Blood Cells, Molecules, and Diseases xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Blood Cells, Molecules, and Diseases

journal homepage: www.elsevier.com/locate/ybcmd

Strong association between a new marker of hemolysis and glomerulopathy in sickle cell anemia

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ARTICLE INFO

Article history:
Submitted 22 June 2010
Revised 16 July 2010
Available online xxxx

(Communicated by H.F. Bunn, M.D.,
21 July 2010)

Keywords:
Sickle cell anemia
Red blood cells
Hemolysis
Glomerulopathy

ABSTRACT

To perform a precise evaluation of the hemolytic status of patients with sickle cell anemia (SCA), advanced red blood cell parameters provided by the last generation analyzers were investigated in a series of SCA patients. The search for precise markers of hemolysis was performed to identify if patients so exposed develop organic complications related to a postulated hemolysis-linked endothelial dysfunction. Red blood cell survival was evaluated by the ratio between mature red blood cell (RBC) and reticulocyte (RET) hemoglobin (RBC-Hb/RET-Hb). In comparison with serum lactate dehydrogenase (LDH) and total bilirubin, the log (RBC-Hb/RET-Hb) was identified as the most discriminant hematological parameter to evaluate hemolysis. Furthermore, by combining this parameter with LDH, we defined a composite variable, which we called CVar, that is highly correlated with albuminuria and might constitute a powerful new marker of risk for this complication.

Introduction

Chronic organ failure, including renal dysfunction, is a major problem in adult patients affected with sickle cell anemia (SCA) [1,2]. Indeed, sickle cell anemia-associated nephropathy (SCAN) is a growing matter of concern as renal failure affects 80% of aging patients [3]. Its natural history is not established, but is likely similar to type 1 diabetic nephropathy. In patients with SCA, two clinical overlapping subphenotypes have been postulated [4]. The first one involves hyperviscosity, related to primary polymerization of the sickle hemoglobin (Hb), to which vaso-occlusive pain crises, acute chest syndrome and osteonecrosis are associated. The second one relates to hyperhemolysis and its potentially associated decreased nitric oxide bioavaibility and might be associated with pulmonary hypertension, priapism or leg ulcers [4–9]. Until recently, renal dysfunction was not clearly related to either one of these two subphenotypes. Findings in adult SCA patients suggested that chronic hemolysis may be a relevant pathologic feature accounting for a high risk of glomerular hyperfiltration, which is proposed as the first step of SCAN [10]. In SCA children, it has been reported that those with microalbuminuria had a lower Hb level [11] and a relationship has been identified between LDH and proteinuria [12] suggesting a relationship between renal dysfunction and hemolysis. However, other studies found no relationship between micro/macroalbuminuria and biological markers of hemolysis in adult SCA patients [13,14]. Thus, the potential relation between hemolysis and SCAN is controversial and a precise evaluation of erythropoietic activity and hemolytic status of patients is needed to classify patients and identify those at risk for the specific complication.

Abbreviations: ACR, albumin-to-creatinine; CVar, composite variable; FRC, fragmented red cells; Hb, hemoglobin; IRF, immature reticulocyte fraction; LDH, lactate dehydrogenase; RBC, red blood cell; RET, reticulocyte; SCA, sickle cell anemia; SCAN, sickle cell anemia-associated nephropathy.

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doi:10.1016/j.bcmd.2010.08.001

Blood counts, Hb, red blood cell (RBC) indices and reticulocyte (RET) counts are routine tests essential to the follow-up of patients, unusual variations of these parameters from their baseline value in a given patient may signify occurrence of complication and/or organ damage [15–17]. However, in SCA samples, the counting imprecision of the RET enumeration either by light microscopy or automated instruments hampers evaluation of the hemolysis rate in a patient. Indeed, when using non-fluorescent RNA binding agents, cytoplasmic particles other than RNA can be confused with ribosomes causing overestimation of RET counts [15]. The automated count using fluorochromes, also, may be altered by erroneous setting of thresholds to exclude fluorescing nucleated cells and background fluorescence [16]. Reticulocytosis is a reflection of extravascular and intravascular hemolysis and, thus, should not be considered a specific indicator to identify patients exposed to hemolysis-related endothelial dysfunction [18]. With progress of automated analyzers, additional parameters provide information on erythropoietic maturation, more specifically on "stress" erythropoiesis which reflects the chronic state of erythroid expansion due to hemolysis [19,20]. Moreover, from the measurement of RET-Hb content, calculation of RBC-Hb/RET-Hb, the ratio between the Hb contained in mature RBCs and that contained in RETs provides an estimation of red blood cell survival [21].

The objectives of this study were (i) to investigate these parameters in a series of SCA patients and to compare them with the standard biochemical markers of hemolysis in order to identify the most relevant hematological parameter to appreciate hemolysis and (ii) to look for its potential relationship with albuminuria, a marker of glomerular damage, the pathogenesis of which is not well understood in SCA.

Materials and methods

Patients

One hundred-thirty adults with SCA (SS homozygotes) previously characterized for the α gene status [22], 4 α genes ($n=74$), 3 α genes ($n=45$) and 2 α genes ($n=12$), were selected for the study. SCA was diagnosed for each patient on the basis of Hb analysis and DNA analysis. Informed consent was obtained from all patients. Patients had to be at steady-state (not experiencing any painful crisis or other acute medical condition for at least 1 month), without iron deficiency, and not treated with hydroxyurea. Clinical investigation was conducted making groups of patients affected ($n=62$) or not ($n=51$) with albuminuria characterized by macroalbuminuria [albumin-to-creatinine (ACR)>30 mg/mmol creatinine] or microalbuminuria [ACR from 3 to 30 mg/mmol creatinine].

Laboratory data

Samples were studied on a Sysmex™ XE-2100 (Sysmex Corporation, Kobe, Japan). Classical parameters were RBC count, Hb and RBC indices. Specific parameters measured in the RET channel, analyzed on a cell-by-cell basis, by laser light scattering and fluorescence were RET count with quantification of the immature fraction of RETs called IRF, mean values of the forward scatter signal in RBC populations proportional to cell size and Hb content (IRF-Y, RET-Y and RBC-Y) [23], and fragmented RBCs (FRCs) [24]. The estimation of RBC survival was evaluated by variations of RBC-Hb/RET-Hb. RET-Hb was calculated by multiplying the absolute RET count by the RET-Hb content (RET-He) calculated from RET-Y by the analyzer, applying the regression formula, $\text{RET-He}=5.5569e^{0.001\text{Ret-Y}}$ [25]. The RBC-Hb was calculated by subtracting the RET-Hb from the total Hb. It has to be noted that a good correlation was previously demonstrated between RET-Hb content measurements by the Sysmex™ XE-2100 (RET-He) and the H*3 RTX (CHr) [24,26]. Results in SCA patients were compared to those of 51 consecutive samples from adults with a normal blood count, hospitalized in different departments for diseases with no hematological consequences.

Routine biochemical parameters, including blood LDH activity, total bilirubin and urine albumin and creatinine, were determined using an Architech ci8200 (Abbott Diagnostics, Abbott Park, Il, USA). Plasma creatinine was measured by enzymatic assay using a Kone creatinine analyzer (Thermo Clinical Labsystems Oy, Finland). Urinary tests were performed on 24 hrs urine samples.

Statistical analyses

Statistical analysis was conducted with Statview (v. 5.0). We used descending step-wise multivariate analyses (linear and logistic regressions). The univariate analyses were performed using Pearson correlation coefficient or Mann–Whitney test for quantitative variables, and chi-square or exact test of Fisher for qualitative variables. The significance level was 0.2 for the univariate phase and 0.05 for the multivariate phase.

Results and discussion

RBC parameters

Hematological and biochemical data in controls and SCA patients are summarized in Table 1. The majority of patients had the expected variable hemolytic anemia and "stress" erythropoiesis with high proportions of IRF, reflecting the chronic state of erythroid expansion due to hemolysis [19,20]. The lack of 1 or 2 of the 4 α globin genes caused higher Hb levels with less RETs and a marked microcytosis. IRF-Y and RET-Y were highly correlated to GR-Y in each group ($p<0.0001$), but when comparing controls and SCA patients, a greater variability was observed in SCA patients, illustrating the extreme heterogeneity of RBCs in terms of volume and Hb content. This finding is consistent with repeated cycles of Hb S polymerization and influence of the α globin gene number. In SCA patients, cells counted in the area of FRCs were higher than in controls and variable among patients, with significant differences when comparing SCA patients with and without α thalassemia ($p<0.0001$). Starting with a model including RBC volume, Hb content, markers of hemolysis, RETs, bilirubin and RBC-Hb/RET-Hb as covariates and then after performing a step-wise multiple linear regression analysis, only RBC volume and Hb content remained significantly and independently associated with FRCs in the model ($p=0.0005$ and 0.005, respectively). Mean values of RBC-Hb/RET-Hb were lower in SCA patients than in controls, with significant differences when comparing values according to the number of α genes ($p<0.05$). This is in agreement with the more reduced RBC survival and the more severe hemolysis in this group and concordant with previous publications [21,27]. As RET-Hb content and RBC-Hb/RET-Hb are highly dependent on iron metabolism [21,25], we made sure that none of the selected patients was iron deficient.

Correlation between hematological parameters and biochemical markers of hemolysis

The linear regressions, taking bilirubin or LDH as dependent variables and Hb, RET count, FRCs, IRF, IRF-Y, RET-Y, RBC-Y and the log (RBC-Hb/RET-Hb) as independent variables showed that the only remaining variable was the log (RBC-Hb/RET-Hb), $p<0.0001$ in both cases. It could be argued that parameters we used are total bilirubin and total LDH and that they can be confounded by concurrent liver disease that commonly occurs in SCA patients. Analysis of conjugated bilirubin and of fractionated LDH1 and LDH2, measurements not affected by a concurrent liver disease, might have been of a great interest. However, the first parameter is highly dependent of the functional repetitive (TA) polymorphism in the *UTG1A1* gene

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Table 1
Hematological and biochemical data in controls and SCA patients.

Patients (n)	Controls (51)	SCA patients (131)		
α Gene status	ND	2 α (12)	3 α (45)	4 α (74)
Hb (g/dL)	13.9 ± 1.3	9.4 ± 1.4	8.6 ± 1.0	8.2 ± 1.1
MCV (fL)	89.9 ± 3.6	68.4 ± 4.5	78.7 ± 5.8	88.1 ± 6.2
RET ($\times 10^9$/1)	65.2 ± 18.2	246.5 ± 52.9	297.7 ± 58.4	364.2 ± 108.3
IRF (%)	4.9 ± 2.8	26.1 ± 6.7	28.4 ± 7.6	25.8 ± 7.4
RBC-Y (channel No)	177 ± 4	140 ± 9	157 ± 11	171 ± 11
RET-Y (channel No)	187 ± 5	156 ± 9	174 ± 9	186 ± 8
IRF-Y (channel No)	195 ± 6	162 ± 10	180 ± 10	191 ± 9
FRC (%)	0.07 ± 0.13	4.7 ± 1.7	3.6 ± 2.1	2.6 ± 2.0
RBC-Hb/RET-Hb	71.2 ± 20.7	16.2 ± 4.5	9.7 ± 2.9	6.9 ± 2.7
Log (RBC-Hb/RET-Hb)	4.3 ± 0.3	2.7 ± 0.3	2.2 ± 0.3	1.8 ± 0.4
LDH (U/L)	ND	320 ± 85	395 ± 100	419 ± 164
Bilirubin (mmol/l)	ND	34 ± 12	56 ± 36	71 ± 36

Data are reported as mean ± 1 SD.
ND: not determined; Hb: hemoglobin; MCV: mean cell volume; RET: reticulocytes; IRF: immature fraction of reticulocytes; RBC-Y, RET-Y and IRF-Y: mean values of the forward scatter signal in RBCs, RETs and IRFs in arbitrary units (channel numbers); FRC: fragmented red cells; LDH: serum lactic dehydrogenase.

Table 2
Step-wise logistic regression analyses of SCA patients affected ($n = 62$) or non-affected ($n = 51$) with albuminuria.

	P	OR	95% CI
(A) Univariate analysis			
Bilirubin	0.06		
LDH	0.0009		
Log (RBC-Hb/RET-Hb)	0.0005		
(B) Multivariate analysis			
(1) Independent variables			
(a) LDH	0.009	1.005	1.001–1.009
Log (RBC-Hb/RET-Hb)	0.0005	0.233	0.073–0.741
(b) LDH > 390 U/L	0.036	2.710	1.04–6.72
Log (RBC-Hb/RET-Hb) < 2.2	0.002	4.313	1.69–10.62
(2) Composite variable (CVar)			
Constant	0.005	0.273	0.11–0.67
CVar = 1 [LDH > 390 U/L and log (RBC-Hb/RET-Hb) > 2.2]	0.016	5.867*	1.39–24.68
CVar = 2 [LDH < 390 U/L and log (RBC-Hb/RET-Hb) < 2.2]	0.001	7.857*	2.20–28.06
CVar = 3 [LDH > 390 U/L and log (RBC-Hb/RET-Hb) < 2.2]	0.0002	11.524*	3.18–38.15

LDH: serum lactate dehydrogenase; RBC-Hb: hemoglobin contained in mature red blood cells; RET-Hb: hemoglobin contained in reticulocytes; CVar: composite variable; OR: odds-ratio; CI: confidence interval.
*All OR are calculated according to the reference group having a Cvar = 0 [LDH < 390 U/L and log (RBC-Hb/RET-Hb) > 2.2].

promoter and genotyping for this polymorphism is, most often, not a routine test performed in SCA patients, as is LDH isoenzymes measurement. Thus, based on routinely available parameters, the log (RBC-Hb/RET-Hb) appears to be the appropriate hematological marker to evaluate hemolysis in SCA patients.

Clinical correlations

Looking for a potential relationship with albuminuria, variations of bilirubin, LDH and the log (RBC-Hb/RET-Hb) were compared in 62 affected and 51 non-affected patients. The following values were obtained, bilirubin = 64 ± 33 versus 54 ± 36 mmol/L, LDH = 452 ± 147 versus 356 ± 117 U/L and log (RBC-Hb/RET-Hb) = 2.0 ± 0.4 versus 2.3 ± 0.5, respectively (p values in Table 2A). The logistic regression analyses showed the association of the log (RBC-Hb/RET-Hb) and LDH with the complication (Table 2B1a). Note that according to the above results, these two parameters are associated; however, the percentage of explained variance is weak (12%). After testing several values, we defined thresholds of 2.2 for log (RBC-Hb/RET-Hb) and 390 U/L for LDH, and these choices made associations with the complication more explicit (Table 2B1b). In an attempt to be more discriminating, we associated these 2 thresholds by setting up a composite variable (CVar) varying from 0 to 3, as described in Table 2B2. A new logistic regression analysis showed that the risk of albuminuria increased as a function of CVar, multiplying the risk by 6, 8 or 11 in regards to the zero value (Table 2B2). Distribution of patients according to the CVar value is illustrated in Fig. 1, which shows that the CVar can be considered as a powerful marker of albuminuria in SCA.

Hemolysis, which has been long discounted in regard to vaso-occlusion in SCD, has now gained interest with the postulation of its direct implication in specific and invalidating complications [1,4–8]. This hypothesis has been challenged [28]. Nevertheless, the search for new and potentially more precise biomarkers of hemolysis is of interest. The steady-state LDH, a biochemical marker of intravascular hemolysis, was identified as a relevant prognosis marker in patients at risk to develop pulmonary hypertension, leg ulcers, priapism and risk of death [6,18,29]. The calculated ratio RBC-Hb/RET-Hb provides an estimation of the reduction in RBC survival [21]. In this study, we found that the log (RBC-Hb/RET-Hb), highly correlated with LDH and bilirubin, is the most relevant hematological parameter to evaluate hemolysis. Novel data are provided here by studying a large group of SCA patients affected with albuminuria. Various structural and/or functional renal abnormalities are described in SCA [2]. Albuminuria is the expression of glomerular damage preceding the development of renal insufficiency and occurs in 68% of adult SCA patients [13]. A strong correlation of albuminuria with log (RBC-Hb/RET-Hb) and LDH was found. By creating a novel parameter, CVar, we show that the association of these 2 markers of hemolysis provides a powerful marker of the complication. As others before [30,31], we found a negative correlation between α thalassemia and albuminuria. Nevertheless, our logistic regression analysis performed between the complication and the variables α thalassemia and CVar showed that CVar remained the only variable significantly associated with albuminuria (data not shown). Rather, our results suggest that the "renoprotective" effect of α thalassemia is related to decreased hemolysis, well established in α thalassemic patients [21,27].

In conclusion, our study, although in no way establishing a cause-to-effect link between hyperhemolysis and renal complications in SCA, describes a novel marker of hemolysis strongly associated with albuminuria. Longitudinal studies are now needed to determine whether CVar should be considered as a useful predictive marker of SCAN. Furthermore, we think that this new parameter should be evaluated in other complications of SCA.

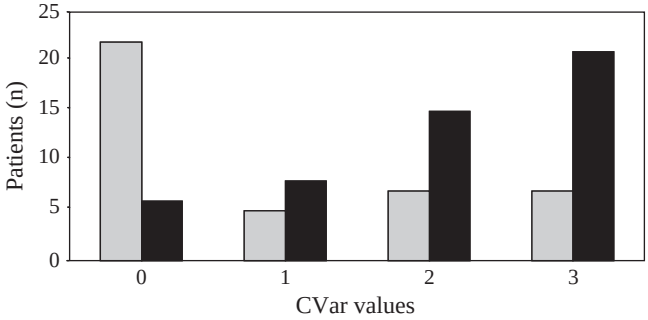

Fig. 1. Distribution of 113 SCA patients, affected and non-affected with albuminuria, according to the CVar values. By associating thresholds of 2.2 for the log (RBC-Hb/RET-Hb) and 390 U/L for LDH, we defined a composite variable (CVar) varying from 0 to 3 (0, log (RBC-Hb/RET-Hb) > 2.2 and LDH < 390 U/L; 1, RBC-Hb/RET-Hb) > 2.2 and LDH > 390 U/L; 2, RBC-Hb/RET-Hb) < 2.2 and LDH < 390 U/L; 3, RBC-Hb/RET-Hb) < 2.2 and LDH > 390 U/L) and stratified the patients according to the CVar values. Histogram shows distribution of the 2 groups of patients, affected ($n = 62$) or non-affected ($n = 51$) with albuminuria, according to the CVar values. Most non-affected patients (▭) have a CVar at 0, the number of affected patients (■) increases with the CVar value. Thus, indeed, the CVar is a marker of risk to develop albuminuria in SCA.

Please cite this article as: M. Maier-Redelsperger, et al., Strong association between a new marker of hemolysis and glomerulopathy in sickle cell anemia, Blood Cells Mol. Diseases (2010), doi:10.1016/j.bcmd.2010.08.001

Authorship and disclosures

MMR designed the study, analyzed results and wrote the paper; JE critically discussed the results and gave a significant contribution in writing the paper; PL was responsible for the statistical analysis, the definition of the composite variable, and approved the paper; FL, KS, JPH and RG managed the patients, provided clinical data and approved the paper; GL provided biochemical data and approved the paper; VA collected clinical data; JPP gave valuable help concerning the use of the Sysmex XE-2100.

The authors reported no potential conflicts of interest.

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Please cite this article as: M. Maier-Redelsperger, et al., Strong association between a new marker of hemolysis and glomerulopathy in sickle cell anemia, Blood Cells Mol. Diseases (2010), doi:10.1016/j.bcmd.2010.08.001