Available online at www.sciencedirect.com
ScienceDirect
Clinica Chimica Acta 383 (2007) 153–157
www.elsevier.com/locate/clinchim

Short communication

Quantitative flow cytometric analysis of transferrin receptor expression on reticulocytes

Mari Ervasti *, Irma Matinlauri, Kari Punnonen

Department of Clinical Chemistry and Hematology, Kuopio University Hospital, PL 1777, 70211 Kuopio, Finland

Received 2 November 2006; received in revised form 16 April 2007; accepted 16 April 2007
Available online 25 April 2007

Abstract

Background: A quantitative flow cytometric (FCM) method for transferrin receptor (TfR) expression on reticulocytes was developed and the results were compared with the markers of iron status.
Methods: A quantitative FCM analysis was performed using the Quantum™ Simply Cellular® kit, according to which the antibody binding capacity (ABC) of TfR expression on reticulocytes was measured using a monoclonal antibody (CD71). Thiazole orange dye was used to identify reticulocytes. The proportion of TfR positive reticulocytes (%TfR^+Ret) of all reticulocytes was also analyzed. The population consisted of 46 patients and 12 controls. Patients were categorized (based on Advia 120 cellular indices and serum iron status parameters) as having replete iron status, functional iron deficiency (FID), and as FID combined with depletion of iron stores (FID+ID).
Results: The TfR expression (ABC values) were higher in FID (1763±922, $p<0.001$) and FID+ID (1441±727, $p=0.05$) groups in comparison with the controls (663±110). Also, the %TfR^+Rets were significantly higher in iron deficiency states than in controls.
Conclusions: The quantitative FCM method for TfR expression on reticulocytes was found to reflect iron status at the cellular level. The potential usefulness of this method should be evaluated further in larger and more defined study populations.

Keywords: Iron status; Reticulocyte; Quantitative flow cytometry; Transferrin receptor

1. Introduction

The transferrin receptor (TfR) is a cell surface protein involved in the transport of iron bound to transferrin from the extracellular space into the cells by endocytosis, after which a fragment of the TfR is shed in a circulating soluble form into plasma (soluble TfR, sTfR) [1,2]. The expression of the TfR is by far the most abundant on the surface of the erythroid precursors in the bone marrow [3,4]. TfRs exist on the erythroid cells at all stages of maturation till the latest immature reticulocytes that enter the blood stream [5–7]. TfR expression on the cells and, thereby, also the level of plasma sTfR is increased in iron deficiency [2,8]. Plasma sTfR is also increased as a mass effect under conditions of accelerated erythropoiesis [2,9]. One could potentially eliminate the contribution of erythropoietic mass to the increase in TfR level by measuring the essential iron requirement at the cellular level. The Quantum™ Simply Cellular® (QSC) assay, designed for quantitative flow cytometry (FCM), enables the evaluation of the cell surface antigen expression [as a unit of antibody binding capacity (ABC)] using directly immunofluorescence-labeled monoclonal antibodies (mAb) [10,11]. In the present study, the FCM analysis of TfR (CD71) expression on reticulocytes was developed and the results were compared with iron status measurements.

2. Materials and methods

The study protocol was approved by the local ethics committee of the Pohjois-Savo Health Center District. The studied blood count samples ($n=46$) had been drawn during three months in 2005 and in 2007 for clinical purposes from patients treated at Kuopio University Hospital. The samples were selected on the basis of high percentage of hypochromic red blood cells (RBC) (%HYPOm) (>3.4%) or reticulocytes (%HYPOr) (>43.5%) [12], or a low mean cellular volume (MCV) (<82 fl) or a hemoglobin concentration (Hb) <100 g/l. Two of the patients were selected because their bone marrow samples were examined on the same day as FCM analyses were performed, and one patient had increased proportion of

* Corresponding author. Tel.: +358 50 3458925; fax: +358 17 173200.
E-mail address: mervasti@hytti.uku.fi (M. Ervasti).

0009-8981/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2007.04.012

reticulocytes (>2%). The population was completed with control samples from healthy volunteers ($n=12$).

The whole population consisted of 22 men and 36 women. The mean (±SD) age of the subjects was 54 (±22). Only one patient was 15 years old, and the remaining patients were over 18 years old. The patients had variable diagnoses: surgical procedures ($n=13$), hematological malignancies ($n=3$), solid tumors ($n=7$), cardiovascular diseases ($n=5$), hemodialysis ($n=3$), polycytemia vera ($n=1$), and the rest ($n=14$) had other diseases. The study groups were divided retrospectively on the basis of %HYPOm, %HYPOr, ferritin concentration, and TfR-F Index (sTfR/LogFerritin). Of the 46 patients, 24 were assigned to the functional iron deficiency (FID) group (having %HYPOm > 3.4% or %HYPOr > 43%), while the combined FID and iron deficiency (FID + ID) ($n=10$) comprised those patients, who fulfilled either one or both of the former criteria, and ferritin concentration was below 12 μg/l or TfR-F Index was above 2. The remaining 12 patients were assigned to the group with "replete iron status".

Venous blood samples had been collected in EDTA tubes (Vacutainer™, Becton Dickinson Vacutainer Systems, Plymouth, UK) for the analysis of the red blood cell and reticulocyte indices, which were analyzed within 2 h using the Advia 120 Hematology System (Bayer Diagnostics [Co.], Tarrytown, NY, USA) [12]. The reticulocyte counting method was based on the staining of reticulocyte RNA using the dye oxazine 750 (Bayer Diagnostics [Co.], Tarrytown, NY, USA). The Advia 120 analyzer was calibrated using OptiPoint (TO3-3682-01) calibration beads.

The analysis of the quantitative expression of TfR on the reticulocytes by FCM was performed within 6 h after collection of the samples. The amount of CD71-PE mAb (mouse anti-human-CD71-PE, clone M-A712, BD-Pharmingen, San Diego, CA) that was needed for saturation of 25 μl Quantum™ Simply Cellular® beads (QSC mouse Ab binding standards, Bangs Laboratories Inc., IN, USA), and 50 μl blood was achieved 30 μl CD71-PE mAb.

25 μl of QSC kit beads or 50 μl blood with 50 μl phosphate buffered saline (PBS) (Isoton® II diluent, Coulter Corporation, Miami, FL, USA) was incubated with 30 μl of CD71-PE mAb for 20 min in the dark at room temperature (rt). After staining, the beads and the samples were centrifuged (300 g, 5 min, rt) and washed twice with 3 ml PBS. The beads were resuspended in 1.0 ml PBS and kept in the dark before analysis. For thiazole orange (TO) labeling, 5 μl of CD71-PE-labeled cells was pipetted into new separate tubes, after which 1.0 ml PBS and a 1.0 ml TO-FITC solution (Retic-COUNT reagent, BD Biosciences, San Jose, CA, USA) were added and the cells were incubated for a further 30 min in the dark at rt.

At least 1000 events from blank beads and 4000 events from standard beads and at least 200,000 cells from blood samples were acquired using the same FCM (Coulter Epics XL MCL FCM [Coulter Corporation, Miami, FL, USA]) and the same settings. EXPO32 ADC software (XL 4 color and Analysis [build 219.1], Coulter Corporation, Miami, FL, USA) was used for data acquisition and analysis. Blood samples were gated in order to obtain peripheral erythroid cells (A) and TO positive reticulocytes (B) (Fig. 1). Gate C was created to evaluate the proportion of the TfR positive reticulocytes of all reticulocytes (gate B) (%TfR$^+$Ret). Platelets (P) were excluded [13,14]. The median channel values of the B-gated CD71-PE positive reticulocytes were compared to the calibration curve based on QSC beads to calculate the ABC values of cell samples using QuickCal software (version 2.3, Bangs Laboratories Inc., IN, USA).

After the FCM analyses, the samples were finally centrifuged and plasma was separated and stored frozen at −20 °C. Plasma sTfR was measured using an automated immunoturbidometric IDeA TfR-IT assay (Orion Diagnostica, Espoo, Finland) with a Konelab 60i instrument (Labsystems Ltd., Espoo, Finland). Plasma ferritin and high sensitivity C-reactive protein (hsCRP) were measured using a chemiluminescent immunometric method with an automated Immulite2000-analyzer (IEMA) (Diagnostic Products Corporation, Los Angeles, CA, USA).

The intra-assay coefficient of variation (CV%) was computed from the results of three different samples that were analyzed eight times. Intra-assay variability (CV%) ranged from 4.2% to 12.4% for ABC of TfR expression and from 2.4% to 10.6% for %TfR$^+$Ret. The day-to-day variation was calculated between the three days in one individual and the CV% ranged from 3.3% to 8.7% for ABC of TfR expression and from 3.3% to 22.8% for %TfR$^+$Ret.

The analysis of variance between the control and patient groups was performed using One-Way ANOVA. The pair wise comparisons between the different groups were based on Tukey's test. When analyzing correlations the association between the iron status measurements, the TfR expression (ABC values) and %TfR$^+$Ret was examined by the Pearson correlations with the statistical significance as $p<0.05$. Statistical analyses were performed using Microsoft Excel 2000 for Windows (Microsoft Office, Microsoft Corporation, USA), GraphPad Prism 4.0 for Windows (GraphPad Software Inc., San Diego, CA, USA) and SPSS 11.5 for Windows (SPSS Inc, Chicago, IL).

3. Results and discussion

The quantitative expression of the TfR on reticulocytes was feasibly measured by FCM. The data that summarize the results are shown in Table 1. In the present study, low ABC levels and a low %TfR$^+$Ret were seen especially in the control group (without iron deficiency). However, subjects whose iron requirement seemed to be more substantial (FID and FID + ID) had more Ab binding sites on the surface of their reticulocytes and their %TfR$^+$Ret was increased (Figs. 1 and 2). Thus, in the FID and the FID + ID groups a lot of reticulocytes with high CD71 expression (Fig. 1) were seen. The %TfR$^+$Ret in the studied samples varied between 8.8 and 61.0%. This is different from an earlier study in which less than 10% of all reticulocytes had TfRs on their cell surface [13].

TfR expression (ABC values) on reticulocytes correlated significantly with the reticulocyte indices [%HYPOr ($r=0.54$, $p<0.001$) and cellular hemoglobin content in reticulocytes (CHr) ($r=0.44$, $p<0.001$)], that reflect characteristics of newly formed cells of erythropoietic tissue. Furthermore, the correlations were also significant between the %TfR$^+$Ret and reticulocyte indices [%HYPOr ($r=0.73$, $p<0.001$) and CHr ($r=0.55$, $p<0.001$)]. %TfR$^+$Ret correlated also significantly with %HYPOm ($r=0.27$, $p=0.04$).

Significant correlations between the sTfR ($r=0.09$, $p=0.50$), ferritin ($r=0.14$, $p=0.29$) or TfR-F Index ($r=0.03$, $p=0.80$) and TfR expression (ABC values) on reticulocytes were not found. Also, %TfR$^+$Rets did not correlate significantly with sTfR ($r=0.21$, $p=0.12$), ferritin ($r=0.15$, $p=0.25$) or TfR-F Index ($r=0.14$, $p=0.31$). TfR expression on reticulocytes presumably reflects a real-time requirement for iron, while plasma sTfR responds to a more sustained ID. It might also be due to the plasma sTfR concentration also being dependent on the whole mass of erythropoietic tissue. As ferritin is the marker of iron stores, it is apparent that our subjects in the FID group had enough storage iron but not enough accessible iron to satisfy the need of erythropoiesis, as seen in the higher %HYPOr, %TfR$^+$Rets or ABC level, and lower CHr.

Classification of the patients into the FID category was based on two cellular indices (%HYPOm and %HYPOr) and ID was categorized on the basis of serum ferritin and TfR measurements. We found significant differences in the cellular or serum iron status parameters between the studied groups. However, the body's iron balance has more nuanced gradations, and the high variability of the ABC values and the %TfR$^+$Ret might at least partly reflect the heterogeneous patient populations. Because acute phase reaction could increase ferritin concentration, we used also TfR-F Index in patient classification. The usage of TfR-F Index [8] diminishes the contribution of the ferritin value and that has been suggested to reflect iron status more precisely than e.g. hsCRP combined with strict cut-off values of ferritin.