Hirosaki Med. J. 71 : 113—119, 2021

ORIGINAL ARTICLE

Relationship between red blood cell aging and intracellular chemokine storage

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Abstract

Background: The Duffy antigen receptor for chemokines (DARC) is expressed on RBCs and is a non-signaling receptor for multiple chemokines. In the previous study, we had shown that DARC-affinity chemokines are stored in the RBCs. However, the fate of intracellularly stored chemokines has yet to be investigated. This study investigated whether RBCs retain the ability to accumulate these chemokines as they age.

Methods: Peripheral blood from five healthy volunteers were collected, and then transferred to hematocrit capillary tubes. After centrifugation, the RBC layer was divided to compare the top, upper, middle and bottom fractions. HbA1c, eotaxin-1 and RANTES were measured in each fraction.

Results: HbA1c increased significantly from the top fraction to the bottom fraction. Eotaxin-1 decreased significantly from the top fraction to the bottom fraction. RANTES exhibited a similar decrease, although no significant differences were observed. Eotaxin-1 showed a negative correlation ($r = -0.51$, $p = 0.03$) with HbA1c. Although not statistically significant, RANTES showed a decreasing tendency ($r = -0.40$, $p = 0.08$) with the increase of HbA1c levels. These results indicate that RANTES and eotaxin-1 decrease rather than increase with RBC aging.

Conclusion: Eotaxin-1 and RANTES stored in RBCs tended to decrease as RBCs age.

Hirosaki Med. J. 71 : 113—119, 2021

Key words: RBC; DARC; chemokine; HbA1c; accumulation.

Introduction

Duffy antigen receptor for chemokines (DARC) is expressed on red blood cells (RBCs) and micro-vascular endothelial cells. DARC binds to several chemokines and can transport functionally intact chemokines across endothelial cells for presentation to leukocytes[1]. Although RBCs are understood to be mainly involved in respiratory gas exchange, they are thought to play a role in inflammatory reactions by regulating chemokine concentrations in the serum[2, 3]. DARC is thought to capture chemokines at times of high serum chemokine levels, subsequently releasing them when chemokine abundance is low[1, 2]. In contrast to the Duffy antigen expressed on endothelial cells, which internalizes and mediates intracellular translocation of the ligands, no internalization of DARC has been observed on RBCs after ligand binding[2]. Despite this lack of direct evidence, Yamamoto[4] and Karsten[3] have found the storage of some DARC-affinity chemokines including eotaxin-1 and regulated on activation, normal T cell expressed and secreted (RANTES) occurs in RBCs. The mechanism of chemokine storage in RBCs and the fate of intracellularly stored chemokines have yet to be elucidated. In this study, we investigated whether RBCs retain the ability to accumulate DARC-affinity chemokines as they age in the bloodstream.

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Received for publication, October 6, 2020
Accepted for publication, November 27, 2020

Materials and methods

Blood donors

The degree of aging of RBCs in the circulating blood is positively correlated with its specific gravity; immature RBCs are less dense and aged RBCs are more dense[5]. Moreover, when RBCs are fractionated using a specific gravity solvent or a microhematocrit tube, young/light RBCs are observed to express low levels of HbA1c and old/heavy RBCs express high levels of HbA1c[6–8]. Thus, HbA1c expression was used as an indicator of RBC age in this study. Several clinical conditions, such as anemia and pregnancy, influence RBC lifespan. Therefore, only samples from male subjects were examined in this study in order to avoid gender-associated effects on RBC lifespan. Blood samples were donated by five healthy male volunteers aged between 30 and 62 years, and were confirmed to be of the DARC-positive (Fya+/Fyb−) blood type. Written informed consent was obtained from every donor before sampling.

Fractionation of RBC layers and measurement of HbA1c and chemokines

Fractionation of RBCs by age was performed by centrifugation using a microhematocrit capillary tube[9]. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (1.5 mg/mL blood). To separate RBCs by age, a plastic microhematocrit capillary tube (1.8 φ × 75 mm) was filled with blood (45 μL) and centrifuged (1000 g, 10 min, 25 ℃). After centrifugation, the supernatant and the RBC layer were separated. At the time of separation, in order to avoid contamination of white blood cells and platelets, the microhematocrit capillary was cut at a distance of 2 mm from the boundary with the supernatant on the RBC layer side. The RBC layer in micro-capillaries was divided equally into 8 fractions by cutting the micro-capillary tube. HbA1c level in each fraction gradually increased from fraction 2 to 8 in order. Therefore, fraction 2, 5 and 8 were selected as the upper, middle and bottom fraction, respectively. Fraction 1 was also selected as the top. Because fraction 1, rich in young RBCs, had the lowest level of HbA1c with a statistically significant difference from that of fraction 2. The RBCs in each fraction were washed with 3.0 mL of saline (400 g, 5 min × 2). After washing, blood counts were performed with a XN-20® hematology analyzer (Sysmex, Kobe, Japan) to normalize the RBC counts (1.0×10^4 cells/μL) for HbA1C and chemokine measurements. A purity of > 99.99% was achieved. The washed RBCs were then lysed in 0.2 mL distilled water. HbA1c was measured immediately after hemolysis. Eotaxin-1 and RANTES were stored at −80° C until measurement. HbA1C was measured using an HLC-723 G11® Automated Glycohemoglobin Analyzer (TOSOH, Tokyo, Japan). The concentration of eotaxin-1 and RANTES was measured using ELISA kits (Quantikine®, R&D Systems, Inc., MN, USA) following the manufacturer's instructions. In brief, the samples were added in duplicate to appropriate pre-coated plates. After the plates were washed, the conjugated detection antibody was added. The substrate used for color development was tetramethylbenzidine. The optical density was measured at 450 nm with a Thermo Scientific Multiskan FC (ThermoFisher Scientific, PA, USA). The lower limit of quantitation for each measurement item was as follows; eotaxin-1, 5 pg/mL; RANTES, 2.0 pg/mL.

Statistical analysis

Data were analyzed using SPSS software version 23.0 (IBM, Chicago, IL, USA) and Statcel (3rd ed., OMS, Tokyo, Japan). Data were expressed as mean ± standard deviation (SD) for continuous data. The statistical significance of differences was determined using the Tukey-Kramer test and Spearman's rank correlation

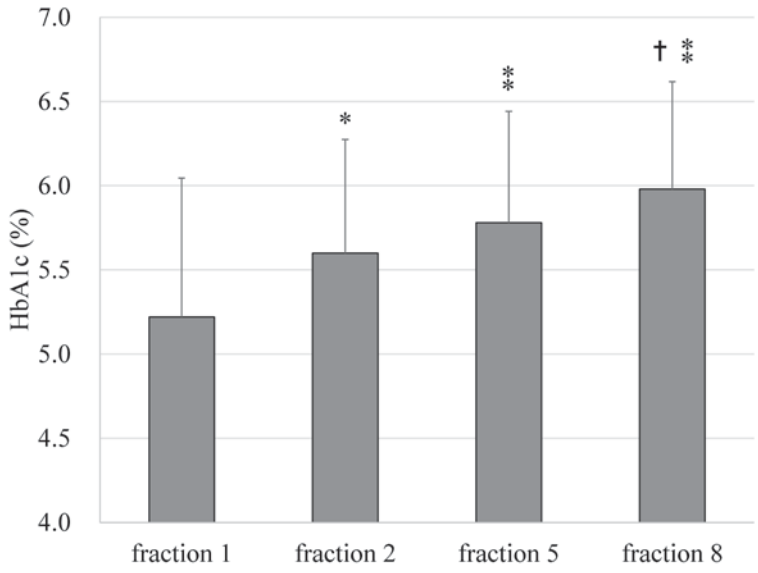

Figure 1 HbA1c levels in RBC fractions

HbA1c levels in RBC fractions. HbA1c was used as an indicator of RBC aging. Fractionation of aged RBCs was performed by centrifugation using a microhematocrit capillary tube. Blood samples were donated by five healthy male volunteers. After centrifugation, the RBC layer of the microcapillaries was divided equally into 8 fractions. Fractions 1, 2, 5 and 8 were selected for measurements in order to compare the top, upper, middle and bottom fractions, respectively. HbA1c increased significantly from the top fraction to the bottom fraction (*P<0.05; fraction 1 vs. 2, †P<0.05; fraction 2 vs. 8, ‡P<0.01; fraction 1 vs. 5, fraction 1 vs. 8).

coefficient test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

Ethical approval

The study protocol was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine (approval number: 2016-269).

Results

HbA1c and chemokine levels in red blood cell fractions

The HbA1C values (%) in fractions 1, 2, 5 and 8 were 5.2 ± 0.8, 5.6 ± 0.7, 5.8 ± 0.7 and 6.0 ± 0.6, respectively. HbA1c increased significantly from the top fraction to the bottom fraction (P<0.05; fraction 1 vs. 2, fraction 2 vs. 8, P<0.01; fraction 1 vs. 5, fraction 1 vs. 8) (Figure 1). Eotaxin-1 concentrations (pg/mL) in fractions 1, 2, 5 and 8 were 7.2 ± 2.5, 5.1 ± 0.3, 5.1 ± 0.2 and 3.5 ± 1.8, respectively. The concentration of eotaxin-1 decreased from the top fraction to the bottom fraction, and a significant decrease was observed between fraction 1 and 8 (P<0.05) (Figure 2). RANTES concentrations (pg/mL) in fractions 1, 2, 5 and 8 were 16.4 ± 17.9, 12.5 ± 10.3, 8.6 ± 9.4 and 8.4 ± 5.1, respectively. Although no significant differences in RANTES levels were detected, a trend toward decreased levels from the top fraction to the bottom fraction was observed (Figure 3). Correlation analysis between HbA1c levels and eotaxin-1 or RANTES levels was performed. When HbA1c and chemokine were ranked in ascending order of concentration, RANTES tended to decrease as HbA1c increased ($r = -0.40$, $p = 0.08$). On the other hand, eotaxin-1 showed a significant correlation ($r = -0.51$, $p = 0.03$).

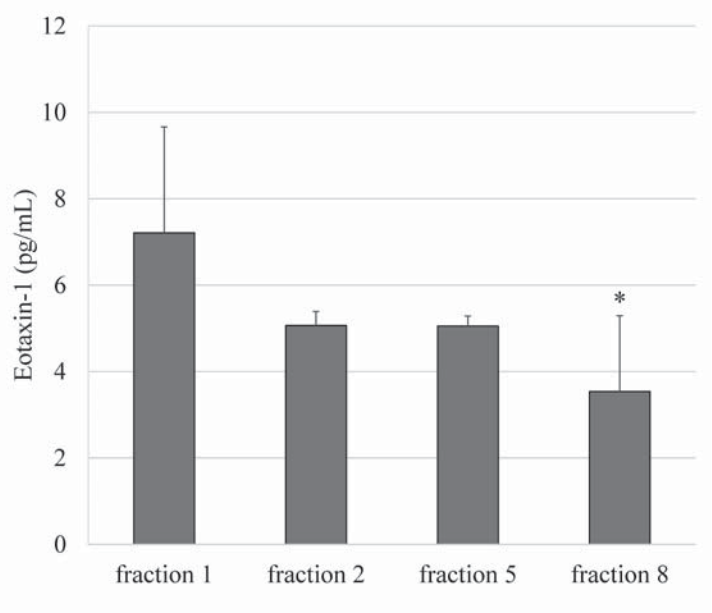

Figure 2 Eotaxin-1 levels in RBC fractions
Eotaxin-1 levels in RBC fractions (n = 5). The concentration of eotaxin-1 decreased from the top fraction to the bottom fraction. A significant decrease was observed between fractions 1 and 8 (*P<0.05).

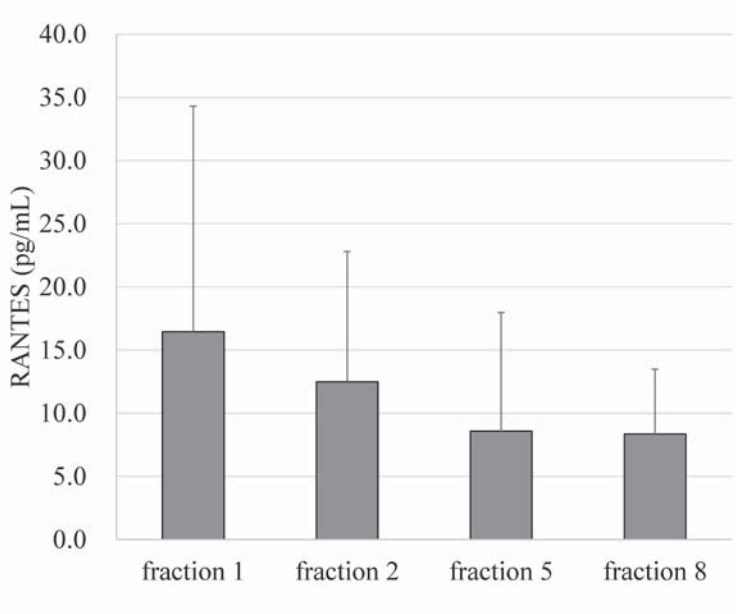

Figure 3 RANTES levels in RBC fractions
RANTES levels in RBC fractions (n = 5). RANTES decreased from the top fraction to the bottom fraction. There were no significant differences between the fractions.

Discussion

In this study, we investigated whether RBCs gradually accumulated chemokines during their lifespan and analyzed the concentrations of two chemokines to address this issue. However, eotaxin-1 and RANTES stored in RBC tended to decrease as RBCs age. Chemokines are cytokines that mainly regulate leukocyte migration and localization. They play a fundamental role in the

regulation of immune and inflammatory responses. Chemokine receptors belong to the family of seven transmembrane-spanning G protein-coupled receptors (GPCRs), with about 50 distinct ligands reported in humans[10, 11]. While chemokines transmit their signals through GPCRs, they also interact with scavenger/decoy receptors lacking the capability to elicit intracellular signal transduction[1]. Duffy antigen receptor for chemokines (DARC) is one such decoy receptor. The Duffy antigen first came to light in a report describing a determinant of the Duffy blood group system in 1950[12]. This antigen is also the receptor exploited by the malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi* for their entry into human RBCs[13, 14]. DARC is expressed not only on RBCs, but also on capillary endothelial cells in normal tissues such as the spleen, kidney, and lung[1, 15]. DARC also binds to multiple inflammatory CC and CXC chemokines such as RANTES (CCL5), monocyte chemotactic protein-1 (MCP-1/CCL2), eotaxin-1 (CCL11), thymus and activation-regulated chemokine (TARC/CCL17), and interleukin-8 (IL-8/CXCL8) [1, 16]. Yamamoto[4] showed that among the DARC-binding chemokines assayed, the levels of eotaxin-1, RANTES, and MCP-1 significantly increased after RBC hemolysis. Furthermore, when RBCs were stained on the surface and intracellular with FITC- or PE-conjugated antibodies, the fluorescence intensity showed the presence of intracellular eotaxin-1, RANTES, and MCP-1, but was not detected by surface staining. We hypothesized that if RBCs could accumulate chemokines throughout their life in the bloodstream, the amount of chemokines stored in RBCs would increase as RBCs age, and could be utilized as clinical indicators of chronic inflammatory diseases. Contrary to our expectation, we observed that eotaxin-1 and RANTES did not increase but rather decreased as RBCs aged. These results suggest the possibility of chemokines being released from RBCs as they age. In the study of blood transfusion, there are reports that transfusion of RBCs that had been stored for more than 2 weeks was associated with a significantly increased risk of postoperative complications as well as reduced short-term and long-term survival in patients undergoing cardiac surgery[17]. In addition, in experiments with mice, transfusion of old RBCs can cause a cytokine storms such as IL-6, CXCL1 and MCP-1[18]. However, the relationship between cytokine decrease due to RBC aging and specific diseases has not been fully elucidated. Chemokine release by aged RBCs may be one of the reasons for the duration of RBCs storage and adverse events. Therefore, the washing of RBCs may be useful for avoiding these risks. However, since the amount of chemokines contained in RBCs is very small compared such as platelets[19], adverse reactions to the use of old RBCs are caused by a variety of factors, not just the chemokines in the RBCs. On the other hand, regarding the relationship between aging (actual age) and chemokine, Seidler[20] showed that serum MCP-1 concentrations increased with age in 181 healthy volunteers (median age 42, range 18-88). However, there are few reports of chemokine in RBCs aging, and further studies are needed. In a study using DARC knockout mice, the plasma concentrations of eotaxin-1 and MCP-1 were significantly lower in knockout mice relative to wild-type mice. In contrast to wild-type mice, infusion of these chemokines resulted in the rapid clearance of the chemokines in plasma of knockout mice[21]. Kersten et al. reported 46 cytokines were detected in RBC lysates, and the median concentration in RBCs was 12-fold higher than in the plasma[3]. These studies suggest that RBCs function as a chemokine sink. Furthermore, another study reported that RBCs lose about 20% of their volume during their 120-day life due to the release of RBC microparticulates[22]. In this study, HbA1c was used as an index of RBC

aging, and its relationship with the accumulation of DARC-affinity chemokines was investigated. The primary limitation of this study is that HbA1C was used in the study as an indicator of RBC aging; however, there are differences in RBC turnover among individuals. Eotaxin-1 and RANTES stored in RBC tended to decrease rather than to increase as RBC ages. The mechanism of the decrease needs further study such as to investigate change in DARC expression and the ability to absorb chemokines in aged RBCs.

Authorship

SO was responsible for analysis and interpretation of the data, and drafting the manuscript. NS was responsible for analysis and interpretation of the data. SM was responsible for data collection, statistical analysis and interpretation. TA shared responsibility for study design and interpretation of the results of the study. HK was responsible for the design of the study and approval of the manuscript version prior to submission. All authors read and approved the final manuscript.

Acknowledgement

The authors have no conflict of interest related to this study. The authors are thankful to Mrs. Mihoko Kushibiki for help with sample preparation.

Declaration of Interest

All authors have no conflicts of interest directly relevant to the content of this article.

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