyzed samples together with 4 spots of LysoPC in the range 250–2,500 ng as a standard curve. As a standard L-α-LysoPCs from egg yolk (approx. 99% purity, Sigma, Ref. 4129), containing predominantly LysoPC 16:0 or LysoPC 18:0 were used. Subsequently, lipids were separated by chloroform:methanol:water (65:35:8; v/v/v). Phospholipids were detected after uniform spraying of the plate with 0.005% (w/v) primulin solution in acetone: water (4:1; v/v); (5 mg in 100 ml of acetone/water, 80:20, v/v). The fluorescence intensity of the LysoPCs spots in UV light was analyzed by photodensitometric scanning (Gel Doc 2000, BioRad) and quantified using Quantity One software version 4 (BioRad). Applying HPTLC procedure of LysoPCs quantification gives a detection limit of less than 20 µM LysoPC.

Statistical Analysis

Data are expressed as mean ± SD. The significance of differences was assessed using analysis of variance (ANOVA), the least significant difference test and Student's t test. Values of $p < 0.05$ were considered significant.

Results

The LysoPCs content in control donor plasma samples (159.6 ± 33.3 µmol/L, range 77.3–178 µmol/L) was similar to that in FFP (186.2 ± 54.9 µmol/L, range 119.2–216.9 µmol/L).

The LysoPCs content in the supernatants from platelet concentrates on day 0 was within the same range as in donor plasma. After 1 day of storage it significantly increased ($p < 0.05$) and remained on the same level until the expiration day (Table 1).

In non-L-RBCs collected between day 0 and day 10, the average LysoPCs concentration was lower than in donor plasma ($p < 0.05$) (in five samples the LysoPCs concentration was below detection limit). From storage day 11 to 40, the LysoPCs content decreased and was found below detection limit in 28 samples (Table 2).

The LysoPCs amount in L-RBCs during the whole storage period was below detection limit in 33 samples and extremely low in 7 (Table 2). According to statistical

Table 1 Lysophosphatidylcholines (LysoPCs, μmol/L) level in supernatants of stored platelet concentrates (PLTs)

Storage time (days)						
0	1	2	3	4	5	6
161.4	224.4	276.3	304.2	324.2	332.6	323.8
121.1	145.6	180.7	200.7	201.7	198.6	197.8
177.0	274.9	307.7	338.1	363.6	368.1	419.2
	338.5	492.8	297.4	339.0	209.2	
	363.2	460.2	463.1	298.9	220.5	
	344.8	339.0			234.2	
	272.5	433.5			410.4	
		336.6				
		296.5				
153.2 ± 28.8[a]	280.6 ± 77.2	347.0 ± 99.0	320.7 ± 94.5	307.1 ± 72.1	281.9 ± 86.4	313.6 ± 111.3

[a] Mean ± SD

Table 2 Lysophosphatidylcholines (LysoPCs, μmol/L) level in supernatants of stored packed red blood concentrates (RBCs)

Storage time (days)							
Non-leukoreduced RBCs				Leukoreduced RBCs			
0–10	11–20	21–30	31–40	0–10	11–20	21–30	31–40
102.7	90.2	79.8	66.9	47.9	42.1	30.5	21.7
104.4	156.4	61.0	118.3	42.3	22.3	19.1	*BDL*
160.1	198.6	150.3	59.7	*BDL*	*BDL*	*BDL*	*BDL*
157.1	78.2	127.7	*BDL*	*BDL*	*BDL*	*BDL*	*BDL*
38.5	39.0	54.5	*BDL*	*BDL*	*BDL*	*BDL*	*BDL*
57.7	29.7	110.3	*BDL*	*BDL*	*BDL*	*BDL*	*BDL*
90.8	79.6	92.0	*BDL*	*BDL*	*BDL*	*BDL*	
108.2	77.6	56.0	*BDL*	*BDL*	*BDL*	*BDL*	
103.5	*BDL*	120.4	*BDL*	*BDL*	*BDL*	*BDL*	
BDL	*BDL*	*BDL*	*BDL*	*BDL*	*BDL*	*BDL*	
BDL	*BDL*	*BDL*	*BDL*		*BDL*		
BDL	*BDL*	*BDL*	*BDL*		*BDL*		
BDL	*BDL*	*BDL*	*BDL*		*BDL*		
BDL	*BDL*	*BDL*			*BDL*		
	BDL	*BDL*					
	BDL	*BDL*					
	BDL	*BDL*					
		BDL					
66.0 ± 59.7[a]	44.1 ± 61.2	47.3 ± 54.2	18.8 ± 38.1	9.0 ± 19.0	4.0 ± 11.6	5.0 ± 10.8	3.6 ± 8.8

BDL below detection limit

[a] Mean ± SD

analysis, the LysoPCs content in L-RBCs was also significantly lower than in non-L-RBCs ($p < 0.05$).

In addition, HPTLC enables the detection of other lipid fractions present in the analyzed material. In non-L-RBCs, only traces of phospholipids and neutral lipids were observed on the HPTLC plates while no lipids were detected in most cases of L-RBCs.

Discussion

All blood components may be implicated in TRALI. However in the plasma-rich components, such as plasma and platelets, the risk per component is the highest because they often contain anti-leukocyte antibodies implicated in TRALI, particularly in fatal TRALI cases (Khan et al.

2007; Silliman et al. 2003b, 2009; Van Stein et al. 2010). The antibody-mediated (immune) TRALI mechanism postulates passive transfer of leukoagglutinating antibodies via transfusion of blood components containing plasma which results in antibody binding to recipient neutrophils. Despite strong experimental and clinical evidence to support an antibody TRALI mechanism there are frequent arguments against antibody-mediated TRALI as some transfused patients never developed TRALI even though their leukocytes expressed cognate antigens (Kopko et al. 2002; Popovsky and Haley 2000; Żupańska et al. 2007).

On the other hand, the study results published by the Denver work group indicate that TRALI in predisposed patients may be triggered not only by leukocyte antibodies but also by bioactive lipids Silliman et al. (1994, 1996, 1997, 2003b).

Blood components are human biological material and during storage may accumulate metabolic products such as bioactive lipids. Silliman et al. (1994, 1996, 1998, 2003a) and Looney and Matthay (2006) demonstrated on an animal model that these breakdown products of membrane lipids, LysoPCs included, can prime neutrophils to trigger a respiratory burst reaction. No such priming activity was observed either in fresh cellular blood components or in acellular plasma. These data are in line with our findings that the LysoPCs level in acellular FFP was similar to that in plasma of random donors. Additionally, the level of LysoPCs in supernatants of fresh PLTs (day 0) was similar to that in donor plasma.

The LysoPCs amount in stored PLTs increased almost two-fold. Our observations were confirmed by other authors who also reported that the LysoPC concentration increased in human PLT components stored for 5–7 days (Silliman et al. 1996, 2003a; Vlaar et al. 2010a). It should be noted that we evaluated the LysoPCs in the supernatant from apheresis platelets using HPTLC method; the concentration of LysoPCs was the same as reported by Vlaar et al. (2010a) after high-performance liquid chromatography (HPLC) tandem mass spectrometry. Our study was also in accordance with the results of Ruebsaamen et al. (2010) who used electrospray-ionization tandem mass spectrometry to describe lipid species profiles of platelets and plasma during storage of PLTs. This agrees with the pathophysiological animal model of neutrophil priming by lipids from stored PLTs (Silliman et al. 1996, 2003a). Recently Vlaar et al. (2010a) also proved that the supernatant of stored PLTs caused lung inflammation and coagulopathy in a syngeneic in vivo transfusion model.

In both immune and non-immune TRALI the neutrophil is postulated as the effector cell. For this reason the neutrophil priming assays are crucial for demonstrating the role of LysoPCs in stored blood components for development of TRALI. Further investigations are necessary to determine the precise concentration of LysoPCs in human pulmonary circulation sufficient to prime neutrophils to lung damage. Bux and Sachs (2007) suggested that TRALI envolves once the neutrophil priming overcomes a certain threshold value.

The LysoPCs content in packed RBCs, mostly leukodepleted RBCs, was observed to be very low or undetectable not only on collection day but also during storage. In the process of preparation of packed non-L- and L-RBCs, plasma, which is the main source of LysoPCs was removed and substituted by storage solution. The low LysoPC content in packed L-RBCs was confirmed by Silliman et al. (2000), Sachs et al. (2010) and Vlaar et al. (2011b). The latter also proved that human RBC supernatant stored in SAGM had no neutrophil priming capacity even after 42 days of storage.

The United States group (Silliman et al. 1994, 1998, 2003b) reported an accumulation of bioactive lipids (LysoPCs) in stored RBCs. The authors observed that these lipids may play a key role in the onset of neutrophil priming and/or activation in the pathogenesis of TRALI because lipids extracted from plasma of outdated RBCs caused pulmonary damage in a rat lung model. The European group (Sachs et al. 2010, Vlaar et al. 2011a, b) observed no such effect in their experimental and preclinical studies. The discrepancy of the results of the United States group may be explained by the differences in product preparation and RBCs storage or in evaluation of neutrophil priming assays. The Silliman group primed neutrophils for 5 min with plasma and lipids from stored packed RBCs and measured the maximal rate of superoxide anion production as a marker of neutrophil priming (Silliman et al. 1994). The Vlaar group incubated neutrophils with supernatant from stored RBCs for 30 min and used H_2O_2 release as a measure of neutrophil priming (Vlaar et al. 2010a, 2011b). However we did not perform such test, our data support the results of the European's group as we found no LysoPCs in most of the packed RBCs samples.

The approach to the role of LysoPCs in neutrophil priming varies but some new data were forwarded in a paper by Vlaar et al. (2011b) who described the effect of various solutions used for the storage of blood components on LysoPCs accumulation and neutrophil priming capacity. The authors prove that LysoPC accumulation is not cell- but plasma- derived and in vitro neutrophil priming by aged PLTs is LysoPCs independent. It is their opinion that LysoPCs accumulation does not occur at 4°C, which is the temperature routinely used for RBCs storage. They suggest that LysoPCs are merely markers or mediators of product deterioration. In line with this information, they demonstrated that aged blood products may cause TRALI in rats both in the presence and absence of bioactive lipid

accumulation and suggest that the link between LysoPCs and lung injury is rather weak.

It is worth noting that biochemical changes that occur in the supernatants of blood components during storage are caused not only by LysoPCs but also by other factors such as elevated potassium level, pH decrease, accumulation of lysophosphtidic acid, proinflammatory cytokines or microparticles with procoagulant activity all of which may contribute to the inflammatory process of TRALI.

Moreover, the authors of the recently published paper (Silliman et al. 2011) pay attention on the role of the non-polar bioactive lipids (arachidonic acid, 5-HETE, 12-HETE) which they identified in packed leuko- and platelet reduced RBCs stored in AS-5 preservation solution. The maximum concentration of these lipids was observed on storage day 42 and the authors suppose that they were RBC membrane derived. The authors of the present paper did not investigate the non-polar lipids in RBCs stored in AS-5 preservation solution.

Finally, we would like to draw attention to the very important role of LysoPCs as regulators of immunologic cell functions, especially in priming neutrophils which are the main cells of innate immunity. Fuchs and Schiller (2009) indicate numerous important biological functions of LysoPCs. Moreover, reduced plasma LysoPCs levels have been noted in sepsis patients and systematic treatment with LysoPCs proved therapeutic in rodent model of sepsis (Cunningham et al. 2008). The above observations may suggest that elevated LysoPCs plasma levels in long-stored apheresis platelets may relieve serious inflammatory conditions or according to Lin et al. (2005) may protect against lung vascular injury. Further studies are however necessary to understand the various roles which this "enigmatic" lysolipid plays in neutrophil activation as the major contributor to acute lung injury and sepsis.

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