Biochemical and Biophysical Research Communications 390 (2009) 161–164

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Plasma annexin A5 and microparticle phosphatidylserine levels are elevated in sickle cell disease and increase further during painful crisis

L.J. van Tits [a,*], W.L. van Heerde [b], P.P. Landburg [c,d], M.J. Boderie [c,d], F.A.J. Muskiet [c,e], N. Jacobs [b], A.J. Duits [c,d], J.B. Schnog [c,d,f]

[a] Department of General Internal Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[b] Central Laboratory for Hematology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[c] CURAMA Study Group, Immunology Laboratory Department, Curaçao, Netherlands Antilles
[d] Immunology Laboratory Department, Red Cross Blood Bank Foundation, Curaçao, Netherlands Antilles
[e] Department of Pathology and Laboratory Medicine, University Hospital Groningen, Groningen, The Netherlands
[f] Department of Internal Medicine, Slotervaart Hospital, Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 21 September 2009
Available online 30 September 2009

Keywords:
Annexin A5
Hypercoagulable state
Microparticles
Phosphatidylserine
Sickle cell disease
Thrombin generation

ABSTRACT

Expression of phosphatidylserine (PS) on the membrane surface of red blood cells and circulating microparticles (MP) plays an important role in etiology of the hypercoagulable state of sickle cell disease (SCD), as well as in the reduced red cell life span and adhesive interactions between red cells and endothelium. Annexin A5, an intracellular protein abundantly present in endothelial cells and platelets, exhibits high affinity for PS and has been shown to inhibit several of these PS-mediated pathophysiological processes. We determined plasma annexin A5 levels and MP-associated procoagulant activity, a measure of MP–PS exposure, in 17 sickle cell patients (12 HbSS and 5 HbSC) in steady state and at presentation with a painful crisis. Twenty-five HbAA blood donors served as controls.

Both annexin A5 and MP–PS were highest in HbSS patients (5.7 ng/mL, IQR 3.7–7.6 and 37.9 nM, IQR 31.9–69.8) as compared to HbSC patients (1.8 ng/mL, IQR 1.7–7.6 and 20.9 nM, IQR 10.9–29.6) and healthy controls (2.5 ng/mL, IQR 1.4–4.4 and 13.1 nM, IQR 9.5–18.5) ($p = 0.01$ and $p < 0.001$, respectively). At presentation with a painful crisis, annexin A5 and MP–PS had increased in 16 of 17 patients ($p = 0.001$ and $p < 0.001$, respectively). Most interestingly, in 7 HbSS patients the proportional increase in MP–PS exposure was higher than the proportional increase in plasma annexin A5 concentration, leading to lower annexin A5/MP–PS ratio of HbSS patients during crisis than HbAA controls (0.0027 (0.0017–0.0049) vs 0.0048 (0.0027–0.0085), $p = 0.05$). In conclusion, patients with SCD have elevated plasma levels of annexin A5- and PS-exposing MP. During crisis both levels increase, but in most HbSS patients MP–PS exposure increases more than annexin A5. Future studies must address a potential role of annexin A5 in modulating PS-related pathophysiological processes in SCD.

Introduction

In contrast to normal hemoglobin, sickle hemoglobin (HbS) polymerizes upon de-oxygenation and causes the erythrocyte to take on its characteristic sickle shape. A cardinal down stream event from HbS polymerization is the loss of red cell membrane phospholipid asymmetry, leading to an at random intra- and extracellular expression of phosphatidylserine (PS) [1]. Increased PS expression on sickle red blood cells is of importance in several processes involved in SCD pathology. Sickle red blood cell PS is involved in the adhesion of sickle erythrocytes to activated endothelial cells [2,3], thereby contributing to micro-vascular occlusion. Also, PS exposure contributes to shortened erythrocyte life span due to complement recognition. Furthermore, once initiated by tissue factor, thrombin generation is greatly accelerated in the presence of PS (normally presented by activated platelets) [1,4,5], and the increased extracellular PS exposure surely contributes to the characteristic hypercoagulable state of sickle cell disease [6]. During a painful crisis, a further dramatic rise of coagulation activation occurs, partly due to an increased formation of microparticles (MP), derived from erythrocytes [7], endothelial

Abbreviations: HbS, sickle hemoglobin; MP, microparticle; PS, phosphatidylserine; SCD, sickle cell disease

* Corresponding author. Address: Radboud University Nijmegen Medical Centre, Department of General Internal Medicine 441, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Fax: +31 24 3541346.
E-mail address: B.vantits@aig.umcn.nl (L.J. van Tits).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.09.102

cells and monocytes [8]. MP expose PS on the membrane and stimulate thrombin generation [9]. In addition, MP derived from monocytes and endothelial cells express tissue factor [8]. Moreover, hydrolysis of PS by secretory phospholipase A_2 leads to generation of vasoactive prostaglandins, leukotrienes and thromboxanes [10,11], which are of importance in the development of the acute chest syndrome [12,13].

Annexin A5 is a protein with high affinity for membrane-bound anionic phospholipids [14]. It is abundantly present in endothelial cells and platelets [15,16] and is released upon (traumatic) tissue injury. Plasma annexin A5 levels are low (1–5 ng/mL) in healthy individuals, but are elevated during acute myocardial infarction or unstable angina [17]. Through formation of highly ordered two-dimensional crystals that coat the external leaflet of phospholipid bilayers, annexin A5 shields these molecules from availability for phospholipid-dependent coagulation reactions [18,19]. Indeed, annexin A5 was recently shown to markedly diminish endothelial MP-driven thrombin generation through PS binding [20]. To our knowledge, annexin A5 levels in SCD have not been studied before. Given the importance of PS expression in the pathophysiology of SCD and the potency of annexin A5 to block PS-mediated processes, we set out to investigate plasma levels of annexin A5 in SCD.

Materials and methods

Subjects. For the present study, blood was collected from the antecubital vein from 17 sickle cell patients presenting at the emergency room department of the St. Elisabeth Hospital (Curaçao, Netherlands Antilles) with a painful crisis (defined as an episode of acute pain in the extremities and/or abdomen not otherwise explained [21]) between March and November 2006. At least 3 weeks after resolution of symptoms, blood was again collected during the clinically asymptomatic state (defined as being free from any symptoms indicating acute SCD related complications). None of the patients were on any kind of treatment (apart from folic acid supplementation), nor had they received blood transfusions during the 3 months prior to sample collection. Twenty-five race- (but not age- and sex-) matched HbAA blood donors served as healthy controls. All blood was collected in EDTA tubes and immediately centrifuged at 4000 rpm for 20 min at 4 °C. Plasma was stored at −80 °C until further analysis. Patients and controls gave written informed consent before sample collection. The study was approved by the local Ethical Review Board and conducted in agreement with the Helsinki Declaration of 2000.

Biochemical measurements. Standard blood counts and clinical biochemistry were determined according to local protocols. Plasma annexin A5 was measured with commercial enzyme immunoassay (Hyphen BioMed, Neuville-sur-Oise, France) according to the instructions of the manufacturer. Intra- and interassay coefficients of variation amounted 1.4% and 3.8%.

MP-associated procoagulant activity was estimated with the Zymuphen MP Activity assay from Hyphen BioMed (Neuville-sur-Oise, France). The assay determines the generation of thrombin in wells of a microtiterplate by MP isolated from samples by binding to immobilized annexin A5. Because the presence of phospholipids on the MP is the rate-limiting factor for thrombin generation in the assay, the amount of thrombin generated is a measure of the procoagulant activity associated with MP present in the sample. A washed and lysed platelet concentrate was used for calibration; results are expressed as nanomolar phosphatidylserine equivalents. Intra- and interassay coefficients of variation of the assay are 3–8% and 5–10%, respectively, as provided by the manufacturer.

Prothrombin fragment 1 + 2 (F1 + 2) was determined by enzyme immunoassay according to the instructions of the manufacturer (Enzygnost, Dade Behring, Marburg, Germany).

Statistical analysis. Data are presented as medians with corresponding interquartile ranges. For between multiple group comparisons the Kruskal–Wallis test was employed. The Mann–Whitney *U*-test was used for comparison between two groups. For paired sample analysis the Wilcoxons' Signed Rank test was used. Statistical software Package for the Social Science version 16.0 (SPSS Inc., Chicago, IL, USA) was used and *P*-values < 0.05 were considered statistically significant.

Results

Characteristics and laboratory data of healthy controls and of sickle cell patients (classified by genotype) in steady state are presented in Table 1. MP–PS exposure was higher in HbSS compared to HbSC ($p = 0.007$) and controls ($p < 0.001$), but did not differ between HbSC and controls. Plasma annexin A5 concentration of HbSC also did not differ from that of controls but in HbSS patients plasma annexin A5 was higher than in controls ($p = 0.005$). The ratios of annexin A5/MP–PS were not different between groups.

At presentation with a painful crisis, MP–PS exposure was significantly higher compared to steady state for both HbSS and HbSC sickle cell patients (Table 2). Simultaneously with the increase in MP–PS exposure, plasma concentration of annexin A5 increased in HbSS and in HbSC sickle cell patients. Even though annexin A5/MP–PS ratios did not differ significantly at presentation with a painful crisis as compared to the steady state when analyzed as a group, in 9 patients (7 HbSS and 2 HbSC) the proportional increase in MP–PS exposure was higher than the proportional increase in plasma annexin A5 concentration. Consequently, in these patients the annexin A5/MP–PS ratio decreased following development of painful crisis. In 3 HbSS patients and in 1 HbSC patient the ratio did not change, and in 2 patients of each genotype the ratio increased. Compared to HbAA controls, the ratio of annexin A5/MP–PS exposure of HbSS sickle cell patients during painful crisis was significantly lower ($p = 0.05$).

At presentation with a painful crisis, F1 + 2 was significantly higher compared to the steady state for HbSS patients but not for HbSC patients (Table 2). Annexin A5/MP–PS ratios of the SCD patients did not significantly correlate to F1 + 2 concentrations or to the length of admission to the hospital, neither when analyzed as genotype subgroups nor when analyzed together (data not shown). Furthermore, none of the measured parameters differed significantly between those sickle cell patients admitted to the hospital

Table 1
Characteristics and laboratory data of healthy controls and of sickle cell patients in steady state.

	HbAA ($n = 25$)	HbSS ($n = 12$)	HbSC ($n = 5$)	*P*-value
Age (year)	47 (42–54)	23 (17–34)	25 (18–50)	<0.001
Gender (m:f)	18:7	8:4	4:1	
Hemoglobin (g/dL)	15.2 (13.4–16.2)	8.8 (7.7–9.4)	11.2 (10.1–11.9)	<0.001
MP–PS (nM PS)	13.1 (9.5–18.5)	37.9 (31.9–69.8)	20.9 (10.9–29.6)	<0.001
Annexin A5 (μg/L)	2.5 (1.4–4.4)	5.7 (3.7–7.6)	1.8 (1.7–7.6)	0.014
Ratio annexin A5/ MP–PS	0.0048 (0.0027–0.0085)	0.0040 (0.0020–0.0055)	0.0040 (0.0021–0.0225)	0.34

Data are depicted as medians with interquartile ranges. The Kruskal–Wallis test was employed for between group analyses. MP, microparticles; PS, phosphatidylserine.

Table 2
Microparticle-associated PS exposure and plasma annexin A5 in the clinically asymptomatic state and at presentation with a painful crisis.

	HbSS (n = 12)			HbSC (n = 5)		
	Asymptomatic state	Painful crisis	P-value	Asymptomatic state	Painful crisis	P-value
MP–PS (nM PS)	37.9 (31.9–69.8)	124.3 (94.6–151.5)	0.003	20.9 (10.9–29.6)	106.8 (53.0–168.6)	0.043
Annexin A5 (μg/L)	5.7 (3.7–7.6)	12.6 (5.5–27.4)	0.004	1.8 (1.7–7.6)	12.1 (6.9–26.1)	0.043
Ratio annexin A5/MP–PS	0.0040(0.0020–0.0055)	0.0027(0.0017–0.0049)	0.39	0.0040(0.0021–0.0225)	0.0051(0.0020–0.0060)	0.69
F1 + 2 (pmol/L)	318 (231–426)	344 (234–574)	0.047	300 (143–477)	233 (215–427)	0.69

Data are depicted as medians with interquartile ranges. Wilcoxons' Signed Rank test was used for paired sample analyses.

for treatment of their painful crisis (n = 8) as compared to the patients whose crisis was managed at the Emergency ward (data not shown).

Discussion

In this study, we show that HbSS sickle cell patients have elevated plasma concentrations of annexin A5 compared to healthy controls, with increments in both HbSS and HbSC patients at presentation with a painful crisis. Simultaneously with the rise of plasma annexin A5, MP–PS exposure increased.

Extracellular PS expression plays an important role in etiology of several pathophysiological processes in SCD. Abnormal adhesive interactions between sickle red blood cells and activated endothelium are, at least in part, orchestrated by PS on red cells and thrombospondin on activated endothelium. Such adhesive interactions have been recognized as key events initiating vaso-occlusion in SCD [22]. In addition, PS exposed on sickle red blood cells (but also on platelets and cell derived MP) act as a docking site for serine proteases of the tenase and pro-thrombinase complexes of blood coagulation, thereby augmenting thrombin generation [1] and promoting the development of a hypercoagulable state [6]. Because annexin A5 through high-affinity binding to PS may block interaction of PS with other plasma proteins, it may modulate SCD-related pathophysiological processes and contribute to the heterogeneity of this monogenic disease.

In support of a potential role for annexin A5 in pathophysiology of SCD, it was previously shown that annexin A5 inhibits the adhesion of sickle erythrocytes to the endothelium [3,23], displaces blood coagulation factors from procoagulant phospholipid surfaces [14,18], and markedly diminishes endothelial MP-driven thrombin generation in vitro [20]. Moreover, annexin A5 has been shown to inhibit phospholipid hydrolysis of PS-exposing cells by secretory phospholipase A_2, thereby reducing the formation of lysophospholipids and free fatty acids (e.g. arachidonic acid), which in turn are a source of mediators of blood coagulation and reperfusion injury such as prostaglandins and thromboxanes [24,25]. Secretory phospholipase A_2 was recently recognized to play an important role in the pathophysiology of the acute chest syndrome, the leading cause of death in SCD [12,13], and it would be interesting to hypothesize that patients with insufficient annexin A5 release could be more prone to developing an acute chest syndrome as opposed to patients with high annexin A5 levels.

Apart from the aforementioned effects of annexin A5, all of which are related to shielding PS from interaction with other ligands, annexin A5 has recently been shown to induce downregulation of tissue factor expression by apoptotic THP-1 macrophages in vitro and by smooth muscle cells in mechanically injured mouse carotid arteries in vivo, perhaps due to internalization of PS-expressing membrane patches and the receptors embedded within them [26].

The present study is the first to report on plasma levels of annexin A5 in SCD. We observed elevated concentrations of annexin A5 in the plasma of sickle cell patients and further increases during sickle cell crisis, possibly due to release from activated platelets and endothelial cells [6,27–30]. Although we observed no significant differences in the annexin A5/MP–PS ratios at presentation with a painful crisis as compared to the steady state when analyzed as groups, in half of our patients annexin A5 decreased relative to the amount of PS exposed by MP. For HbSS patients but not for HbSC patients this resulted in a significantly lower ratio of annexin A5/MP–PS than HbAA controls. Most interestingly, HbSS patients but not HbSC patients showed an increase in prothrombotic activity, as depicted by increased F1 + 2 concentrations. Whether a low annexin A5/MP–PS ratio is indicative of an annexin A5 shortage cannot be deduced from these data and is subject of further investigation. Furthermore, prospective studies including larger numbers of patients are being carried out in order to determine whether annexin A5 is related to SCD severity and whether it could be used as a biomarker of disease severity.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Heidi Lemmers, Helga Toenhake-Dijkstra, Luuk van den Goor and Paul Lap.

References

[1] R.F. Zwaal, A.J. Schroit, Pathophysiologic implications of membrane phospholipid asymmetry in blood cells, Blood 89 (1997) 1121–1132.
[2] B.N. Setty, S. Kulkarni, A.K. Rao, M.J. Stuart, Fetal hemoglobin in sickle cell disease: relationship to erythrocyte phosphatidylserine exposure and coagulation activation, Blood 96 (2000) 1119–1124.
[3] B.N. Setty, S. Kulkarni, M.J. Stuart, Role of erythrocyte phosphatidylserine in sickle red cell–endothelial adhesion, Blood 99 (2002) 1564–1571.
[4] K.G. Mann, Biochemistry and physiology of blood coagulation, Thromb. Haemost. 82 (1999) 165–174.
[5] B.N. Setty, A.K. Rao, M.J. Stuart, Thrombophilia in sickle cell disease: the red cell connection, Blood 98 (2001) 3228–3233.
[6] K.I. Ataga, N.S. Key, Hypercoagulability in sickle cell disease: new approaches to an old problem, Hematology (Am. Soc. Hematol. Educ. Program) 2007 (2007) 91–96.
[7] D. Allan, A.R. Limbrick, P. Thomas, M.P. Westerman, Release of spectrin-free spicules on reoxygenation of sickled erythrocytes, Nature 295 (1982) 612–613.
[8] A.S. Shet, O. Aras, K. Gupta, et al., Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes, Blood 102 (2003) 2678–2683.
[9] R.J. Berckmans, R. Nieuwland, A.N. Boing, et al., Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation, Thromb. Haemost. 85 (2001) 639–646.
[10] N.A. Neidlinger, E.R. Hirvela, R.A. Skinner, et al., Postinjury serum secretory phospholipase A2 correlates with hypoxemia and clinical status at 72 hours, J. Am. Coll. Surg. 200 (2005) 173–178.
[11] N.A. Neidlinger, S.K. Larkin, A. Bhagat, et al., Hydrolysis of phosphatidylserine-exposing red blood cells by secretory phospholipase A2 generates lysophosphatidic acid and results in vascular dysfunction, J. Biol. Chem. 281 (2006) 775–781.
[12] L.A. Styles, C.G. Schalkwijk, A.J. Aarsman, et al., Phospholipase A2 levels in acute chest syndrome of sickle cell disease, Blood 87 (1996) 2573–2578.
[13] L.A. Styles, A.J. Aarsman, E.P. Vichinsky, F.A. Kuypers, Secretory phospholipase A(2) predicts impending acute chest syndrome in sickle cell disease, Blood 96 (2000) 3276–3278.
[14] H.A. Andree, C.P. Reutelingsperger, R. Hauptmann, et al., Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers, J. Biol. Chem. 265 (1990) 4923–4928.

[15] M.J. Flaherty, S. West, R.L. Heimark, et al., Placental anticoagulant protein-I: measurement in extracellular fluids and cells of the hemostatic system, J. Lab. Clin. Med. 115 (1990) 174–181.
[16] J. Romisch, E. Schuler, B. Bastian, et al., Annexins I to VI: quantitative determination in different human cell types and in plasma after myocardial infarction, Blood Coagul. Fibrinolysis 3 (1992) 11–17.
[17] N. Kaneko, R. Matsuda, S. Hosoda, et al., Measurement of plasma annexin V by ELISA in the early detection of acute myocardial infarction, Clin. Chim. Acta 251 (1996) 65–80.
[18] H.A. Andree, M.C. Stuart, W.T. Hermens, et al., Clustering of lipid-bound annexin V may explain its anticoagulant effect, J. Biol. Chem. 267 (1992) 17907–17912.
[19] F. Oling, W. Bergsma-Schutter, A. Brisson, Trimers, dimers of trimers, and trimers of trimers are common building blocks of annexin a5 two-dimensional crystals, J. Struct. Biol. 133 (2001) 55–63.
[20] D. Lechner, M. Kollars, A. Gleiss, et al., Chemotherapy-induced thrombin generation via procoagulant endothelial microparticles is independent of tissue factor activity, J. Thromb. Haemost. 5 (2007) 2445–2452.
[21] G.R. Serjeant, C.D. Ceulaer, R. Lethbridge, et al., The painful crisis of homozygous sickle cell disease: clinical features, Br. J. Haematol. 87 (1994) 586–591.
[22] R.P. Hebbel, M.A. Boogaerts, J.W. Eaton, M.H. Steinberg, Erythrocyte adherence to endothelium in sickle-cell anemia. A possible determinant of disease severity, N. Engl. J. Med. 302 (1980) 992–995.
[23] B.N. Setty, S.G. Betal, Microvascular endothelial cells express a phosphatidylserine receptor: a functionally active receptor for phosphatidylserine-positive erythrocytes, Blood 111 (2008) 905–914.
[24] F.A. Kuypers, S.K. Larkin, J.J. Emeis, A.C. Allison, Interaction of an annexin V homodimer (Diannexin) with phosphatidylserine on cell surfaces and consequent antithrombotic activity, Thromb. Haemost. 97 (2007) 478–486.
[25] J. Romisch, M. Grote, K.U. Weithmann, et al., Annexin proteins PP4 and PP4-X. Comparative characterization of biological activities of placental and recombinant proteins, Biochem. J. 272 (1990) 223–229.
[26] S. Ravassa, A. Bennaghmouch, H. Kenis, et al., Annexin A5 down-regulates surface expression of tissue factor: a novel mechanism of regulating the membrane receptor repertoire, J. Biol. Chem. 280 (2005) 6028–6035.
[27] K.I. Ataga, C.G. Moore, C.A. Hillery, et al., Coagulation activation and inflammation in sickle cell disease-associated pulmonary hypertension, Haematologica 93 (2008) 20–26.
[28] A. Solovey, Y. Lin, P. Browne, et al., Circulating activated endothelial cells in sickle cell anemia, N. Engl. J. Med. 337 (1997) 1584–1590.
[29] A. Solovey, L. Gui, N.S. Key, R.P. Hebbel, Tissue factor expression by endothelial cells in sickle cell anemia, J. Clin. Invest. 101 (1998) 1899–1904.
[30] C. Sultana, Y. Shen, V. Rattan, et al., Interaction of sickle erythrocytes with endothelial cells in the presence of endothelial cell conditioned medium induces oxidant stress leading to transendothelial migration of monocytes, Blood 92 (1998) 3924–3935.