Title:

Intracellular storage of Duffy antigen-binding chemokines by Duffy-positive red blood cells

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Declaration of Interest

All authors declare that they have no conflict of interest.

Running title: Chemokines stored in red blood cells

Summary

Background: Duffy antigen/chemokine receptor (DARC**)** is a non-signaling receptor for multiple chemokines. The role of DARC on red blood cells (RBCs) has remained elusive. The purpose of this study was to analyze selective storage of DARC-binding chemokines in RBCs.

Methods: Peripheral blood from healthy volunteers of DARC-positive blood type was collected in EDTA tubes. The concentration of DARC binding chemokines (i.e., MCP-1, RANTES, eotaxin-1, TARC and IL-8), of DARC non-binding chemokines (i.e., MIP-1α, IP-10) and of several cytokines in the supernatant of purified RBCs before and after hemolysis was measured using Bio-Plex and ELISA assays. Storage of chemokines in RBCs and the expression of DARC were evaluated using flow-cytometry.

Results: The levels of all DARC-binding chemokines except TARC and IL-8 increased significantly after hemolysis. There was no significant increase in any of the DARC-non-binding chemokines or in the other cytokines after hemolysis. RANTES, eotaxin-1 and MCP-1 were detectable intracellularly but not on the RBC surface. RANTES was absorbed by RBCs. DARC was expressed intracellularly in RBCs as well as on the surface.

Conclusions: These data suggested that DARC-positive RBCs store RANTES, MCP-1 and eotaxin-1. DARC on RBC may be internalized from the surface in the process of chemokine absorption.

Key words: Chemokine, DARC, RBC, RANTES, IL-8

Introduction

The Duffy antigen first came to light in a report describing a determinant of the Duffy blood group system in 1950[1]. This antigen is also the receptor exploited by the malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi* for their entry into human red blood cells (RBC) [2, 3]. Another function of the Duffy antigen is as a receptor for binding to multiple inflammatory CXC and CC chemokines such as interleukin-8 (IL-8/CXCL8), monocyte chemotactic protein-1 (MCP-1/CCL2), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), eotaxin-1 (CCL11), and thymus and activation-regulated chemokine (TARC/CCL17)[4]. Duffy antigen receptor for chemokines (DARC) does not produce intracellular signaling events [5]. DARC is expressed not only on erythrocytes, but also on endothelial cells of capillaries in normal tissues such as the spleen, kidney and lung [6]. It has been suggested that the function of DARC on endothelium might be to transport abluminal chemokines transcellularly, leading to their subsequent presentation to blood leukocytes [7]. In contrast, DARC on RBC was suggested to scavenge chemokines in plasma, leading to the hypothesis of DARC on RBC as a chemokine sink [8]. In contrast to the Duffy antigen expressed on endothelial cells, which internalizes and carries the ligands intracellularly, it has been reported that the DARC on RBCs cannot be internalized after binding to its ligands [8]. However, in our preliminary study we showed the presence of intracellularly stored RANTES in RBCs. In the present study, selective storage of some of the chemokines with high affinity to DARC in RBCs was shown. Furthermore, intracellular expression of DARC was confirmed by flow cytometry.

Materials and methods

Blood donors

Blood samples were donated by healthy volunteers aged between 22 and 25 years whose blood type was confirmed to be DARC-positive. Written informed consent was obtained from every donor before sampling.

Measurement item

The concentration of five DARC-binding chemokines (IL-8, MCP-1, RANTES, eotaxin-1, TARC), two DARC non- or low-binding chemokines (macrophage inflammatory protein: MIP-1α, interferon gamma inducible protein 10: IP-10 and other cytokines (tumor necrosis factor-α: TNF-α, IL-1β, vascular endothelial growth factor: VEGF) was measured in phosphate buffered saline (PBS)-suspended purified RBCs before and after hemolysis.

Isolation of RBCs

Blood samples were collected in ethylenediaminetetracetate (EDTA) as an anticoagulant (1.5 mg/mL blood). Samples were immediately diluted with PBS. After centrifugation for 5 minutes at

400 *g*, 0.5 mL of RBCs were re-suspended and washed twice in PBS. To minimize contamination of platelets and leukocytes, RBCs were taken from the bottom of the tubes. The RBCs were then layered gently on top of Percol® gradient medium (Amersham Pharmacia Biotech AB, Sweden) adjusted to a gradient of 1.100 g/mL. After centrifugation for 15 minutes at 800 *g*, the RBCs were collected from the bottom of the tubes. A purity of > 99.99% was achieved.

Quantification of chemokines and cytokines

The purified RBCs were suspended in PBS (or distilled water for hemolysis) using three times the volume of the purified RBCs. After centrifugation for 5 minutes at 400 *g*, the supernatant was kept at -20 °C before analysis. The supernatant of RBCs suspended in PBS and distilled water was used as the sample before and after hemolysis, respectively. The concentration of IL-1β, TNF-α, IL-8, eotaxin-1 and VEGF was measured using the Bio-Plex® suspension array system 171-000001 (Bio-Rad, CA, USA) following the manufacturer's instructions. In brief, samples were incubated with capture antibody-coupled magnetic beads for 60 minutes. Following three washes, samples were then incubated with biotinylated detection antibody for 30 minutes. Each captured analyte was detected by the addition of streptavidin-phycoerythrin and was quantified using a Bio-plex® array reader and Bio-Plex® manager software. The concentration of MCP-1, RANTES, MIP-1α, TARC and IP-10 in the RBC supernatant before and after hemolysis was assayed 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 microplate reader (AP-960, Kyowa medics, Japan). The lower limit of quantitation for each measurement item was as follows; eotaxin-1, 40.9 pg/mL; IL-1β, 3.2 pg/mL; IL-8, 1.9 pg/mL; TNF-α, 0.2 pg/mL; VEGF, 5.5 pg/mL, RANTES, 31.2 pg/mL; TARC, 31.2 pg/mL; MCP-1, 31.2 pg/mL; IP-10, 7.8 pg/mL; and MIP-1α, 31.2 pg/mL.

Measurement of lactate dehydrogenase concentration

With the aim of confirming that intracellularly stored RANTES was the source of the increased RANTES in the RBC supernatant after hemolysis, the correlation between the concentration of RANTES and lactate dehydrogenase (LDH) in the supernatant was evaluated using RBCs from one donor. LDH concentration was used as an indicator of the extent of hemolysis. The extent of hemolysis could be controlled by suspending the RBCs in solutions with various osmotic pressures ranging between 30-50% of that of normal serum. The concentration of LDH in the supernatant of various percentages of hemolyzed RBCs was measured with the BioMajesty JCA-BM2250 auto-analyzer (JEOL, Japan) using an enzymatic method.

In vitro culture of RBCs with RANTES for the study of RANTES absorption

To examine RANTES absorption by RBCs, 100 µL of purified RBCs were suspended in 300 µL of PBS supplemented with human-recombinant RANTES (SIGMA-ALDRICH, MO, USA) at a concentration of 0 or 6.0 ng/mL. This mixture was then incubated at 37 °C. The supernatant was then separated from the RBCs by centrifugation for 5 minutes at 400 *g*. The obtained RBCs were washed three times with 3 mL of PBS per wash. One hundred micro liters of washed RBCs were hemolyzed by adding 300 µL of distilled water. The concentration of RANTES in the supernatant and in the RBC lysate was monitored at 10 minutes, 1, 3 and 6 hours after incubation. The measured value was quadrupled in hemolyzed samples to adjust for the 4-fold dilution with distilled water that occurred in the process of hemolyzation.

Flow cytometry (FCM)

Surface and intracellular staining of RBCs

The expression of DARC and CD47 by RBCs, and the presence of RANTES, MCP-1, eotaxin-1 and IL-8 in RBCs were investigated using FCM. FACSCan™ and FACSCanto™II (Becton, Dickinson and Company, NJ) were used for the FCM analysis. The following antibodies (Ab) and control Igs were used for flow cytometry: fluorescein conjugated mouse anti-human IL-8 (# 6217), fluorescein conjugated mouse IgG_1 isotype control (# 11711), fluorescein conjugated mouse anti-human DARC (# 350387), Carboxy fluorescein (CFS) conjugated mouse IgG_{2A} isotype control (# 20102), CFS conjugated mouse IgG_{2B} isotype control (#133303) , fluorescein conjugated mouse anti-human MCP-1 (# 23002), Phycoerythrin (PE) conjugated mouse anti-human eotaxin-1 (#43915), and PE conjugated mouse IgG1(#11711), all from R&D Systems, Inc., MN, USA; PE conjugated mouse anti-human RANTES (#2D5), all from BD Pharmingen, CA, USA. Intracellular staining of RBCs was performed in accordance with the method proposed by Davis et al. in 1988 [9]. In brief, the RBCs were fixed in 1 mL of 0.05% glutaraldehyde for 10 minutes at room temperature and were then washed three times with PBS. The RBCs were permeabilized by re-suspending in 0.5 mL of 0.15% Triton X-100 (Sigma, MO, USA) for 3 minutes before intracellular staining.

RBCs were cultured with recombinant human IL-8 (208-IL-010, R&D Systems, Inc., MN, USA) before the intracellular staining for IL-8 in some experiments.

Surface DARC and surface plus intracellular DARC were measured as follows. For surface DARC staining, cells were incubated with FITC-conjugated anti-DARC Ab or isotype-matched negative control Ab, and the RBCs were then fixed, permeabilized, and incubated with FITC-conjugated isotype-matched negative control Ab again to measure non-specific intracellular antibody binding. For both surface and intracellular DARC staining, intracellular DARC was stained with anti-DARC Ab following surface staining and permeabilization. Control samples were stained with

isotype-matched negative control Ab both on the surface and intracellularly. In brief, the sample for surface staining was surface stained with FITC-conjugated anti-DARC Ab and was intracellularly stained with isotype-matched negative control Ab. Whole cell (i.e., surface and intracellular) staining was performed with FITC-conjugated anti-DARC Ab before and after permeabilization. Considering the differential expression of DARC according to RBC FY genotype [10, 11], the samples were obtained from five healthy volunteers with Fya(+) Fyb(-) blood type.

Calculation of delta-mean fluorescent intensity (MFI)

The ΔMFI was calculated as an indicator of the quantity of the measurement items. ΔMFI was calculated by subtracting the mean fluorescence of the sample stained with an isotype matched negative control antibody from that of the sample stained with a specific antibody.

Statistical analysis

The data are expressed as means ± standard error of the mean (SEM). The statistical significance of differences was determined by Student's t-test for comparison between two groups or by one-way ANOVA–Tukey–Kramer post hoc test for multiple comparisons. Correlation between two variables was investigated by calculating the Pearson product-moment correlation coefficient. A p value of less than 0.05 was considered statistically significant. Statistical analysis was performed using the Microsoft Excel software program with the add-in software Statcel3 (OMS Inc. Japan).

Ethics

This study was approved by the ethics committee of Hirosaki University Graduate School of Medicine.

Results

Intracellular chemokines in RBC

Among the five DARC-binding chemokines assayed, the levels of eotaxin-1, RANTES and MCP-1 increased significantly after RBC hemolysis. IL-8 and TARC were not detectable before or after hemolysis. The levels of the two DARC non-binding chemokines (MIP-1α and IP-10) and of the other cytokines (TNF-α, IL-1β and VEGF) showed no significant increase after hemolysis (Table 1). FITC- or PE-conjugated antibodies were used for intracellular staining of RANTES, eotaxin-1, MCP-1 and IL-8 in three to five samples from different donors. The fluorescent intensity histograms of these samples stained with specific antibodies indicated the presence of intracellular RANTES, eotaxin-1 and MCP-1, but not of intracellular IL-8, in all of the samples (Figure 1). RANTES, eotaxin-1 and MCP-1 were not detected by surface staining. These data suggested that RANTES, eotaxin-1 and MCP-1 were stored intracellularly in RBC. In a series of hemolyzed samples (n=12),

the RANTES concentration ranged from 237 to 5,820 pg/mL (median; 1,150 pg/mL).

Correlation between LDH and RANTES in a series of samples with various level of hemolysis

A high correlation (ρ=0.87, p<0.01) was observed between RANTES and LDH concentration, which was used as an indicator of the extent of RBC hemolysis, in a series of samples obtained from one donor that were adjusted to various levels of hemolysis. This result was compatible with the flow cytometric finding that indicated intracellular storage of RANTES in RBCs.

RANTES absorption by RBC

We next determined if RBCs in PBS could absorb RANTES that was added to the PBS. When 100 μL of RBCs were added to 300 μL of PBS supplemented with RANTES at a concentration of 6 ng/mL, the concentration of RANTES in the PBS showed a significant (p < 0.01) decrease within 10 minutes of incubation at 37 ℃ (Figure 2). To confirm that the RANTES in the PBS had been absorbed by the RBCs, the intracellular concentration of RANTES was measured. The RANTES concentration in RBCs increased within 10 minutes after the RBCs were added to PBS containing RANTES (Figure 2). Considering the wide range of serum RANTES concentration [12, 13], we confirmed RANTES absorption by RBCs at RANTES concentrations of 1 ng/mL and 10 ng/mL. Rapid absorption of RANTES by RBCs was observed at both of these concentrations (data not shown).

Surface and intracellular expression of DARC by RBCs

The storage of DARC-binding chemokines in RBCs demonstrated above suggested the possible involvement of DARC internalization as previously reported in endothelial cells [7]. The presence of DARC in RBCs was therefore investigated using FCM. DARC was detected both on the RBC surface and intracellularly in every sample (Figure 3). The surface expression level of DARC differed from one sample to another. The lowest value of ΔMFI for the surface expression of DARC among the samples was 43.3% of the highest value.

Intracellular staining for IL-8 in RBCs cultured with IL-8

Based on the results that IL-8 was not stained intracellularly using RBCs sampled from healthy volunteers, we then performed intracellular staining for IL-8 using RBCs cultured with a high concentration (0.3 ng/mL) of IL-8 for 24 hours. IL-8 was not detected intracellularly even after exposure of the RBCs to a high concentration of IL-8 (n=3, data not shown).

Discussion

DARC expression on RBC has been hypothesized to modulate chemokine bioavailability by acting as a chemokine sink [8] or as a reservoir [14]. However, it is still not fully understood where chemokines are kept after binding to DARC on RBC. DARC has a promiscuous chemokine binding profile with high affinity for multiple chemokines such as RANTES, eotaxin-1, TARC, MCP-1, MCP-2, MCP-3, GRO-α and IL-8 [15]. In the present study, it was shown that the concentration of RANTES, eotaxin-1and MCP-1in the RBC suspension solution increased after RBC hemolysis. On the other hand, no significant change in the concentration of DARC non-binding chemokines and cytokines was observed after hemolysis. Rapid absorption and storage of RANTES by RBC were also shown in this study. These findings suggested the selective absorption and storage of DARC-binding chemokines by DARC-expressing RBCs. IL-8, MCP-1 and RANTES bind to DARC with K_d binding values of 20.0±4.7, 33.9±7.0 and 41.9±12.8 nM, respectively [16]. Although IL-8 has a higher affinity for DARC than RANTES and MCP-1, the levels of IL-8 did not increase in the RBC suspension solution after RBC hemolysis, nor was IL-8 stained in RBCs from healthy subjects in the present study. These results regarding IL-8 could be explained by the lack of internalization of IL-8 after binding to DARC [8], or by the much lower physiological concentration of IL-8 (24.4-35.9pg/mL) in the serum of healthy subjects than that of RANTES (5,147-6,089pg/mL) [17]; however, IL-8 was not observed in RBCs even after RBCs were exposed to a high concentration of IL-8 in this study. Middleton et al. [18] observed IL-8 transport by DARC internalization in endothelial cells. We could not observe IL-8 transport and intracellular storage by RBCs. The interaction between IL-8, CXC chemokine, and DARC may be different from that of DARC-binding CC chemokines such as RANTES, eotaxin-1 and MCP-1. DARC internalization and chemokine transport in RBCs is not yet understood. The average serum TARC level of healthy adults has been reported as 215.34 ± 26.79 pg/mL by Kakinuma et al. [19]. Further study is required to determine whether the RBCs of patients with atopic dermatitis collect TARC intracellularly at a higher concentration than non-atopic individuals.

In the present study, it was shown that DARC was expressed intracellularly as well as on the surface of RBCs. DARC is expressed on erythroid cells in the bone marrow [20, 21] and is exposed to DARC-binding chemokines in the circulating blood through their lifespan**.** The presence of DARC and DARC-binding chemokines in RBCs suggest that DARC may suggest that DARC-binding chemokines are absorbed by RBCs through DARC internalization.

This study has several limitations. The RBCs used in this study were taken from volunteers with a DARC-positive blood type. Further experiment using DARC-negative RBCs would be required to understand the role of DARC in the selective absorption and storage of some of the DARC-binding chemokines. The fate and the role of the absorbed chemokines have yet to be determined. The clinical relevance of DARC-binding chemokines stored in RBCs could not be discussed because the

blood donors were all young and healthy volunteers. Internalization of DARC was not directly shown in this study. Further study is required to clarify DARC internalization in RBCs.

Conclusions

The data of this study suggested that Duffy-positive RBCs absorb and store some of the DARC-binding chemokines. The clinical relevance of DARC-binding chemokines stored in RBCs needs to be clarified.

Acknowledgements

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Figure legends

Figure 1. Fluorescence intensity histograms of RBCs stained for RANTES, IL-8, MCP-1 and eotaxin-1

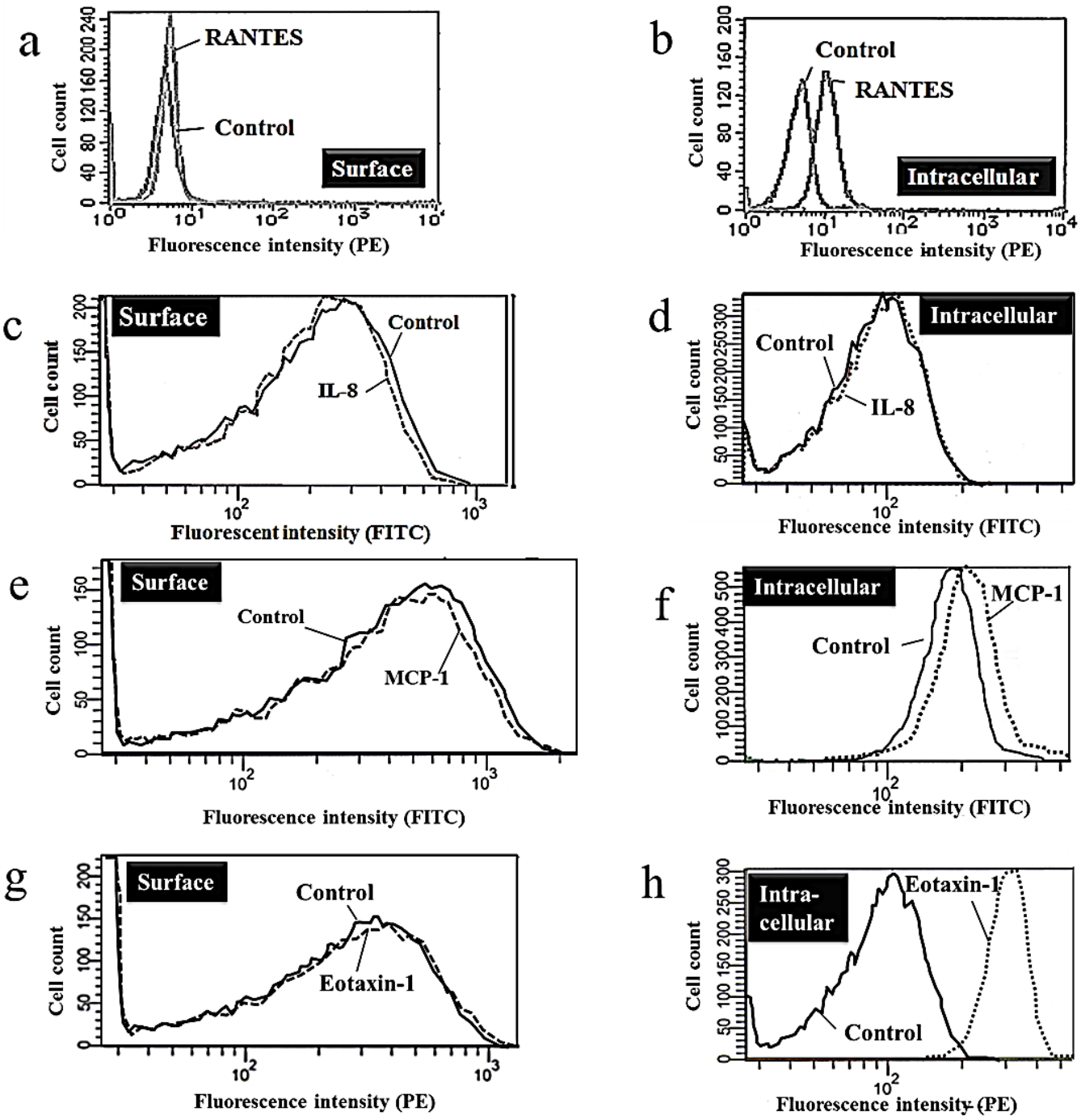
The surface (a) and intracellular (b) expression of RANTES were analyzed by FACSCan. Surface and intracellular levels of IL-8 (c and d), MCP-1 (e and f) and eotaxin-1 (g and h) were also analyzed by FACSCanto^{TM}II. Representative results from three to five independent experiments using blood from different donors were shown for each chemokine. PE conjugated mouse anti-human RANTES (#2D5), FITC conjugated mouse anti-human IL-8 (# 6217), FITC conjugated mouse anti-human MCP-1 (# 23002) and PE conjugated mouse anti-human eotaxin-1 (#43915) were used for the detection of each chemokine.

Figure 2. Fig.2 shows the release of RANTES from hemolyzed RBC as well as the adsorption of RANTES by intact RBC.

The sequential change of RANTES concentration in RBCs cultured with (solid squares) or without (open squares) RANTES are shown. RBCs from different donors were used in the experiments. Solid triangles indicate the change in RANTES concentration in PBS with prior supplementation of 6 ng/ml of RANTES. (n=3, *: $p<0.05$, **: $p<0.01$, vs. before culture; bars represent standard error of the mean)

Figure 3. Overlay presentation of fluorescent intensity histograms for DARC expressed by RBCs

Surface and total (surface plus intracellular) DARC expression by RBCs were shown using flowcytometry. The dotted line indicates the surface expression, and the broken line indicates the total expression of DARC. The shaded histogram indicates the control. Representative results from seven independent experiments using different donors were shown.


a
RANTES
Control
Surface
Cell count
Fluorescence intensity (PE)
b
Control
RANTES
Intracellular
Cell count
Fluorescence intensity (PE)
c
Surface
Control
IL-8
Cell count
Fluorescent intensity (FITC)
d
Intracellular
Control
IL-8
Cell count
Fluorescence intensity (FITC)
e
Surface
Control
MCP-1
Cell count
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Intra-
cellular
Eotaxin-1
Control
Cell count
Fluorescence intensity (PE)

Figure 1

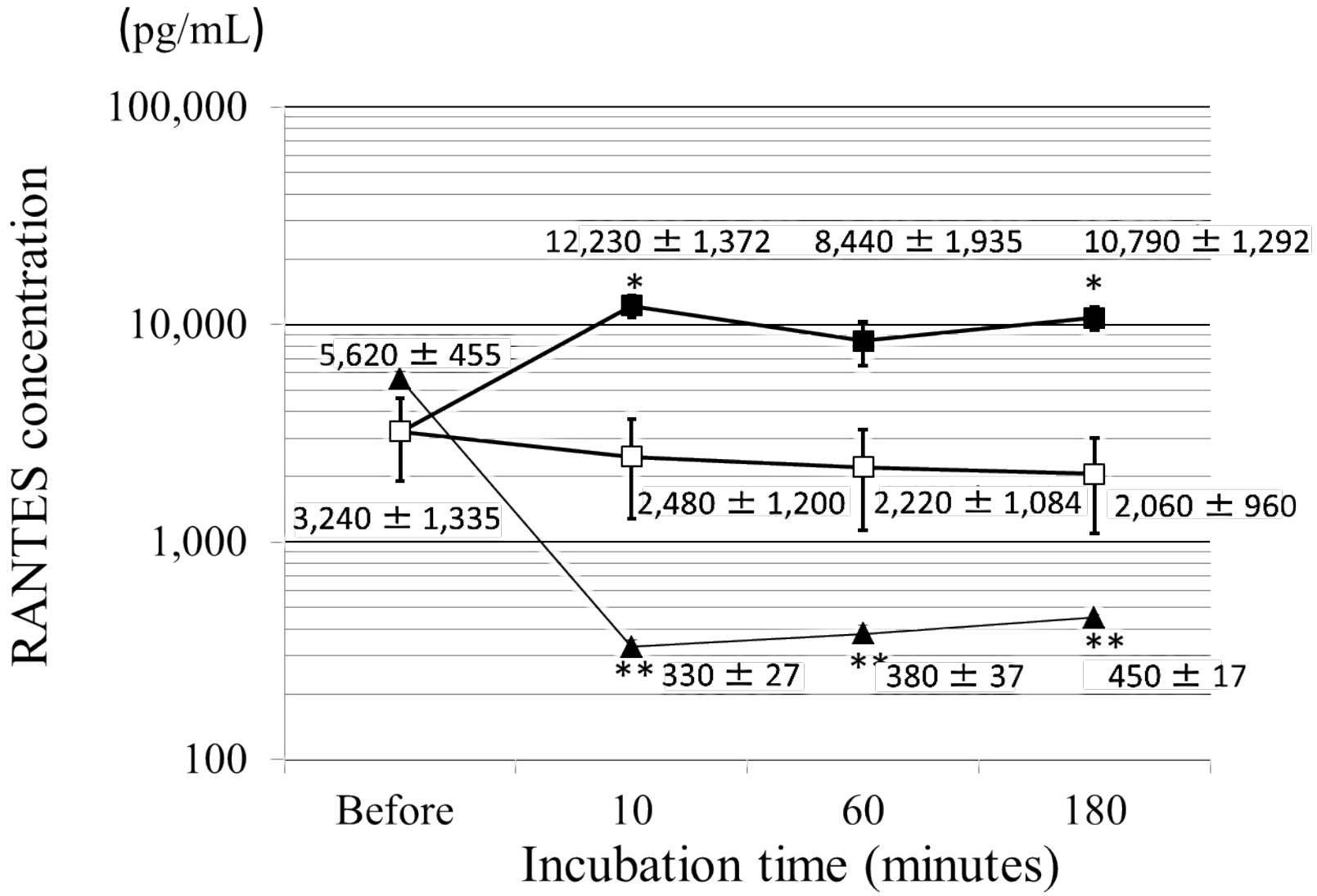
(pg/mL)
100,000
10,000
1,000
100
RANTES concentration
12,230 ± 1,372
8,440 ± 1,935
10,790 ± 1,292
5,620 ± 455
3,240 ± 1,335
2,480 ± 1,200
2,220 ± 1,084
2,060 ± 960
** 330 ± 27
** 380 ± 37
** 450 ± 17
Before
10
60
180
Incubation time (minutes)

Figure 2

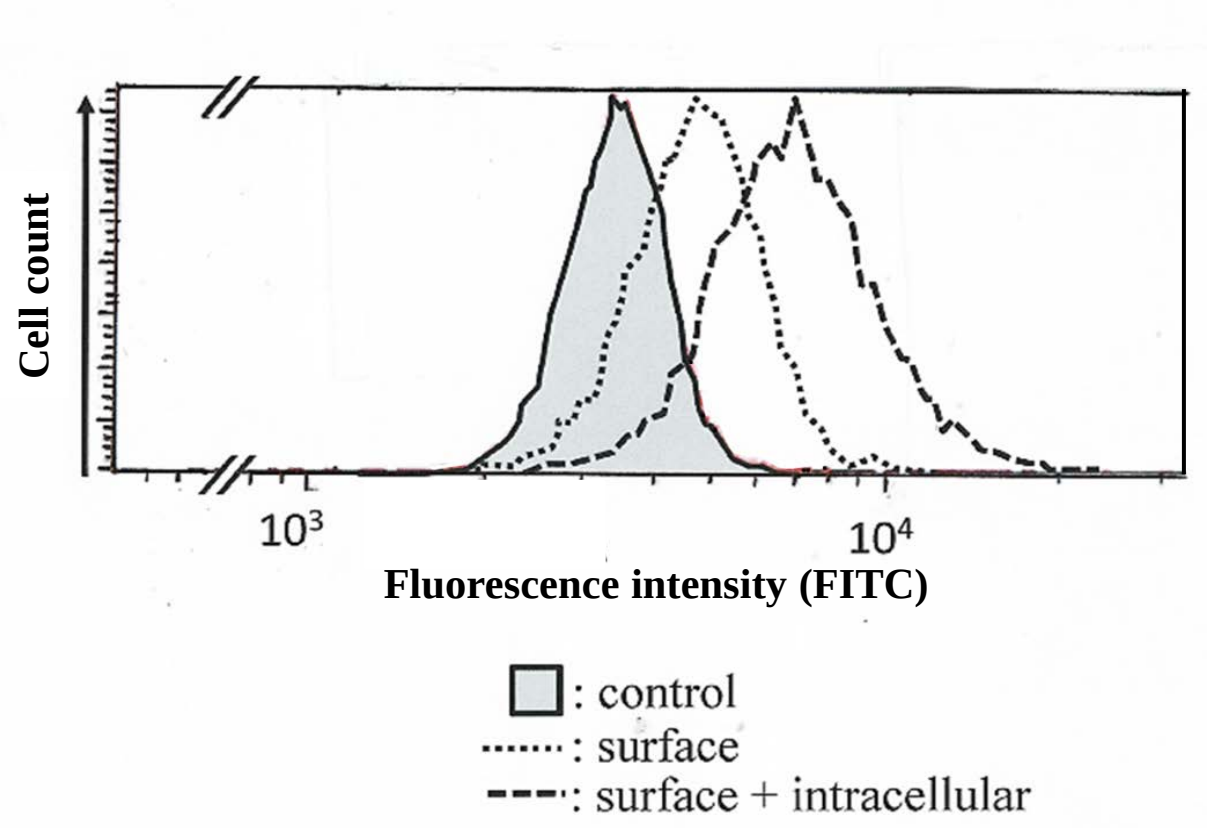
Cell count
10³
10⁴
Fluorescence intensity (FITC)
: control
: surface
: surface + intracellular

Figure 3

Table 1. Comparison between chemokine concentrations before and after hemolysis

DARC affinity	Substance	Before hemolysis (pg/mL)	After hemolysis (pg/mL)	n	*p* -value	Assay
High	Eotaxin-1	147.4 ± 4.4	310.4 ± 36.2	6	$p < 0.01$	BioPlex
	RANTES	ND*	398.0 ± 119.7	5	$p < 0.01$	ELISA
	MCP-1	ND*	99.4 ± 18.3	6	$p < 0.01$	ELISA
	TARC	ND*	ND*	5	NA†	ELISA
	IL-8	ND*	ND*	6	NA†	BioPlex
Low	IP-10	ND*	ND*	5	NA†	ELISA
No	MIP-1α	ND*	ND*	5	NA†	ELISA
	IL-1β	ND*	ND*	6	NA†	BioPlex
	TNF-α	10.9 ± 1.0	9.7 ± 0.8	6	NS‡	BioPlex
	VEGF	26.7 ± 6.0	37.1 ± 6.0	6	NS‡	BioPlex

Data are expressed as mean ± standard error of mean.
*: Not detectable, †: Not applicable, ‡: Not significant.