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Potential contribution of mitochondrial (mt) DNA damage associated molecular patterns (DAMPs) in transfusion products to the development of acute respiratory distress syndrome (ARDS) after multiple transfusions

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Abstract

Introduction—Massive transfusions are accompanied by an increased incidence of a particularly aggressive and lethal form of acute lung injury (delayed TRALI) which occurs >24 hours after transfusions. In light of recent reports showing that mtDNA DAMPs are potent pro-inflammatory mediators, and that their abundance in the sera of severely injured or septic patients is predictive of clinical outcomes, we explored the idea that mtDNA DAMPs are present in transfusion products and are associated with the occurrence of delayed TRALI.

Methods—We prospectively enrolled fourteen consecutive severely injured patients that received greater than three units of blood transfusion products and determined if the total amount of mtDNA DAMPs delivered during transfusion correlated with serum mtDNA DAMPs measured after the last transfusion, and whether the quantity of mtDNA DAMPs in the serum predicted development of ARDS.

Results—We found detectable levels of mtDNA DAMPs in PRBCs (3 ± 0.4 ng/mL), FFP (213.7 ± 65 ng/mL), and platelets (94.8 ± 69.2), with the latter two transfusion products containing significant amounts of mtDNA fragments. There was a linear relationship between the mtDNA DAMPs given during transfusion and the serum concentration of mtDNA fragments ($R^2 = 0.0.74$,

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Contributions: JDS: Principle investigator, study design, data interpretation, writing of manuscript. YLL: Study design, data interpretation, writing of manuscript. VMP: PCR, data analysis. GC: Data interpretation, writing of manuscript. CAM: collection of samples, PCR, data analysis. DCM: PCR, data analysis. MLM: PCR, data analysis, writing of manuscript. SBB: data analysis, writing of manuscript. MNG: study design, data interpretation, writing of manuscript.

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$p<0.01$). The quantity of mtDNA DAMPs in serum measured at 24 hours after transfusion predicted the occurrence of ARDS (9.9 ± 1.4 vs 3.3 ± 0.9 , $p<0.01$).

Conclusion—These data show that FFP and platelets contain large amounts of extracellular mtDNA, that the amount of mtDNA DAMPs administered during transfusion may be a determinant of serum mtDNA DAMP levels, and that serum levels of mtDNA DAMPs after multiple transfusions may predict the development of ARDS. Collectively, these findings support the idea that mtDNA DAMPs in transfusion products significantly contribute to the incidence of ARDS after massive transfusions.

Level of Evidence—Level 1. (Prognostic and Diagnostic).

Keywords

TRALI; Transfusion Related Acute Lung Injury; mitochondrial DNA; DAMPs; ARDS; Acute Respiratory Distress Syndrome

INTRODUCTION

The recent paradigm shift in resuscitation of patients in hemorrhagic shock, using blood components in a manner which reconstitutes whole blood and limits the use of crystalloid, has given rise to a new form of Transfusion Related Acute Lung Injury (TRALI) termed “delayed” TRALI. (1, 2) The “traditional” form of TRALI is rare (1 in 5,000 transfusions), occurs after only one unit of transfusion, has immediate onset, and is often self-limiting with complete resolution. (3, 4) In contrast, the “delayed” form of TRALI is seen 48–72 hours after massive transfusions, occurs in over 50% of patients receiving multiple transfusions, and progresses to a non-resolving severe respiratory failure (i.e. Acute Respiratory Distress Syndrome, ARDS). (2) Regardless of the clinical presentation, ARDS has emerged as the most frequent manifestation of multi-organ failure after massive transfusions. A better understanding of the mechanisms of delayed TRALI leading to the identification of discrete pharmacological targets for intervention is urgently needed and is the overall goal of this research.

Although traditional TRALI is often attributed to leukocyte antibodies in the donor blood directed against the recipient, the stark differences between the “traditional” and “delayed” forms of TRALI suggest a different pathogenesis. The notion that there are critical mediators present in transfused blood products which accumulate after massive transfusions is intuitively appealing in light of the direct correlation between the occurrence of ARDS (i.e. delayed TRALI) and the number of transfusions and/or storage-life of the transfused products. (2, 5) In support of this “mediator concept,” we recently reported that pro-inflammatory fragments of the mitochondrial (mt) genome, termed mtDNA Damage Associated Molecular Patterns (DAMPs), can be detected in variable amounts in stored blood products (6).

Evidence that endogenously-formed mitochondrial DAMPs – including mtDNA fragments and other mt-associated proteins – contribute to human disease is compelling (7). Because of its proteobacterial origin, the structure and pro-inflammatory effects of mtDNA are similar if not identical to bacterial DNA. Both propagate damage through TLR9-mediated activation

of itinerant and resident inflammatory and other target cells. Administration of exogenous mtDAMPs to experimental animals, isolated lungs, and cultured cells recapitulates many elements of SIRS and multi-organ system failure, including acute lung injury (8, 9). Finally, we and others have previously shown that serum levels of mtDNA DAMPs correlate with outcome in multiple patient populations (9–12). Against this background, we tested the hypothesis that in severely injured patients, ARDS following multiple transfusions is accompanied by a higher quantity of serum mtDNA DAMPs which is also associated with the quantity of pro-inflammatory mtDNA DAMPs transfused to the patient.

METHODS

Quantification of mtDNA in Transfused Blood Products

Fourteen consecutive severely injured patients (Injury Severity Score, ISS >15) of age >18 years requiring greater than three units of blood product transfusion within 48 hours of presentation were prospectively enrolled. Once blood product units were allocated for transfusion, aliquots of each unit was collected and stored at –20°C until processing, after which mtDNA quantification was performed for up to the first ten units per patient. ABO blood type, donor gender, and product storage times were recorded. Within twelve hours of collection, samples from the transfusion products were then centrifuged at 1200 $\times g$ for 25mins at 21°C, and 200 μ L of the supernatant was decanted and processed using the Qiagen DNEasy kit (Qiagen Inc., CA, USA) as previously described (6, 10). After DNA elution, quantitative polymerase chain reaction (qPCR) was performed using the USB VeriQuest Fast SYBR Green qPCR Master Mix (Affymetrix, CA, USA) per manufacturer's protocol, amplifying a ~200 bp sequence corresponding to the ND6 region of the mitochondrial genome as previously described (6, 10) (ND6 Primers: Forward CCATCGCTGTAGTATATCCAA, Reverse TCGGGTGTGTTATTATTCTGA). Cumulative transfused mtDNA burden was determined by summing the mtDNA content for all transfused products calculated as the concentration in each tested aliquot multiplied by transfusion volume of the respective unit (350mL for packed red blood cells (PRBC), 300mL for fresh frozen plasma (FFP), and 250mL for platelets).

Measurement of Serum mtDNA after Transfusion

A waiver of informed consent was approved by the University of South Alabama IRB per DHHS 45 CFR 46.116; all enrolled patients or their legal proxy were provided an information sheet describing the study protocol at earliest availability with the option to withdraw from the study. A 4mL sample of peripheral blood was collected and placed into a citrated tube within 8 hours of initial transfusion and 24–48 hours afterward. Samples were immediately centrifuged at 1200 $\times g$ for 25mins at 21°C, after which 200 μ L of the supernatant was decanted and processed for DNA quantification as described above. Patients were monitored during the course of care for development of Acute Respiratory Distress Syndrome per the Berlin definition (13).

Data were analyzed using Graphpad Prism software by ANOVA with Newman-Keuls post-test, unpaired t-test, or linear regression, as dictated by the specific experimental design.

Results are displayed as mean $\pm$ standard error and statistical significance was defined as $p < 0.05$.

RESULTS

Characteristics of mtDNA DAMPs in transfusion products

Mitochondrial DNA was detected by qPCR in all three blood components examined (Figure 1), with FFP and platelets containing significantly more mtDNA than PRBCs (PRBC 3.0 ± 0.4 , FFP 195.8 ± 58.8 , Plt 93.9 ± 24.3 , $p < 0.01$) corroborating our initial report. (6) To determine the effect of storage life on extracellular mtDNA concentration, blood products were classified as young, moderate, or old based on relative shelf-life; for PRBCs 0–2 weeks, 2–4 weeks, and 4–6 weeks respectively; for plasma 0–4 months, 4–8 months, and 8–12 months respectively; for platelets 1–2 days, 3–4 days, and 5–7 days respectively. PRBC and FFP differed by shelf-life in mtDNA concentration but individual strata did not significantly differ by Newman-Keuls post-test (PRBC: Young 2.2 ± 0.6 , Moderate 3.5 ± 0.8 , Old 2.9 ± 0.6 , $p = 0.009$; FFP: Young 265.2 ± 83.5 , Moderate 87.1 ± 39.1 , Old 19.3 ± 7.6 , $p < 0.001$). Blood products were also categorized based on ABO donor blood type (Figure 2). Variances significantly differed for all products by ANOVA; however, ABO types did not differ by Newman-Keuls post-test (A = 189.4 ± 81.1 , B = 33.7 ± 21.1 , AB = 15.0 ± 5.4 , O = 49.3 ± 16.2 , $p < 0.001$). ABO type exhibited similar trends in total mtDNA content when PRBC, FFP, and platelets were individually analyzed (data not shown). Blood products were also examined based on donor gender (Figure 3). Packed red cells did not differ between male and female (Male 2.5 ± 0.5 vs. Female 3.4 ± 0.7). Female FFP had greater mtDNA content relative to males (Male 178.6 ± 67.3 vs. Female 690.5 ± 128.1 , $p = 0.046$), and female platelets had a similar tendency towards higher mtDNA content, but this did not reach statistical significance (Male 33.86 ± 5.412 vs. Female 198.5 ± 172.5 , $p = 0.29$).

Characteristics of enrolled patients and serum mtDNA concentrations

Enrolled patients were followed for development of ARDS after > 3 transfusions with the intent of determining if the development of ARDS (i.e. delayed TRALI) was associated with the amount of mtDNA DAMPs transfused to the patient and/or serum concentration. There were fourteen patients enrolled and five met the criteria for ARDS. Patient characteristics did not significantly differ between groups (Table 1). Within eight hours of presentation, serum mtDNA DAMPs did not differ significantly between groups; however, by 24–48 hrs after presentation, those who developed ARDS had higher levels of serum mtDNA DAMPs than patients who did not (Figure 4).

Association of transfused mtDNA DAMPs and serum concentration

Patients who developed ARDS received significantly more mtDNA DAMPs during transfusion than patients who did not (No ARDS $1.9 \times 10^4 \pm 6.6 \times 10^3$ ng/mL vs. ARDS $3.8 \times 10^4 \pm 9.6 \times 10^3$ ng/mL, $p < 0.05$) despite receiving similar volumes of transfusion products (Table 1). The amount of transfused mtDNA was then compared to serum levels. As depicted in Figure 5, the serum mtDNA concentration following transfusion correlated with cumulative transfused mtDNA ($R^2 = 0.74$, $p < 0.01$).

DISCUSSION

Despite improvements in resuscitation and supportive care, pharmacologic strategies to prevent or reverse ARDS have to date been unsuccessful. This deficiency has been ascribed to the prospect that key pathways integrating cellular responses to systemic inflammation have not yet been identified. In this context, multiple lines of emerging evidence converge on the idea that mitochondria orchestrate responses to injurious and inflammatory stimuli by releasing a family of mediators known collectively as mtDAMPs, including formyl and other peptides as well as mtDNA fragments (14). The latter is conspicuous in light of observational studies in human subjects showing that serum levels of mtDNA DAMPs are predictive of outcomes in critically ill, septic or severely injured patients (10–12, 15, 16). In addition, a number of experiments in animal and cell culture models show that mtDNA is elaborated into the extracellular environment by inflammatory injury and that administration of exogenously-produced mtDNA DAMPs recapitulates many of the attributes of ARDS and multi-organ failure seen in human subjects (8, 9, 17, 18). Interventions that block the actions of exogenously-administered mtDNA DAMPs, such as inhibition of the receptor on which mtDNA acts, TLR9, or DNase to enhance DNA degradation, also prevent lung injury evoked by inflammatory stimuli (8). Finally, exogenous mtDNA DAMPs cause mtDNA damage and release of more mtDNA DAMPs, thus pointing to the intriguing prospect that accumulation of serum mtDNA DAMPs, regardless of the inciting event, could activate a feed-forward cycle that persists even after termination of the initiating stimulus (8, 19).

Here we tested the idea that the amount of mtDNA DAMPs administered via transfusion to patients after severe injury is associated with the occurrence of ARDS. Of particular importance to our conclusions is the fact that not only were patients prospectively enrolled and displayed comparable severities of injury, but all patients enrolled in this proof-of-concept study received similar total volumes of blood products. Against this background, although serum concentrations of mtDNA DAMPs measured within eight hours of admission were similar between groups, mtDNA DAMPs measured 24–48h after admission were significantly higher in individuals developing ARDS relative to those who did not (Figure 4). Because the serum concentration of mtDNA DAMPs was closely correlated with the amount of mtDNA given during transfusion (Figure 5), and since observations in multiple animal and cell culture models show that exogenous mtDNA DAMPs mimic key attributes of ARDS, these data provide perhaps the most persuasive support in human subjects heretofore available that mtDNA DAMPs may be associated with the development of ARDS after massive transfusions.

Our data do not permit conclusions about which mtDNA fragment, or indeed, which mt-derived DAMP contributes to ARDS in the setting of massive transfusion. There are previous reports of synergy between mtDNA and other mitochondrial proteins (20). It is also unknown how much the quantity of crystalloid used during resuscitation, prior ischemia-reperfusion (IR) injury, or other inflammatory inciting events contribute to the development of ARDS. Other inflammatory mediators interacting with mtDNA DAMPs also present in transfusion products or generated in the patient as a result of transfusion are other possible events adding complexity to the proposal that mtDNA DAMPs are important in the evolution of transfusion-related ARDS (21–23). Finally, the pathways governing the disposition of

circulating mtDNA are almost completely unknown, as are the determinants of the response to transfused mtDNA DAMPs. Circulating DNase is one possible pathway of degradation, but other mechanisms of clearance cannot be discounted. Addressing these issues will require studies of a different design than those used in the present report.

We detected impressive differences in the amount of mtDNA in the three transfusion products. These differences are likely attributable to whether the product was leuko-reduced and whether the constituents of the product contained mitochondria. For example, because RBCs do not contain mitochondria and are subjected to leukocyte reduction it is not surprising that the concentration of mtDNA detected in this transfusion product was lowest among the three tested. The highest concentrations of mtDNA DAMPs were detected in FFP. In this instance, we postulate that the high levels of mtDNA DAMPs are likely due to the fact that FFP is not subjected to leukocyte reduction and thus contain a large number of WBCs. In addition, because FFP is frozen immediately after procurement and thawed immediately prior to transfusion, we suspect that mtDNA DAMPs are formed as a result of leukocyte rupture during thawing.

Substantial amounts of mtDNA DAMPs were also detected in platelets, a finding not particularly surprising since platelets contain large numbers of mitochondria DNA. In addition, because the mature platelet does not contain a nucleus, it lacks the machinery to repair oxidative mtDNA damage, thereby creating a condition wherein platelet mtDNA may undergo cycles of replication and degradation (24). In light of these considerations, it is tempting to speculate that an oxidant-enriched environment in stored platelets (25, 26) leads to oxidative mtDNA damage and attendant elaboration of mtDNA DAMPs (8, 17), which may, in turn, contribute to the limited shelf-life of platelets (27).

The impact of blood donor gender on complications after transfusion has been investigated for decades. Older publications on this subject tend to focus on antibody-mediated TRALI, which may have little bearing on understanding the relationship between transfusion and the phenotype of delayed TRALI. However, recent large retrospective studies have demonstrated gender-based differences in mortality after high plasma ratio transfusions, and that female recipients of transfusion have worse outcomes following the newer guidelines (28). Provocatively, we found that FFP from females displayed higher mtDNA DAMP concentrations; no gender-related differences were detected in LR-PRBCs. These findings, in concert with the previously mentioned report, confirm that a gender-based predilection towards increased inflammatory responses likely exists and that mtDNA DAMPs may contribute to this reaction. Another interesting finding in our study was that mtDNA DAMP concentrations differ between ABO blood types. For example, type A donors tended to have higher concentrations of mtDNA DAMPs compared to other ABO types, regardless of the specific stored blood component. Indeed, previous studies have shown that patients with blood type A are more likely to develop ARDS after sepsis and trauma (29).

In conclusion, these data show that FFP and platelets contain large amounts of extracellular mtDNA, that the amount of mtDNA DAMPs administered during transfusion may be a contributor of serum mtDNA DAMP levels, and that serum levels of mtDNA DAMPs after multiple transfusions may predict the development of ARDS. Collectively, these findings

support the idea that mtDNA DAMPs in transfusion products are associated with the incidence of ARDS after massive transfusions. Finally, to determine the true value of serum mtDNA DAMP measurements as predictive biomarkers for the evolution of ARDS and MODS in the setting of severe injury, it will be ultimately be necessary to standardize means for measuring and reporting mtDNA abundance and apply these to a substantial patient cohort.

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Figure 1.

Extracellular mitochondrial DNA was detected in all blood product types. Variances were significantly different by ANOVA ($p < 0.001$). FFP contained significantly more mtDNA compared to PRBCs by Newman-Keuls post test. (PRBC 2.99 ± 0.41 , $n = 114$; FFP 195.8 ± 58.8 , $n = 81$; Plt 93.8 ± 24.3 , $n = 17$)

Figure 2.

Extracellular mitochondrial DNA was measured in blood products based on ABO type. Though variances significantly differed ($p < 0.01$), mtDNA content did not significantly differ by blood type by Newman-Keuls post-test. (A 189.4 ± 81.1 , $n=56$; B 33.7 ± 21.1 , $n=15$; AB 15.0 ± 5.4 , $n=17$; O 49.3 ± 16.2 , $n=108$)

Figure 3.

Transfused blood products were categorized based on gender of the donor. Male plasma had significantly less extracellular mtDNA than female; other products did not significantly differ by Mann-Whitney test. (PRBC Male 2.54 ± 0.52 , $n=50$; Female 3.39 ± 0.65 , $n=60$; FFP Male 178.6 ± 67.3 , $n=68$; Female 690.5 ± 128.1 , $n=5$; Plt Male 33.9 ± 5.4 , $n=3$; Female 198.5 ± 172.5 , $n=2$)

Figure 4.

Serum extracellular mitochondrial DNA was measured within 8 hours of transfusion, and again after 24–48 hours later. Patients were followed clinically for development of ARDS. Though serum mtDNA concentration not differ initially, those who developed ARDS had significantly higher mtDNA levels at 24–48 hours after transfusions compared to those who did not develop ARDS. (No ARDS t0 5.85 ± 2.89 , n=9; t24 2.27 ± 0.44 , n=9; ARDS t0 11.21 ± 3.43 , n=5; t24 16.23 ± 9.02 , n=5)

Figure 5.

Cumulative transfused mtDNA DAMPs were compared to post-transfusion serum quantities. A linear correlation was noted between transfusion burden and serum levels ($R^2=0.74$, $p<0.01$). Those who developed ARDS are marked in red triangles; those who did not are marked in black circles.

Table 1

Patient demographics by development of ARDS

	No ARDS	ARDS	p
N	9	5	
Male	7	5	0.51
ISS	24.5±4.3	23.0±6.3	0.83
Units transfused	16.6±1.8	20.2±5.1	0.44
PRBC	11.0±1.5	11.8±3.4	0.94
FFP	6.3±1.0	6.2±1.6	0.94
Platelets	0.6±0.2	2.2±1.0	0.28
Vent Days	5.1±1.7	9.6±2.9	0.18
ICU Days	8.5±2.1	20.6±6.1	0.08
Mortality	0	1	0.36

Statistical significance defined as $p < 0.05$ by Fisher exact test or Mann-Whitney test as appropriate