Modulation of erythroid adhesion receptors expression by hydroxyurea in children with sickle cell disease

Marie-Hélène Odièvre,[1,8] Viviane Bony,[7] Malika Benkerrou,[2] Claudine Lapouméroulie,[1] Corinne Alberti,[5] Rolande Ducrocq,[3] Evelyne Jacqz-Aigrain,[4,6] Jacques Elion,[1,3] and Jean-Pierre Cartron[7]

[1]INSERM, U763, Paris, Université Paris 7-Denis Diderot, Faculté de médecine, Paris; [2]AP-HP, Hôpital Robert-Debré, Centre de la drépanocytose, Paris; [3]AP-HP, Hôpital Robert-Debré, Laboratoire de biochimie génétique, Paris; [4]AP-HP, Hôpital Robert-Debré, CIC 9202, Paris; [5]INSERM, CIE 5, Unité d'épidémiologie clinique, Paris; [6]AP-HP, Hôpital Robert-Debré, Laboratoire de pharmacologie, Paris; [7]INSERM, U665, Institut National de la Transfusion Sanguine, Paris, and [8]AP-HP, Hôpital Louis-Mourier, Service de Pédiatrie, Colombes, France

ABSTRACT

Background

We investigated adhesion receptor levels on red blood cells, reticulocytes and erythroid progenitors from children with sickle cell disease treated or not with hydroxyurea.

Design and Methods

Four groups of patients were investigated: (i) children receiving hydroxyurea for severe vaso-occlusive events (n=26); (ii) untreated children with a history of vaso-occlusive events (n=20); (iii) children with no history of vaso-occlusive events (n=28); and (iv) healthy African controls (n=27). Expression of adhesion receptors was analyzed by flow cytometry with specific monoclonal antibodies.

Results

Reticulocytes and/or red blood cells from the children with sickle cell disease showed significantly higher expression of CD36, $\alpha 4\beta 1$, Lu/BCAM than those from controls, whatever the severity of the disease, as well as less marked increases in expression of ICAM-4, CD47 and CD147. Under hydroxyurea treatment, the expression of CD36, $\alpha 4\beta 1$ and ICAM-4 (to a lesser extent) was decreased, but surprisingly the expression of Lu/BCAM (and also CD47 and CD147 to a lesser extent) was significantly increased. Alterations of levels of adhesion receptors could be recapitulated in two-phase liquid cultures of erythroid progenitors from controls and untreated children with a history of vaso-occlusive disease, grown in the absence or presence of hydroxyurea.

Conclusions

Our results suggest that hydroxyurea acts during erythroid development and modulates adhesion receptor expression and function differently, possibly by acting on gene expression and the signaling cascade leading to receptor activation.

Key words: sickle cell disease, hydroxyurea, red cell, erythroid progenitor, adhesion.

Citation: Odièvre MH, Bony V, Benkerrou M, Lapouméroulie C, Alberti C, Ducrocq R, Jacqz-Aigrain E, Elion J, and Cartron JP. Modulation of erythroid adhesion receptors expression by hydroxyurea in children with sickle cell disease. Haematologica 2008 Apr; 93(4):502-510. doi: 10.3324/haematol.12070

Acknowledgments: we thank the children and their families for their kind participation in this study. This investigation was supported in part by a Contrat de Recherche et d'Innovation Clinique (CRIC n° 02022) from Assistance Publique, Hôpitaux de Paris and in part by INSERM U763. MHO is a recipient of a doctoral fellowship from the INSERM (Poste d'Accueil), and from the Fondation pour la Recherche Médicale. We thank Claude Gaultier for participating in the follow-up of patients at CIC 9202 and Wassim El Nemer (INSERM U665) for critical reading of the manuscript.

Manuscript received August 2, 2007. Revised version arrived on October 23, 2007. Manuscript accepted November 22, 2007.

Correspondence: Jean-Pierre Cartron, Dr-Sc, INSERM U665 INTS, 6 rue Alexandre Cabanel, 75015 Paris, France. E-mail: cartron@idf.inserm.fr

Introduction

One of the hallmarks of sickle cell disease, resulting from polimerization of hemoglobin S under hypoxic conditions and the formation of sickle red blood cells, is episodic occurrence of painful crises which are believed to originate from vascular occlusion and lead ultimately to organ failure. Growing evidence support the hypothesis that abnormal adhesiveness of sickle red blood cells to endothelial cells slows the transit of blood cells through small vessels and precipitates the occurrence of vaso-occlusive crises (VOC).[1,2] This makes interactions between the sickle cells and endothelial cells central to the pathophysiology of the disease.[3] Adherence of sickle red cells to endothelial cells is a complex process that requires interactions between multiple adhesion molecules and receptors on the red cells and endothelial cells, in which subendothelial matrix components, plasma proteins forming bridges between the red cells and endothelial cells, and leukocytes, all play important roles.[4-6] On the red blood cell side, integrin α4β1 (VLA-4) and CD36 (thrombospondin receptor) were among the first adhesion molecules identified; they are overexpressed on young reticulocytes that emerge prematurely from the bone marrow as a consequence of the anemic stress.[7-9] More recently, two other molecules not restricted to reticulocytes have been implicated, namely Lutheran blood group/basal cell adhesion molecule (Lu/BCAM), a receptor for the extracellular matrix protein laminin α5[10,11] and intercellular adhesion molecule-4 (ICAM-4), otherwhise known as LW blood group glycoprotein, which binds different types of integrins, particularly αVβ3.[12-14] Other molecules may also increase the adhesiveness of sickle red cells.[6] On the endothelial side, the major cell membrane ligands for red blood cell adhesion molecules include vascular cell adhesion molecule-1 (VCAM-1), CD36, αVβ3 integrin and selectins,[4,5] but recently a new interaction that involves endothelial Lu/BCAM and α4β1 integrin on sickle red cells has been described.[15] The relevance of these adhesion proteins in the pathophysiology of sickle cell disease is suggested by the higher expression of α4β1, CD36 and Lu/BCAM on the surface of reticulocytes and red cells from patients with sickle cell disease compared to that of normal subjects,[8-11] the increase of thrombospondin during VOC,[16] and the increased adherence of sickle reticulocytes to VCAM-1,[9,17] which in turn could potentially contribute to VOC in sickle cell disease.

Despite these progresses in understanding the pathophysiology of sickle cell disease, advances in therapy for this disease remain modest. Charache *et al.*[18] first showed the efficacy of hydroxyurea in decreasing the frequency of painful crises in adults. The efficacy of hydroxyurea in sicke cells disease was initially attributed to pharmacological stimulation of fetal hemoglobin.[19,20] However, hydroxyurea therapy was often associated with clinical improvement before any measurable rise in fetal hemoglobin, suggesting that hydroxyurea could also act through other mechanisms, such as a decrease of VCAM-1 expression and release of endothelin-1 from human endothelial cells in culture.[21] Hydroxyurea also decreases the adhesion of sickle red cells and reticulocytes to endothelial cells,[21] and to the subendothelial matrix proteins, thrombospondin and laminin.[22] *In vivo*, hydroxyurea decreases the percentage of reticulocytes expressing α4β1 and CD36,[23] and soluble VCAM-1[24] and plasma endothelin-1 levels.[25] Hydroxyurea could also exert its impact through a reduction in neutrophil activation.[26] Thus, both adhesion receptors on both red blood cells and endothelial cells seem to be a target for hydroxyurea.

Since information on red cell adhesion receptors following hydroxyurea therapy is restricted to α4β1 and CD36, we examined the status of various adhesion molecules on red cells and reticulocytes from symptomatic children with sickle cell disease receiving or undergoing hydroxyurea therapy and a group of healthy children. In parallel, we investigated these receptors on erythroid progenitors exposed to hydroxyurea in a two-phase liquid culture system.

Design and Methods

Patients

One hundred and one subjects of sub-Saharian Africa extraction, living in France, were enrolled at the Sickle Cell Disease Center at Robert-Debré Hospital (Paris, France) and at the pediatric unit of Louis-Mourier Hospital (Colombes, France); all were homozygous for the hemoglobin S mutation. The subjects were divided into three groups; (i) children treated with hydroxyurea (20-25 mg/kg/day) since at least 3 months with clinical efficacy on vaso-occlusive events (acute chest syndrome or recurrent painful crises requiring morphinomimetics drugs) (n=26; median age 10.0 years; range 4.0-19.0 years) (hydroxyurea group); (ii) untreated children with major vaso-occlusive events (more than two episodes of acute chest syndrome or more than three hospital admissions for painful crises during the preceding 12 months) (n=20; median age 9.5 years; range, 6.0-17.0 years) (VOC group); and (iii) children >5 year-old without a history of vaso-occlusive events (n=28; median age 10.5 years; range, 5.0-20.0 years) (non-VOC group). All patients were in a steady-state of disease (free of any infectious or vaso-occlusive events for the 4 weeks prior to and 2 weeks after blood sampling, and transfusion-free for 4 months prior to blood sampling). All were receiving penicillin V and folate supplementation. No patients had a stroke. Seven patients included in the VOC group, were secondarily treated with hydroxyurea. For group comparisons, these patients were considered only in the VOC-group. Longitudinal follow-up of these patients was analyzed independently. A control group of 27 sub-

jets consisted of heterozygous AS parents or siblings of the patients (n= 21; median age 41.0 years; range, 7.0-66.0 years) and AA siblings or healthy African unrelated subjects (n=6, median age 17.0 years, range 10.0-36.0 years). None of the controls had taken any drug on the day of blood sampling. Patients and controls were invited to the Clinical Investigation Center (CIC 9202) at Robert-Debré Hospital between January 2003 and March 2005, and blood samples were taken after checking the inclusion criteria and obtained signed informed consent from the subjects of their parents. Whole blood was sampled and transported immediately to the laboratory. Complete blood counts, including reticulocyte counts and assessment of iron status, were performed; the HbF level was also measured by high performance liquid chromatography (BioRad Laboratories, California, USA) using the β-thal short program. The day of the visit to the Clinical Investigation Center, children treated with hydroxyurea received their daily oral dose from the nurse in order to obtain plasma samples just before (residual) and two hours after (peak) oral hydroxyurea ingestion.

Two-phase liquid cultures of human peripheral blood erythroid progenitors

Mononuclear cells from peripheral blood were isolated and cultured using a two-phase liquid culture system as described before[27] with some modifications during the erythropoietin-dependent phase. Indeed, erythroid progenitors were cultured in normal oxygen conditions (21% O_2) during this phase to avoid sickling of precursors,[28] although it is known that cell growth is favored in hypoxic conditions (5% O_2).[29,30] The dose of hydroxyurea and the day of exposure in the erythropoietin-dependent phase of the culture were selected to avoid toxicity at high concentrations and the paradoxal stimulation of erythropoiesis observed at hydroxyurea concentrations below 50 μM.[31] In these experiments, 50 μM hydroxyurea was added on day 10 of the culture (4 days after the addition of erythropoietin) and the adhesion receptor pattern of erythroid cells was determined on day 20 by flow cytometry analysis by gating glycophorin-A^+ subsets of cells.[27]

Flow cytometry analysis

The expression of adhesion molecules on red blood cells and reticulocytes was evaluated by flow cytometry (FACScan flow cytometer, Becton Dickinson) as described previously,[27,32] using mouse monoclonal antibodies against human CD36, α4β1 and α5β1 (respectively, clones FA6.152, HP2/1 and SAM-1, Beckman-Coulter, Immunotech, Marseille, France), against human LU (clone F241), ICAM-4 (clone 1A1), CD47 (clone 3E12 from our Institute; INTS), and against CD147 (clone TRA-1-85, a gift from P Goodfellow, University of Cambridge) at a concentration of 5 μg/mL or 20 μL phosphate-buffered saline/bovine serum albumin containing mouse IgG_1 monoclonal antibodies (Becton Dickinson, San Jose, CA, USA) as a negative control. The specific antibody-binding capacity per erythroid cell, which is an estimate of the copy number of antigen molecules/cell, was deduced from a calibration curve obtained with Qifikit calibration beads (Dako).[32] For expression of adhesion molecules on erythroid progenitors, 5×10^5 cells were resuspended in 100 μL phosphate-buffered saline/bovine serum albumin and processed as described elsewhere.[27] CD71 (clone YDJ1.22, Beckman-Coulter) and glycophorin-A (R18, generous gift from PAW Edwards) were used for window gating of erythroid cells.

Plasma hydroxyurea assays

Three milliliters of fresh venous blood collected into heparin were kept on ice in the dark, and immediately centrifuged at 3,500 rpm for 10 min at 4°C. Plasma samples (500 μL) were deproteinized with 50 μL of 35% sulfosalicylic acid, centrifuged at 4,000 rpm for 10 min at 4°C and the supernatants stored at -20°C until hydroxyurea assay by high performance liquid chromatography using a modificaton of the procedure described by Havard *et al.*[33]

Statistical analysis

Qualitative variables are described as counts (percentages) and quantitative variables as medians with minimum and maximum values. In a first statistical step, we examined differences in blood cell parameters and status of adhesion molecules on red blood cells and reticulocytes between groups (sickle cell disease patients and controls) using the Kruskall-Wallis test. When the results were statistically significant at an α-risk level of 5%, a second statistical step examined group to group differences with Wilcoxon's tests and Bonferroni's procedure to adjust for multiple comparisons. Results are expressed as *p*-values multiplied by the number of tests. Changes in (i) adhesion modecules on red cells and reticulocytes in the control group, (ii) adhesion molecules during the longitudinal study and (iii) hydroxyurea-cultures were examined with signed-Wilcoxon's tests. All tests were two-sided and results were considered statistically significant at an α-risk level of 5%. Statistical analyses were performed using SAS 9.1 (SAS Inc, Cary, NC, USA) software.

Results

Clinical and laboratory characteristics of the patients with sickle cell disease

The patients in the hydroxyurea group showed the classical variations of blood parameters usually observed under hydroxyurea therapy,[34] indicating that compliance with treatment was acceptable (Table 1). Similar variations in blood cell parameters were also observed before and after hydroxyurea therapy in the subgroup of seven

patients treated secondarily during the study (*not shown*). Residual plasma levels before hydroxyurea ingestion were very low (<50 ng/mL) in 17 of the 23 patients studied in the hydroxyurea group and were in the range of 63 to 1,840 ng/mL for the six others. Two hours after hydroxyurea ingestion, substantial increases in peak levels were observed among the 23 patients studied, ranging from 4,776 to 37,005 ng/mL, except for in one patient whose plasma hydroxyurea level was still below 50 ng/mL at this time point. However, the blood cell parameters of this last patient, studied before and after hydroxyurea therapy, presented the marked variations usually observed under hydroxyurea therapy.

Expression of adhesion molecules on red blood cells and reticulocytes

Control group

Levels of adhesion receptors on red blood cells and reticulocytes from the control group (n=20) were measured by flow cytometry with monoclonal antibodies. As shown in Table 2, adhesion molecules were significantly ($p<10^{-4}$) more abundant (about 1.2 to 2-fold) on reticulocytes than on mature red blood cells, but accurate estimates were limited by the low reticulocyte count in this group (0.9±0.05%) as assessed by thiazole orange staining. CD36 and integrin α4β1 were absent or expressed at very low levels on red blood cells. However, as noted previously,[32] in about 50% of the subjects a small subpopulation of reticulocytes (7%), which stains more strongly with thiazole orange and presumably comprises more immature reticulocytes, expressed higher levels of these molecules (Table 2). Regarding Lu/BCAM expres-

Table 1. Characteristics of the groups of patients with sickle cell disease and the control group.

	Subjects				*p*
Blood cell parameters	*Controls (n=27)*	*NonVOC-group (n=28)*	*VOC-group (n=20)*	*HU-group (n=26)*	
Leukocytes (×10⁹/L)	4.7 (3.1-9.6)	11.4 (6.8-17.5)	12.1 (6.8-20.1)	9.3 (3.4-14.5)¶	*<10⁻⁴*
PMN (×10⁹/L)	2.3 (0.8-6.5)	5.7 (1.9-12.4)	5.5 (1.7-14.7)	4 (0.7-10)	*<10⁻⁴*
Platelets (×10⁹/L)	246 (152-419)	478 (262-854)	434 (253-660)	431 (94-778)	*<10⁻⁴*
Red blood cells (×10¹²/L)	4.7 (3.9-6.1)	3.1 (2.0-4.2)	2.9 (2.2-4.1)	2.8 (2.2-4.5)	*<10⁻⁴*
Hemoglobin (g/L)	129 (89-158)	80 (59-106)	79 (61-97)	87 (62-104)	*<10⁻⁴*
Reticulocytes (×10⁹/L)	50.85 (27.8-108.24)	231.68 (92.4-609.6)	303.45 (164.97-509.4)	227.98 (98.56-360.1)¶	*<10⁻⁴*
MCV (fL)	82.1 (66.3-98.1)	78.7 (61.6-104.5)	84.2 (65.4-100)	87.6 (72.5-112.3)	*=0.01*
Hematocrit (%)	39.9 (27.8-46.2)	24.7 (18.7-32.1)	24.3 (19.1-31.7)	26.6 (17.4-33.1)¶	*<10⁻⁴*
MCH (pg)	27.8 (21.2-31.5)	26.0 (18.6-33.6)	27.6 (20.7-32.6)	29.1 (22.1-36.5)	*=0.006*
MCHC (g/dL)	32.7 (30.1-34.9)	32.4 (29.3-35.0)	32.7 (29.3-36.2)	31.8 (30.5-36.2)	*=0.41*
Hemoglobin F (%)	0.2 (0.2-1.6)	8.8 (3.2-25.5)	6.5 (0.9-18.1)	11.6 (1.8-19.9)	*<10⁻⁴*

Results are expressed as median (range). p values: overall significance between groups. ¶Significantly different from VOC-group (p<0.05) with adjustment for multiple testing. PMN : polymorphonuclear neutrophils; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular hemoglobin concentration.

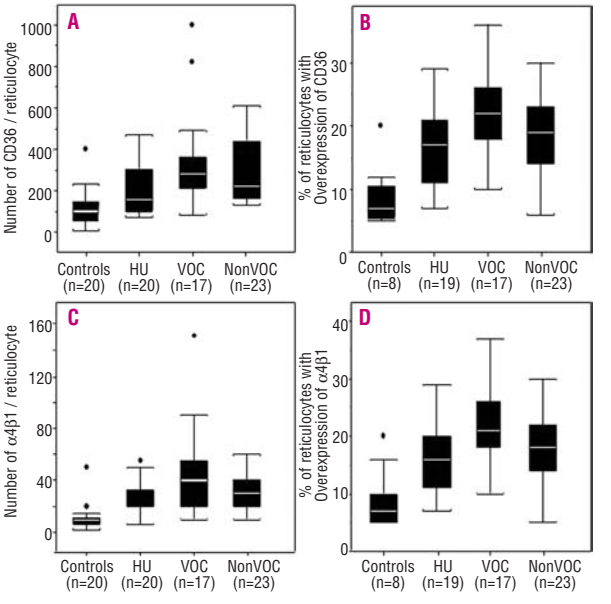

Figure 1. CD36 and α4β1 expression on reticulocytes. For each group under study (Control, hydroxyurea-treated [HU], VOC and Non-VOC), the number of receptors/reticulocyte (A, C), and the percentage of reticulocytes that overexpress receptors (B, D) are indicated. Values are given as median, lower quartile, upper quartile, minimum and maximum for each group of patients and controls.

Table 2. Adhesion molecules on red blood cells (RBC) and reticulocytes from individuals in the control group.

Adhesion molecule	*Copy number/cell*		*p*
	Red blood cells	*Reticulocytes*	
CD36	32 (6-60)	100 (7-400) [1,435 (560-5,290) (7%)]*	*<10⁻⁴*
α4β1	nd	10 (2-50) [425 (320-1,095) (7%)]*	
Lu/BCAM**			
Lu-pos	725 (410-1,050) (68%)	1,260 (720-2,790) (73%)	*<10⁻⁴*
ICAM-4	775 (310-1,080)	920 (500-1,270)	*<10⁻⁴*
CD147	1,695 (1,050-3,220)	2,315 (1,460-5,070)	*<10⁻⁴*
CD47	12,395 (9,395-20,800)	15,490 (10,890-24,860)	*<10⁻⁴*
α5β1	nd	2 (0-20)	

*Copy number of adhesion receptors on red blood cells (RBC) and reticulocytes from volunteers of African origin (control group, n=20) determined by flow cytometry with specific monoclonal antibodies. Values are expressed as median (range). nd: not detectable. *In about 50% of the subjects, there is a small subpopulation (n7%) of reticulocytes that express a higher level of receptors. **From one individual to another, between 15 to 30% of RBC or reticulocytes are Lu-weak (or negative). Lu-pos=cell population strongly stained with anti-Lu.*

sion, staining of red blood cells and reticulocytes with the monoclonal anti-Lu revealed a Lu-positive population (70-85%) and another Lu-weak or negative population (15-30%), the proportions of which varied from one individual to another. Only the Lu-positive population was considered since the Lu-weak/negative population largely overlapped with the isotype control. α5β1 integrin was not significantly expressed on either red blood cells or reticulocytes from the control group.

CD36 and α4β1 integrin in patients with sickle cell disease

CD36 and α4β1 were not expressed at significant levels on sickle red cells (*not shown*). On reticulocytes, the expression of CD36 and α4β1 was significantly higher in untreated sickle cell disease patients (VOC group; p=0.002 for CD36, $p<10^{-3}$ for α4β1; non-VOC group, $p<10^{-3}$ for CD36, $p<10^{-3}$ for α4β1) than in controls, although there were no significant differences between these two groups of patients. An increased expression (about 2-fold) of CD36 and α4β1 was detected when both the copy number of molecules/cell (Figure 1A,C) and the percentage of cells overexpressing the antigens (immature reticulocytes, strong thiazole orange staining) were considered (Figure 1B,D). In hydroxyurea-treated patients, the percentages of reticulocytes overexpressing CD36 (p=0.03) (Figure 1B) and α4β1 (p=0.03) (Figure 1D) were significantly lower than those in untreated patients.

Lu/BCAM and ICAM-4 in patients with sickle cell disease

The expression of Lu/BCAM on red blood cells and reticulocytes was significantly higher (1.5 to 2-fold) in untreated patients (VOC-group: p=0.002 for RBCs, $p<10^{-3}$ for reticulocytes; non-VOC-group: p=0.012 for red cells, p=0.0012 for reticulocytes) than in controls, and both the copy number/cell (Figure 2A,E) and the percentage of Lu/BCAM-positive cells were increased (VOC-group: p=0.0012 for red cells, p=0.0008 for reticulocytes; non-VOC-group: p=0.04 for red cells, p=0.028 for reticulocytes) (Figure 2C,G). Surprisingly, Lu/BCAM expression on red blood cells and on reticulocytes was significantly higher (p=0.012 for red cells, p=0.028 for reticulocytes) in

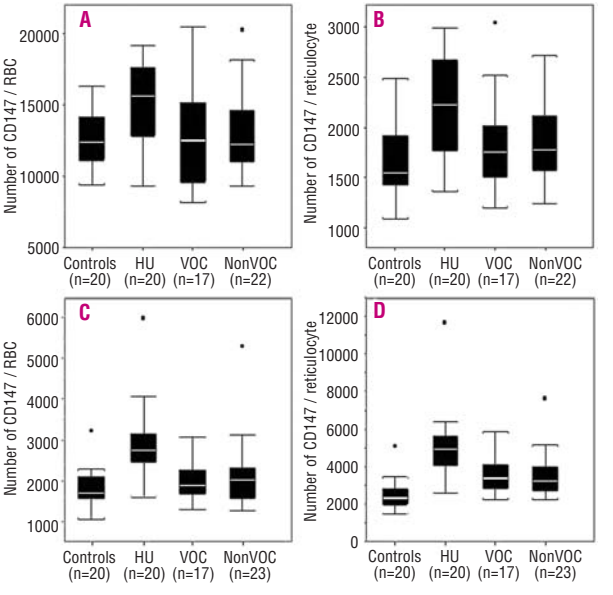

Figure 3. CD47 and CD147 expression on red blood cells (RBC) and on reticulocytes. For each group under study (Control, hydroxyurea-treated [HU], VOC and Non-VOC), the number of receptors/RBC (A, C) or reticulocytes (B, D) are indicated. Values are given as median, lower quartile, upper quartile, minimum and maximum for each group of patients and controls.

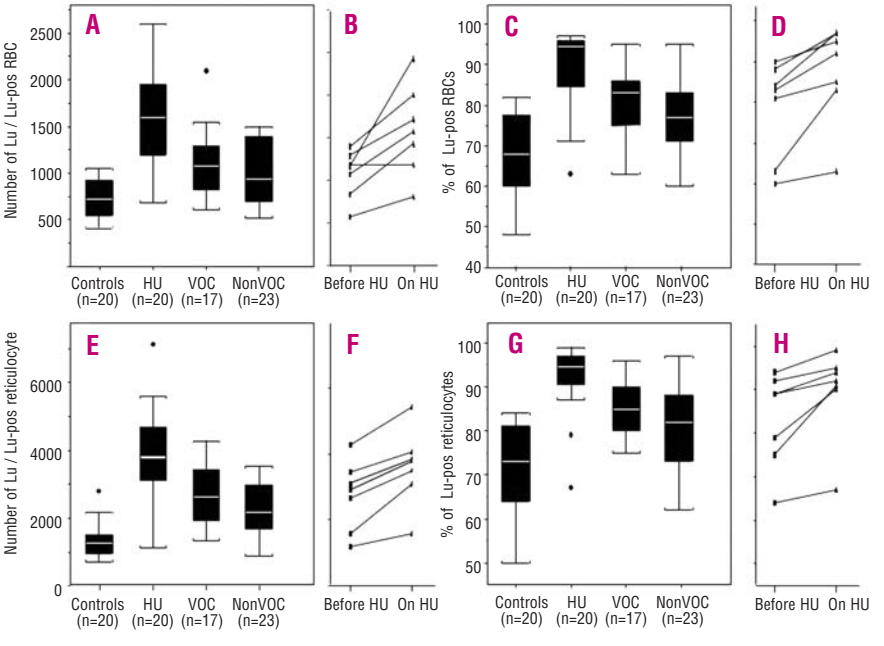

Figure 2. Lu/BCAM expression on red blood cells (RBC) and on reticulocytes. For each group under study (Control, hydroxyurea-treated [HU], VOC and NonVOC), the number of Lu/BCAM molecules/Lu-positive (Lu-pos) RBC or reticulocyte (A,E), and the percentage of Lu/BCAM-positive (Lu-pos) RBC or reticulocytes (C,G) are indicated. Lu/BCAM expression is also shown along a longitudinal follow-up of seven patients (B,D,F,H) initially included in the VOC-group and secondarily re-included in hydroxyurea-group after hydroxyurea therapy. Values are given as median, lower quartile, upper quartile, minimum and maximum for each group of patients and controls

hydroxyurea-treated patients than in untreated patients (Figure 2A, C, E, G), a result also found in the longitudinal study of the seven patients who were treated secondarily with hydroxyurea (p=0.02) (Figure 2B, D, F, H). There was no significant variation of ICAM-4 expression and percentage of ICAM-4 positive cells on red blood cells and reticulocytes between untreated patients, hydroxyurea-treated patients and controls (not shown), although a small but non-significant decrease (1.3-fold, p=0.06, *not shown*) of ICAM-4 expression on reticulocytes and red blood cells was seen for the five RhD-positive patients among the seven who initiated hydroxyurea treatment during the study (two are RhD-negative).

CD47 and CD147 in patients with sickle cells disease

CD47 and CD147 were not significantly increased in untreated patients as compared to in controls (Figure 3). CD47 expression on red blood cells (Figure 3A) and on reticulocytes (Figure 3B) was higher in hydroxyurea-treated patients (hydroxyurea-group) than in untreated patients and controls (the difference was significant for red blood cells, p=0.03); likewise, the expression of CD147 was higher in hydroxyurea-treated patients than in untreated patients and controls (p=0.0004 for red cells, p=0.008 for reticulocytes) (Figure 3C,D). The seven patients who were treated with hydroxyurea during the study also had an increase in CD47 expression (p=0.03) (*not shown*).

a5b1 integrin in patients with sickle cell disease

α5β1 integrin was not expressed at significant levels on red blood cells or reticulocytes from the patients with sickle cell disease (*not shown*).

Expression of adhesion molecules on erythroid progenitors

Control group

The control group included nine healthy African volunteers (AA or AS genotype). In cultures of their cells between day 6 (addition of erythropoietin) and day 20, the number of cells increased 8-fold and at day 20 about 67% of the cells were glycophorin-A$^+$, corresponding to polychromatic and acidophilic erythroblasts, but no significant enucleation occurred (*not shown*). When 50 μM hydroxy-urea were added on day 10 (proerythroblastic stage), both cell proliferation and maturation at day 20 were significantly decreased (3.6-fold, p<0.0001 and 40% glycophorin-A$^+$, p=0.0006, respectively). In contrast to the findings on reticulocytes (see above and Table 2), 100% of the glycophorin-A$^+$ progenitors were strongly CD36- and α4β1-positive (no sub-populations detectable at day 20). In contrast, the Lu/BCAM expression profile of progenitors was similar to those of red blood cells and reticulocytes, and only the Lu-positive population was analyzed, taking into account the percentage of Lu-positive cells and their LU antigen density.

Examination of adhesion molecules on the glycophorin-A$^+$ subsets cultured in the absence and presence of hydroxyurea showed a slight but not significant variation for CD36 (and CD147, *not shown*) (Figure 4A), but α4β1 and ICAM-4 were significantly reduced (median 15,460 range [2,600-23,730] *vs* 12,000 [range, 1,440-14,690] α4β1/cell, respectively, p=0.002 and median 2,940 [range, 600-4,810], *vs* 1,660 [range, 360-3,350] ICAM-4/cell, respectively, p=0.008), as illustrated in Figure 4 C and E. In contrast, in hydroxyurea-containing cultures both the percentage of LU-positive cells and the Lu/BCAM antigen density were increased. Indeed, the percentage of LU-positive cells rose from 31 (range 19-64) to 47 (range 24-75) (p=0.02) in hydroxyurea-containing cultures (*not shown*), and the number of Lu/BCAM molecules/Lu-positive cells increased slightly from 4,100 (range, 3,240-8,040) to 5,610 (range 3,890-8,600) (p=0.02), as illustrated in Fig.4G. Similarly, CD47 expression increased significantly in cultures with HU from 67,550 (range 47,900-103,150) to 92,825 (range 59,710-116,760) (p=0.03) (*not shown*).

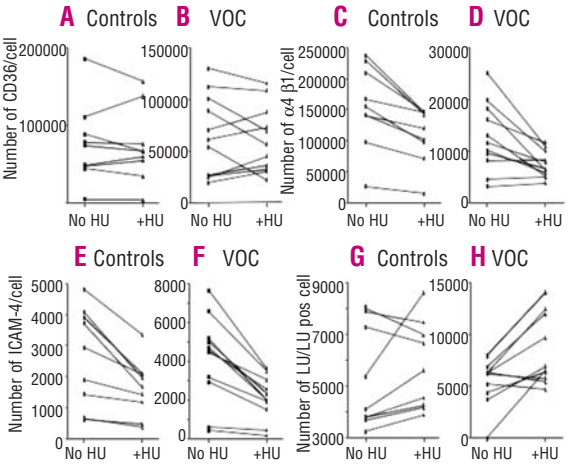

Figure 4. Adhesion receptors on erythroid progenitors from peripheral blood of control (n=9) and sickle cell patients (VOC, n=11) were grown in a two-phase liquid culture system in the absence (No hydroxyurea) or the presence (+ hydroxyurea) of 50 μM hydroxyurea [HU] added at day 10, during the erythropoietin-dependent phase. Adhesion receptors CD36 (A,B), α4β1 (C,D), ICAM-4 (E,F), Lu/BCAM (G,H) on erythroid cells at day 20 of the culture were determined by flow cytometry with monoclonal antibodies on the glycoprotein-A$^+$ gated cell populations.

VOC group

As compared to the control group, essentially similar results were obtained in symptomatic untreated patients (n=11), that is a significant decrease of cell proliferation and maturation at day 20 in hydroxyurea-containing cultures (4-fold and 53% glycophorin-A$^+$, respectively, p=0.0001). Regarding adhesion receptors, there was a significant decrease of α4β1 and ICAM-4 in hydroxyurea-containing cultures (median 11,640 [range, 3,135-25,150] *vs* 6,590 [range, 3,750-11,710] α4β1/cell, respectively, p=0.007 and median 4,530 [range, 430-7,640] *vs* 1,970

[range, 150-3,600] ICAM-4/cell, respectively, p=0.002), as illustrated in Figure 4 D and F, but CD36 did not differ (Figure 4B). The percentage of Lu-positive cells rose from 56 (range, 35-75) to 78 (range, 53-91) (p=0.002) in hydroxyurea-containing cultures (*not shown*) and the number of Lu/BCAM molecules/Lu-positive cell increased significantly from 6,305 (range, 3,690-8,020) to 8,315 (range, 4,690-14,130) (p=0.02), as shown Figure 4H. CD47 expression was also significantly increased from 49,610 (range, 38,550-62,760) to 63,810 (range, 45,630-91,870) (p=0.002), but CD147 was not significantly changed (*not shown*).

Discussion

Given that the clinical improvement after hydroxyurea therapy in patients with sickle cell disease includes a decrease in painful VOC and that adhesion of sickle cells to vascular endothelium is a critical factor in the pathogenesis of vaso-occlusive events, we analyzed the effect of hydroxyurea on adhesion receptors, present on circulating red blood cells and reticulocytes as well as on erythroid progenitors grown in a two-phase liquid culture system. Based on a large series of 74 patients, and in agreement with previous reports, we found an increase of CD36 and α4β1 expression before hydroxyurea[7-9] and reduced expression after hydroxyurea therapy.[23] We significantly extended these findings by analyzing the pattern of expression of other adhesion receptors, such as ICAM-4, Lu/BCAM, CD47 and CD147, on red bloods and reticulocytes before and after hydroxyurea therapy.

ICAM-4 expression and the percentage of red cells and reticulocytes expressing ICAM-4 was not different between patients with sickle cell disease and controls, which contrasts with the findings of another study published only as an abstract.[35] ICAM-4 expression under hydroxyurea treatment was not different, but there was a significant decrease in the longitudinal follow-up of seven patients who started hydroxyurea therapy during the study. Our finding of enhanced Lu/BCAM expression on red blood cells and reticulocytes in untreated patients as compared to controls confirms previous reports.[10,11] However, we found that overexpression of Lu/BCAM did not increase with disease severity since no significant difference was noted between patients in the non-VOC and VOC groups. Much to our surprise, Lu/BCAM expression on red blood cells and reticulocytes was significantly higher in hydroxyurea-treated than untreated patients. Moreover, this increase was also clearly presented in the longitudinal follow-up of seven patients studied before and after hydroxyurea therapy. This is the first report describing the effect of hydroxyurea therapy on Lu/BCAM expression. Our results are unexpected for several reasons: (i) it is well established that *in vitro* adhesion of sickle cells to thrombospondin and laminin[22] and to endothelial cells[36,37] is decreased in patients on hydroxyurea, (ii) there is a direct relationship between Lu/BCAM level and the laminin adhesion of sickle red cells,[10,11] and (iii) other adhesion receptors such as CD36, α4β1 (and ICAM-4 to a lesser extent) are significantly decreased after hydroxyurea therapy (*see above*).

Lu/BCAM was not the only adhesion receptor whose expression on red blood cells and reticulocytes was enhanced following hydroxyurea therapy. This was also found for CD47 and CD147. A notable difference, however, was that the level of these adhesion receptors was not increased in sickle cells patients as compared to other receptors discussed above. The only effect was an increase in hydroxyurea-treated patients. As noted for Lu/BCAM, overexpression of CD47 on red blood cells and reticulocytes of hydroxyurea-treated patients was unexpected as adhesion of sickle cells to thrombospondin through a CD47-induced α4β1 activation mechanism[38] is reduced on hydroxyurea.[22] CD147 (basigin, Oka antigen) is a widely expressed transmembrane glycoprotein implicated in reproduction, neural function, inflammation, and tumor progression.[39] Although anti-CD147 monoclonal antibodies inhibit adhesion of a breast cancer cell line to laminin, fibronectin and type IV collagen,[40] the significance of CD147 increase under hydroxyurea therapy is not clear.

To gain further insights into the mechanism(s) by which sickle cells acquired adhesion receptors we looked at the expression of these molecules on erythroid progenitors cultured in a two-phase liquid culture system in the presence or absence of hydroxyurea. The results obtained with erythroid progenitors from symptomatic untreated patients (VOC group) and controls were concordant with our findings on circulating red blood cells and reticulocytes, although there were some differences. In the presence of hydroxyurea, α4β1 and ICAM-4 expression was decreased whereas Lu/BCAM and CD47 expression was increased within the glycophorin-A$^+$ subsets of erythroid cells. CD36 and CD147 exhibited no significant changes but were still expressed at a high level, consistent with a delayed stage of maturation of the erythroblasts. These data suggest that hydroxyurea probably acts early during erythropoiesis, on erythroid progenitors before their release into the peripheral circulation. Of note, grossly similar changes were found with cultures of erythroid progenitors exposed to hydroxyurea both from sickle cell patients and control individuals, suggesting that hydroxyurea is able to modulate similarly adhesion receptor expression in normal and sickle erythroid progenitors. Although the results performed on circulating cells and erythroid progentitors are in fairly good agreement, they still should be interpreted with caution since hydroxyurea was given *in vivo* to patients but was added *in vitro* into cell cultures, and many factors may influence the *in vivo* action of hydroxyurea therapy, including oral biodisponibility and metabolism. The mechanism(s) by which hydroxyurea modulates adhesion receptor expression is poorly understood. Hydroxyurea might act at least in part through some alteration of proliferation and maturation of erythroid progenitors.[30] It might also modulate gene expression[31] and further studies should indicate whether

CD36, α4β1 and ICAM-4 on one hand, and Lu/BCAM (as well as CD47 and CD147) on the other hand are differently regulated by transcription.

Our current results strongly indicate some uncoupling between membrane expression level of adhesion molecules and substrate adhesion of sickle cells following hydroxyurea therapy, suggesting that hydroxyurea may affect red blood cells adhesiveness by another mechanism. Interestingly, recent studies have revealed that several erythrocyte adhesion receptors consist of molecules that require activation by signal transduction. Examples are α4β1 via CD47,[38] ICAM-4[40] and Lu/BCAM.[41-43] Lu/BCAM adhesion to laminin may occur through two adrenergic activation cAMP-dependent pathways, one being PKA-dependent[41] involving Lu/BCAM phosphorylation[42] and another that occurs independently of PKA, which is promoted by Rap1 (small GTPase) activation.[43] The relative contribution of these pathways to promoting adhesion is unknown. Therefore, a tentative explanation of our results could be that the activation state of these adhesion receptors through the phosphorylation cascade might be the critical factor that modulates adhesiveness rather than their membrane expression level, as previously thought.[23] Studies to test this hypothesis are currently in progress. In conclusion, the critical observation made in this study showing that the expression level of some adhesion receptors, particularly Lu/BCAM, is increased *in vivo* on erythroid cells from sickle cell patients receiving hydroxyurea therapy and that this effect can be seen *in vitro* when erythroid progenitors are grown in the presence of hydroxyurea deserves further studies and provide an interesting model for investigating the mechanism(s) of action of hydroxyurea and the relationship between activation of adhesion receptors and cellular adhesion.

Authorship and Disclosures

All authors meet the criteria for being contributing authors. MHO participated in the patients' recruitment, performed the erythroid cell cultures and participated in the preparation of the manuscript. VB performed the FACS analysis of red cells and erythroid precursors. MB participated in recruitment and follow-up of the patients. CL participated in the erythroid cell cultures. CA performed the statistical analyses. RD and EJA determined HbF concentrations in plasma and hydroxyurea concentrations in erythroid cells. JE and JPC contributed equally to the design of the research, analysis of the results and writing the manuscript. All authors were involved in the discussion and interpretation of data and all approved the final version.

References

1. Hoover R, Rubin R, Wise G, Warren R. Adhesion of normal and sickle erythrocytes to endothelial monolayer cultures. Blood 1979;54:872-6.
2. Hebbel RP, Yamada O, Moldow CF, Jacob HS, White JG. Eaton JW. Abnormal adherence of sickle erythrocytes to cultured vascular endothelium: possible mechanism for microvascular occlusion in sickle cell disease. J Clin Invest 1980;65: 154-60.
3. Hebbel RP. Adhesive interactions of sickle erythrocytes with endothelium. J Clin Invest 1997;99:2561-4.
4. Frenette P. Sickle cell vaso-occlusion: multistep and multicellular paradigm. Curr Op Hematol 2002;9:101-6.
5. Hebbel RP, Osarogiagbon R, Kaul D. The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. Microcirc 2004;11: 129-51.
6. Telen MJ. Erythrocyte adhesion receptors: blood group antigens and related molecules. Transf Med Rev 2005; 19:32-44.
7. Sugihara K, Sugihara T, Mohandas N, Hebbel RP. Thrombospondin mediates adherence of CD36+ sickle reticulocytes to endothelial cells. Blood 1992;80:2634-42.
8. Joneckis CC, Ackley RL, Orringer EP, Wayner EA, Parise LV. Integrin a 4 beta 1 and glycoprotein IV (CD36) are expressed on circulating reticulocytes in sickle cell anemia. Blood 1993; 82:3548-55.
9. Swerlick RA, Eckman JR, Kumar A, Jeitler M, Wick TM. Alpha 4 beta 1-integrin expression on sickle reticulocytes: vascular cell adhesion molecule-1-dependent binding to endothelium. Blood 1993;82:1891-9.
10. El Nemer W, Gane P, Colin Y, Bony V, Rahuel C, Galacteros F, et al. The Lutheran blood group glycoproteins, the erythroid receptors for laminin, are adhesion molecules. J Biol Chem 1998;273:16686-93.
11. Udani M, Zen Q, Cottman M, Leonard N, Jefferson S, Daymont C, et al. Basal cell adhesion molecule/Lutheran protein. The receptor critical for sickle cell adhesion to laminin. J Clin Invest 1998;101:2550-8.
12. Hermand P, Gane P, Callebaut I, Kieffer N, Cartron JP, Bailly P. Integrin receptor specificity for human red cell ICAM-4 ligand. Critical residues for aIIb3 and aVb3 binding. Eur J Biochem 2004; 271:3729-40.
13. Mankelow TJ, Spring FA, Parsons SF, Brady RL, Mohandas N, Chasis JA, et al. Identification of critical amino-acid residues on the erythroid intercellular adhesion molecule-4 (ICAM-4) mediating adhesion to aV-integrins. Blood 2004;103:1503-8.
14. Kaul DK, Liu XD, Zhang X, Mankelow T, Parsons S, Spring F, et al. Peptides based on aV-binding domains of erythrocyte ICAM-4 inhibit sickle red cell-endothelial interactions and vaso-occlusion in the microcirculation. Am J Physiol Cell Physiol 2006;291:C922-30.
15. El Nemer W, Wautier MP, Rahuel C, Gane P, Hermand P, Galacteros F, et al. Endothelial Lu/BCAM glycoproteins are novel ligands for red blood cell a4b1 integrin: role in adhesion of sickle red blood cells to endothelium cells. Blood 2007;109:3544-51.
16. Browne PV, Mosher DF, Steinberg MH, Hebbel RP. Disturbance of plasma and platelet thrombospondin levels in sickle cell disease. Am J Hematol 1996;51: 296-301.
17. Gee BE, Platt OS. Sickle reticulocytes adhere to VCAM-1. Blood 1995;85: 268-74.
18. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. N Engl J Med 1995;332:1317-22.
19. Platt OS, Thorington BD, Brambilla DJ, Milner PF, Rosse WF, Vichinsky E, et al. Pain in sickle cell disease. Rates and risk factors. N Engl J Med 1991; 325:11-6.
20. Charache S, Dover GJ, Moore RD, Eckert S, Ballas SK, Koshy M, et al. Hydroxyurea: effects on hemoglobin F production in patients with sickle cell anemia. Blood 1992;79:2555-65.
21. Brun M, Bourdoulous S, Couraud PO, Elion J, Krishnamoorthy R, Lapoumeroulie C. Hydroxyurea downregulates endothelin-1 gene expression and upregulates ICAM-1 gene expression in cultured human endothelial cells. Pharmacogen J 2003; 3:215-26.

22. Hillery CA, Du MC, Wang WC, Scott JP. Hydroxyurea therapy decreases the in vitro adhesion of sickle erythrocytes to thrombospondin and laminin. Br J Haematol 2000; 109:322-7.
23. Styles LA, Lubin B, Vichinsky E, Lawrence S, Hua M, Test S, Kuypers F. Decrease of very late activation antigen-4 and CD36 on reticulocytes in sickle cell patients treated with hydroxyurea. Blood 1997;89:2554-9.
24. Saleh AW, Hillen HF, Duits AJ. Levels of endothelial, neutrophil and platelet-specific factors in sickle cell anemia patients during hydroxyurea therapy. Acta Haematol 1999;102: 31-7.
25. Lapoumeroulie C, Benkerrou M, Odievre MH, Ducrocq R, Brun M, Elion J. Decreased plasma endothelin-1 levels in children with sickle cell disease treated with hydroxyurea. Haematologica 2005;90:401-3.
26. Benkerrou M, Delarche C, Brahimi L, Fay M, Vilmer E, Elion J, et al. Hydroxyurea corrects the dysregulated L-selectin expression and increased H2O2 production of polymorphonuclear neutrophils from patients with sickle cell anemia. Blood 2002;99: 2297-303.
27. Bony V, Gane P, Bailly P, Cartron JP. Time-course expression of polypeptides carrying blood group antigens during human erythroid differentiation. Br J Haematol 1999;107:263-74.
28. Hasegawa S, Rodgers GP, Dwyer N, Noguchi CT, Blanchette-Mackie EJ, Uyesaka N, et al. Sickling of nucleated erythroid precursors from patients with sickle cell anemia. Exp Hematol 1998;26:314-9.
29. Wada H, Suda T, Miura Y, Kajii E, Ikemoto S, Yawata Y. Expression of major blood group antigens on human erythroid cells in a two-phase liquid culture system. Blood 1990;75: 505-11.
30. Fibach E, Burke LP, Schechter AN, Noguchi CT, Rodgers GP. Hydroxyurea increases fetal hemoglobin in cultured erythroid cells derived from normal individuals and patients with sickle cell anemia or b-thalassemia. Blood 1993; 81:1630-5.
31. Wang M, Tang DC, Liu W, Chin K, Zhu JG, Fibach E, et al. Hydroxyurea exerts bi-modal dose-dependent effects on erythropoiesis in human cultured erythroid cells via distinct pathways. Br J Haematol 2002;119: 1098-105.
32. Lee K, Gane P, Roudot-Thoraval F, Godeau B, Bachir D, Bernaudin F, et al. The nonexpression of CD36 on reticulocytes and mature red blood cells does not modify the clinical course of patients with sickle cell anemia. Blood 2001;98:966-71.
33. Havard J, Grygiel J, Sampson D. Determination by high-performance liquid chromatography of hydroxyurea in human plasma. J Chromatogr 1992;584:270-4.
34. Davies S, Gilmore A. The role of hydroxyurea in the management of sickle cell disease. Blood Rev 2003; 17:99-109.
35. Krishnan R, Brown KA, Pearson TC, Macey M, Spring FA, Parsons SF, et al. Increased expression of Lutheran and LW glycoproteins on red blood cells from patients with sickle cell disease. Br J Haematol 1999;105:64.
36. Bridges KR, Barabino GD, Brugnara C, Cho MR, Christoph GW, Dover G, et al. A multiparameter analysis of sickle erythrocytes in patients undergoing hydroxyurea therapy. Blood 1996; 88:4701-10.
37. Adragna NC, Fonseca P, Lauf. PK. Hydroxyurea affects cell morphology, cation transport, and red blood cell adhesion in cultured vascular endothelial cells. Blood 1994;83:553-60.
38. Brittain JE, Han J, Ataga KI, Orringer EP, Parise LV. Mechanism of CD47-induced α4β1 integrin activation and adhesion in sickle reticulocytes. J Biol Chem 2004;279:42393-402.
39. Nabeshima K, Iwasaki H, Koga K, Hojo H, Suzumiya J, Kikuchi M. Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression. Pathol Intern 2006; 56:359-67.
40. Schiavone EM, Tortora V, Armetta I, Bontempo P, Mosti MR, Pezone L, et al. In: Leukocyte Typing VI (Kishimoto et al., eds), Garland Publishing, Inc., New York, 1997. p. 764-6.
40. Zennadi R, Hines PC, De Castro LM, Cartron JP, Parise LV, Telen MJ. Epinephrine acts through erythroid signaling pathways to activate sickle cell adhesion to endothelium via LW-aVb3 interactions. Blood 2004;104: 3774-81.
41. Hines PC, Zen Q, Burney SN, Shea DA, Ataga KI, Orringer EP, et al. Novel epinephrine and cyclic AMP-mediated activation of BCAM/Lu-dependent sickle (SS) RBC adhesion. Blood 2003;101:3281-7.
42. Gauthier E, Rahuel C, Wautier MP, El Nemer W, Gane P, Wautier JL, et al. Protein kinase A-dependent phosphorylation of Lutheran/basal cell adhesion molecule glycoprotein regulates cell adhesion to laminin a5. J Biol Chem 2005;280:30055-62.
43. Murphy MM, Zayed MA, Evans A, Parker CE, Ataga KI, Telen MJ, et al. Role of Rap1 in promoting sickle red blood cell adhesion to laminin via BCAM/LU. Blood 2005;105:3322-9.