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Metabolomics Profile Comparisons of Irradiated and Non-Irradiated Stored Donor Red Blood Cells

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Abstract

BACKGROUND—Understanding the metabolites that are altered by donor red blood cell (RBC) storage and irradiation may provide insight into the metabolic pathways disrupted by the RBC storage lesion.

STUDY DESIGN AND METHODS—Patterns of metabolites, representing over 11,000 distinct mass-to-charge ratio (m/z) features, were compared between gamma irradiated and non-irradiated CPDA-1split RBCs from 6 human donors over 35 days of storage using multilevel sparse partial least squares discriminant analysis (msPLSDA), hierarchical clustering, pathway enrichment analysis and network analysis.

RESULTS—In msPLSDA analysis, RBC units stored 7 days or less (irradiated or non-irradiated) showed similar metabolomic profiles. By contrast, donor RBCs stored 10 days or more demonstrated distinct clustering as a function of storage time and irradiation. Irradiation shifted metabolic features to those seen in older units. Hierarchical clustering analysis identified at least 2 clusters of metabolites that differentiated between RBC units based on storage time and irradiation exposure, confirming results of the msPLSDA analysis. Pathway enrichment analysis, used to map

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WEB-BASED RESOURCES USED

R statistical software package: <http://www.r-project.org/> (Last accessed 2/14/14)

METLIN metabolite database: <http://metlin.scripps.edu/index.php> (Last accessed 3/1/14)

Kyoto Encyclopedia of Genes and Genomes (KEGG): <http://www.genome.jp/kegg/> (Last accessed 3/1/14)

AABB Circular: <http://www.aabb.org/resources/bct/Documents/doi1113.pdf> (Last accessed 5/14/14)

the discriminatory biochemical features to specific metabolic pathways, identified four pathways significantly affected by irradiation and/or storage including arachidonic acid ($P=3.3 \times 10E-33$) and linoleic acid ($P=1.61 \times 10E-11$) metabolism.

CONCLUSION—RBC storage under blood bank conditions produces numerous metabolic alterations. Gamma irradiation accentuates these differences as the age of blood increases, indicating that at the biochemical level irradiation accelerates metabolic aging of stored RBCs. Metabolites involved in the cellular membrane are prominently affected, and may be useful biomarkers of the RBC storage lesion.

Keywords

Storage lesion; blood bank; red cell survival; metabolites; biomarker

INTRODUCTION

Prolonged storage of donated red blood cells (RBC) is associated with progressive alterations that may reduce RBC function and viability and cause detrimental clinical effects^{1–4}. With increasing duration of storage, the red cell membrane stiffens and becomes less deformable,⁵ develops reduced antioxidant capacity and impaired glutathione homeostasis,⁶ and increased aggregability⁵ and endothelial adherence⁷. Observational studies suggest that transfusion of blood stored for more than 14 days is associated with an increased risk of postoperative complications and mortality in adults undergoing cardiac surgery⁸. By contrast, the recently published ARIPI trial found no difference in neonatal morbidity between preterm infants transfused with fresh compared to old blood⁹. Additional randomized trials investigating the clinical effects of pre-transfusion blood storage are ongoing¹⁰.

Taken by itself, the chronological age of donor RBCs is a relatively imprecise measure of the function and viability of stored RBCs. Post-donation processing can affect stored RBCs and this may not be accounted for in the age of blood. Gamma irradiation, a widespread practice to reduce the risk of graft-versus-host disease in immune-compromised patients such as premature infants^{11,12}, worsens the RBC storage lesion by increasing release of intracellular contents and increasing RBC rigidity with abnormal morphology,^{5,13–15} while pre-storage leukoreduction may alleviate some of the alterations caused by RBC storage¹⁶. Improving our understanding of functional changes that occur with prolonged RBC storage, including the effects of gamma irradiation, may allow for the development of better measures of donor RBC function and viability.

We believe that understanding the changes in metabolic pathways associated with *ex vivo* RBC aging or by RBC irradiation may provide insight into the functional changes accompanying the RBC storage lesion. The objective for this study was to compare patterns of metabolites between irradiated and non-irradiated RBCs from human donors over 35 days of storage through dimension-reduction techniques and hierarchical clustering. Based on these results, we sought to identify specific candidate metabolites whose alterations were most highly associated with blood storage and/or irradiation.

MATERIALS AND METHODS

Donors

We obtained whole blood donations from 6 healthy donors 50 to 57 years of age. Four donors were female; five donors were Caucasian and one was African-American. Volunteer donors were screened by a health history questionnaire and vital signs prior to donation. Written informed consent was obtained from each donor. The study was approved by the Emory University Institutional Review Board, which takes into consideration the guidelines set forth by the Declaration of Helsinki.

Red blood cell processing

Citrate-phosphate-dextrose-adenine-1 (CPDA-1) packed RBC units (Fenwal Inc., Lake Zurich, IL, USA) were prepared from each whole blood donation and split into 2 bags on the day of collection. One of each pair of bags was irradiated on the day of collection (day 0) at a dose of 25 Gy using a Nordion Gamma irradiator (Nordion, Ottawa, ON, Canada). The other bag served as a control. Aliquots were then taken from each divided RBC unit on days 2, 3, 7, 10, 14, 17, 21, 28 and 35 of storage. Donor RBCs were leukodepleted and stored at 2–6 °C under blood bank conditions until the moment of sampling 30 minutes prior to aliquoting. Donor RBC bags were placed on a rotating platform in a cold room to homogeneously re-suspend the RBCs and then fitted with syringe access ports using sterile technique. Ports were cleaned with ethanol and a 5mL syringe fitted with a 16 gauge needle was used to withdraw 5 mL of the whole unit sample including any supernatant using sterile technique. The bags were then returned to the refrigerator and stored as noted above until the next period of sampling. Each 5 mL sample from the bag was split into 5 × 1 mL aliquots that were pipetted into cryovials, immediately snap-frozen on liquid nitrogen within minutes of sampling, and stored at –80 °C until metabolomics analysis.

Metabolomics analysis

Samples were randomized prior to analysis to minimize possible effects due to run order. Samples were treated with 2 volumes of ice-cold acetonitrile containing a mixture of 14 stable isotope internal standards, allowed to stand 30 minutes on ice, and centrifuged for 10 minutes at 13,400 × rpm at 4°C to remove precipitated protein. Samples were maintained in a refrigerated autosampler prior to injection of 10 µl for analysis and each sample was analyzed in three technical replicates. We used a high-resolution Linear Trap Quadrupole Fourier Transform mass spectrometer (LTQ-FT, Thermo Scientific, Waltham, MA, USA) with reverse phase liquid chromatography using a 2.1 × 10cm Targa C18 column (Higgins Analytical Inc., Mountain View, CA, USA), which is good for separation of lipids, peptides with medium to low hydrophobicity, and other semi-polar compounds such as flavonoids, alkaloids, glycosylated steroids, and phenolic acids. The mass spectrometer was set to collect data from m/z 85 to 850 to identify and quantify metabolites as previously described^{17,18}. Briefly, a spray voltage of 6 kV, sheath gas of 60 (arbitrary units), capillary temperature of 275°C, capillary voltage of 44 V and tube lens of 120 V were used. Ion transfer optics were optimized automatically. Maximum injection time was 500 ms, and the maximum number of ions collected for each scan was 3×10^6 . A wide range scan was used for the FT-ICR with mass resolution of 50,000. The C18 chromatography was performed

with an acetonitrile gradient for 10 min. A flow rate of 0.35 ml/min was used for the first 6 min and 0.5 ml/min for the remaining 4 min. The first 2-min period consisted of 5% A, 60% water, 35% acetonitrile, followed by a 4-min linear gradient to 5% A, 0% water, 95% acetonitrile. The final 4-min period was maintained at 5% A, 95% acetonitrile. Raw spectral data files were converted to computable document format (CDF) using Thermo Xcalibur prior to data analysis. Peak detection, noise filtering, mass-to-charge ratio (m/z) and retention time alignment, feature quantification, and data quality filtering was performed using apLCMS¹⁹ with xMSanalyzer¹⁸. Data was extracted as m/z features where an m/z feature was defined by m/z , retention time, and integration ion intensity. We took the average of the three technical replicates for subsequent biostatistical and bioinformatics analyses. Only features with at least 70% signal in either one of the experimental conditions were used for further analysis to identify metabolites with differential expression patterns due to storage age (in days) or gamma irradiation status (yes or no). In addition, we performed targeted analysis that evaluated known metabolites in the glutathione synthesis pathway, based on published findings demonstrating alterations in this pathway in stored murine and human RBCs^{20–22}. The analytical structure included pooled reference plasma samples as every twenty-first sample. This pooled reference sample has been calibrated to the NIST SRM 1950²³. All samples and reference standards included 14 stable isotopes: [¹³C₆]-D-glucose, [¹⁵N]-indole, [2-¹⁵N]-L-lysine dihydrochloride, [¹³C₅]-L-glutamic acid, [¹³C₇]-benzoic acid, [3,4-¹³C₂]-cholesterol, [¹⁵N]-L-tyrosine, [trimethyl-¹³C₃]-caffeine, [¹⁵N₂]-uracil, [3,3-¹³C₂]-cystine, [1,2-¹³C₂]-palmitic acid, [¹⁵N,¹³C₅]-L-methionine, [¹⁵N]-choline chloride, and 2'-deoxyguanosine-¹⁵N₂,¹³C₁₀-5'-monophosphate. The analytical structure allowed for quantification of metabolites, and we reported relative quantification due to limited curation of metabolites.

Statistical analysis

All statistical analysis was performed using R (see web based resources). Raw data underwent logarithmic transformation to reduce heteroscedasticity and normalization, such that each metabolite had a mean of 0 and standard deviation of 1. We performed additional quantile normalization of samples to minimize between sample variability^{24,25}. Quantile normalization is a normalization procedure that aims to make the distributions of feature intensities similar across all the samples. Principal component analysis, an unsupervised dimension reduction technique, was performed using R. Previous studies have highlighted the importance of taking the study design into account to improve the statistical power and data interpretability of high-dimensional data^{26–28}. Multilevel sparse partial least squares discriminant analysis (msPLSDA),²⁹ a supervised multivariate dimensionality reduction method, was used in this study as it performs simultaneous discriminatory analysis and selection of the most informative m/z features while taking into account the dependency structure of the m/z features, the repeated measurements of the subjects over time (within-patient correlation), and the effect of RBC gamma irradiation. Unlike principal component analysis (PCA), msPLSDA is a supervised dimensionality reduction approach that aims to maximize the covariance between the response variables (storage time and irradiation status) and the predictors (m/z features) in combination with variable/feature selection. This process is performed in two levels: a) first a mixed-model is used to split-up the variation according to storage time, stimulation factor (irradiation), and their interaction; b) sparse PLSDA³⁰ is

then used to identify the most discriminative predictors that separate the groups of subjects²⁹. The *tune.multilevel()* function in the mixOmics package was used to optimize the selection of the latent variables (msPLS-dims), representing linear combinations of variables analogous to a principal component in PCA. The tuning function allows optimization of selection of number of significant features (balancing type 1 and type 2 errors) without any loss of information by taking into account the covariance between the feature intensities and sample class information³⁰. The top 200 most significant *m/z* features from the first 3 latent variables (msPLS-dim1, msPLS-dim2, and msPLS-dim 3) were selected for further downstream analysis.

To visualize the patterns of metabolites from the different samples, two-dimensional score plots were used to perform pairwise comparisons between the three latent variables (msPLS-dim1, msPLS-dim2, msPLS-dim3). Additionally, two-way hierarchical clustering analysis (HCA), which is an unsupervised method, was used to evaluate the discriminatory ability of the detected *m/z* features using the *hclust()* function in R. Pearson correlation was used as the distance metric for HCA. Next, we performed pathway enrichment analysis using MetaboAnalyst³¹ after mapping the discriminatory *m/z* features to known metabolites in the METLIN metabolite database (see web based resources) using the positive adducts and a +/ – 10 parts per million *m/z* search threshold to obtain putative identifications. Correction for multiple hypothesis testing in pathway enrichment analysis was performed using an overall false discovery rate (FDR) of 5% in MetaboAnalyst³¹.

To further evaluate specific individual metabolites and candidate pathways that differed significantly between groups, we compared the changes in normalized intensities over time and performed exploratory analysis of the *m/z* (corresponding to the detected discriminatory metabolite) that putatively matched known metabolites in the Kyoto Encyclopedia of Genes and Genomes (KEGG, see web-based resources). We also performed a targeted correlation-based network analysis using the GeneNet package in R³² to detect any correlation between discriminatory metabolites identified by the msPLSDA analysis and those involved in glutathione metabolism. For this analysis, a partial Spearman correlation matrix (after removing the potential confounding effects due to correlation with other additional metabolites) was generated using the corpcor package in R to depict associations between known metabolites involved in glutathione metabolism and those detected by msPLSDA analysis. Network analysis and significance tests of the associations were performed using a false discovery rate (FDR) threshold of 5% using the GeneNet package.

RESULTS

The metabolomic analysis of paired irradiated and control samples from 6 CPDA-1 units stored from 2 to 35 days yielded 11,615 distinct *m/z* features with an average median coefficient of variation (CV) of 34.96%. PCA was performed on the normalized data and the pairwise score plots of the first three principal components showed the separation of samples based on duration of storage (Supplementary Figure 1). To enhance the ability to detect metabolic pathways impacted by length of RBC storage and irradiation status, the top 200 *m/z* features from the loadings of each latent variables were determined from the msPLSDA approach. A total of 599 unique *m/z* features, comprising 200 *m/z* features for each of the 3

latent variables with one redundant feature, were detected by this approach. Across msPLS-dim 1, which accounted for the majority of variability in the discriminatory features, we found a distinct separation between fresh and old RBC samples (Figure 1, **Panel A**), which was consistent with the time-dependent separation observed using PCA. RBCs stored from 2 to 7 days clustered together on msPLS-dim 1 and msPLS-dim 2. However, between 7 and 10 days of storage, there was a marked shift in the 200 m/z features comprising msPLS-dim 1, and thereafter samples from 10 to 35 days clustered together. At later time points (days 17 to 35), msPLS-dim 1 also showed more subtle clustering differences between irradiated and control units. Among msPLS-dim 2 metabolites, as with msPLS-dim 1, there was no clear difference between samples from days 2 to 7 of RBC storage. However, from days 10 to 14, msPLS-dim 2 separated irradiated and non-irradiated units into distinct clusters (Figure 1, **Panel A**). Taken together, msPLS-dim 1 and msPLS-dim 2 metabolites could distinguish between irradiated and non-irradiated RBC units stored between 10 and 35 days.

In a score plot comparing msPLS-dim 1 to msPLS-dim 3, we detected a near complete separation of metabolite patterns between irradiated and control units, regardless of the storage period. Furthermore, the discriminatory separation of msPLS-dim 3 metabolites increased with greater RBC storage age. While substantial differences were seen between irradiated and control units as early as 7 days of storage, significantly greater separation along the msPLS-dim 3 axis was seen between 10 and 35 days of storage (Figure 1, **Panel B**). Direct comparisons were also made between msPLS-dim 2 and msPLS-dim 3, but did not yield any additional information.

As an alternative approach, two-way hierarchical clustering analysis (HCA) was used to evaluate the discriminatory characteristics of the m/z features selected using msPLSDA. The HCA revealed 12 distinct clusters of RBC metabolites (Figure 2). One cluster (A) demonstrated marked separation between RBC samples stored from 2 to 7 days and those samples stored for 10 or more days, which was consistent with the discriminatory characteristics of msPLS-dim 1 metabolites. A second cluster (B) demonstrated separation between irradiated and control units between both 2 to 7 day old blood as well as blood with a longer storage age, consistent with msPLS-dim 3 results.

To further characterize specific metabolite pathways that were altered by storage and irradiation, we performed pathway enrichment analysis utilizing an FDR of 5%. The analysis yielded 4 candidate pathways (Table 1): arachidonic acid metabolism, linolenic acid metabolism, steroid biosynthesis, and alpha-linolenic acid metabolism. We selected the arachidonic acid metabolic pathway for further analysis to identify specific putative biochemical intermediates that were altered by RBC storage and irradiation (Figure 3). Of note, twelve of the 599 most discriminatory m/z features from the msPLSDA analysis mapped to 5 specific metabolites in the arachidonic acid pathway. The m/z matching 15-deoxy- 12,14 -PGJ₂ demonstrated marked increases in relative intensity between 7 and 10 days of storage, whereas relative intensity of the m/z for 20-COOH-leukotriene B₄ significantly declined beginning at 14 days of storage. No effects of irradiation were seen on these two metabolites. By contrast, we detected differences between irradiated and non-irradiated samples in the m/z ions matching two metabolic products of 8-isoprostane: 2,3-dinor-8-iso-PGF1 α and 2,3-dinor-8-iso-PGF2 α .

Finally, in an additional targeted analysis, we detected both positive and negative correlations between metabolites detected by msPLSDA analysis and four known metabolites involved in glutathione synthesis, which are known to be altered in stored RBCs²². The majority of correlation with metabolites detected by msPLSDA analysis occurred with glutathione. The high number of detected connections with different adducts/fragments of glutathione indicate this may be an important “hub” metabolite altered by RBC aging and irradiation.

DISCUSSION

In this study, we observed that RBC storage and gamma irradiation produced distinct metabolomic profiles in CPDA-1 RBCs. While other studies have investigated the effects of storage on global metabolic changes in mouse²⁰ and human^{6,21,22} RBCs, our study is the first to report the effects of gamma irradiation on metabolic patterns. Furthermore, the untargeted metabolomics platform used here identified many more distinct *m/z* features than in previous investigations of stored RBCs.

We found a marked difference in biochemical profiles between RBC units stored from 0 to 7 days and those stored beyond 10 days. This suggests that 7 to 10 days of storage is an important threshold, beyond which normal metabolism is detectably altered. Current US guidelines allow for 35 days of storage shelf life for donor RBCs stored in CPDA-1 anticoagulant preservative solution, with up to 28 total days of storage following gamma irradiation (AABB circular; see web based resources). In addition, there is variability in the capacity for individual centers to perform in-hospital irradiation, with some hospitals receiving blood that has already been irradiated by the supplier. It is interesting to note that while the typical RBC units transfused to the two groups in the ARIPI study (stored 5.1 vs. 14.6 days)⁹ should be distinguishable metabolically by our methodology, most members of the old RBC group received units that were not stored long enough to experience the most dramatic metabolic changes during blood storage. Further, the ARIPI study did not evaluate the additional effects of irradiation on stored RBCs.

A significant finding of this study is that irradiation of RBC units shifts the changes in metabolomic profiles of stored blood towards those of older samples, with this effect seen as early as 7 days of storage. Thus, gamma irradiation of RBCs appears to accelerate metabolic aging and the acquisition of the RBC storage lesion. As this effect is first seen at 10 days of storage, our data suggests that the use of fresh blood (< 7 days of storage) may shield recipients from receiving units that are metabolically altered by irradiation and pre-transfusion storage.

Our analysis yielded 4 candidate biochemical pathways most affected by RBC storage and/or irradiation. Several of these pathways are involved in eicosanoid metabolism, a key component of the cellular membrane. Dysfunction of the red cell membrane through alterations in these pathways may lead to RBC stiffening with loss of deformability and contribute to the clinical effects of the storage lesion³³. Alterations in the eicosanoid pathway in a murine model of RBC storage³⁴ also support the potential for metabolites within these pathways to serve as biomarkers for the RBC storage lesion. These changes in

metabolic pathways may be a consequence of increased oxidative injury to the RBC membrane, exacerbated by altered glutathione homeostasis and concomitant alterations in the RBC proteome with *ex vivo* aging^{21,22,35}. In targeted metabolomics analysis, we also found multiple significant correlations between metabolites altered by the storage conditions and those involved in glutathione metabolism. Although the pathway enrichment analysis did not reveal the GSH pathways to be a significant discriminator of storage and irradiation conditions, the correlation between known metabolites in the GSH pathway and discriminatory metabolites from msPLSDA analysis indicate that alterations in patterns of metabolites we detected may also coincide with similar changes in the GSH pathway and this remains an area for future study. Further study is needed to validate specific biomarkers detected in this study, including identifying metabolites that can identify donor RBCs that are associated with adverse transfusion-related events in recipients. Nevertheless, metabolite biomarkers remain a promising tool to evaluate the viability of donor RBCs that may be a more precise measure than the duration of RBC storage itself.

Our study has several limitations. We were unable to identify quantitative changes in each specific metabolite, as the focus of our analysis was on determining changes in the patterns of metabolite across the various storage conditions. Similarly, we compared the effects of both RBC storage and irradiation. As such, we were unable to determine if candidate pathways identified in enrichment analysis were due to the effects of irradiation, storage, or both. However, our additional analysis of metabolites associated with the arachidonic acid pathway suggests that irradiation and storage may have differential effects on alterations of some metabolites. Finally, our samples were irradiated on the day of RBC donation. Therefore, we were unable to determine if irradiation after a period of storage for 7–10 days would have similar effects on metabolite patterns as pre-storage irradiation.

In conclusion, the metabolomic characteristics of donor RBCs differ between fresh and stored blood and irradiation of RBCs accentuate these differences. Furthermore, we have identified candidate metabolites involved in membrane metabolism that may be involved in pathways related to the RBC storage lesion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Score plots comparing patterns of metabolites by irradiation status and storage age
Panel A. Separation of samples across msPLS-dim 1 is demonstrated between 7 and 10 days of storage, with clustering of both irradiated and control samples from 2 to 7 days of age (red circle). By contrast, separation of samples by both irradiation status and storage age is visible across msPLS-dim 2 from 10 days onwards (orange circles). **Panel B.** Near complete separation between irradiated and control RBC samples across msPLS-dim 3 is seen, independent of storage age. Separation in patterns of metabolites was detected as early as 7

days after storage between irradiated (orange circle) and non-irradiated samples (red samples).

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Figure 2. Dendrogram from two-way hierarchical clustering by irradiation status and storage age Twelve distinct clusters of RBC metabolites are identified along the y-axis. The first cluster (A) demonstrates different metabolite patterns between fresh (storage age of 2–7 days) and old RBCs (storage age >7 days). The second cluster (B) demonstrates differences in metabolite patterns between irradiated and control samples.

Figure 3. Kinetic changes in metabolites involved in the Arachidonate metabolism

Changes in standardized mean intensity over time between irradiated and non-irradiated samples are depicted for metabolites within the arachadonic acid pathway. Whisker bars indicate 95% confidence intervals (CI).

TABLE 1

Candidate pathways of metabolites disrupted by RBC storage and irradiation

Metabolite	Raw P	FDR
Arachidonic acid metabolism	3.28E-33	2.62E-31
Linoleic acid metabolism	1.61E-11	5.00E-10
Steroid hormone biosynthesis	1.88E-11	5.00E-10
alpha-Linolenic acid metabolism	1.75E-05	0.00035
Retinol metabolism	0.078191	1
One carbon pool by folate	0.24998	1
Sphingolipid metabolism	0.51166	1
Primary bile acid biosynthesis	0.57811	1
Glycerophospholipid metabolism	0.61518	1
Terpenoid backbone biosynthesis	0.70368	1
Biotin metabolism	0.71347	1
Lysine degradation	0.75885	1
Glycosylphosphatidylinositol-anchor biosynthesis	0.79645	1
Cyanoamino acid metabolism	0.83799	1
Sulfur metabolism	0.87107	1
Pyrimidine metabolism	0.89969	1
Caffeine metabolism	0.90851	1
Drug metabolism - cytochrome P450	0.91359	1
Ether lipid metabolism	0.92723	1
Inositol phosphate metabolism	0.93327	1

Abbreviations: RBC, red blood cell; FDR, false discovery rate.

Note: The top twenty known metabolites identified through pathway enrichment analysis from 599 detected metabolites are shown.