Increased In Vivo Production of Thromboxane in Patients With Sickle Cell Disease Is Accompanied by an Impairment of Platelet Functions to the Thromboxane A2 Agonist U46619

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Thrombosis represents an important cause of mortality in patients with sickle cell disease, in addition to the complications caused by the primary defect of inherited abnormal hemoglobin. To study the involvement of platelets in these complications, we assessed the biosynthesis of thromboxane A2 in samples from 49 patients with sickle cell disease and in 33 control subjects. The urinary excretion of the major arachidonic acid metabolite of platelet origin (11-dehydrothromboxane B2) and of the vascular endothelial cell (2,3-dinor-6-ketoprostaglandin Flα) were very significantly increased (p<0.0002) in the patients. In a small group of patients (n=14), we further investigated the ex vivo response of their platelets to U46619, a stable analogue of thromboxane A2. We observed decreased aggregation and [14C] serotonin release compared with control (p<0.05); similarly, we found impaired p47 protein phosphorylation (p<0.05). In contrast, platelets from these patients responded normally to thrombin (0.1 unit/mL). In vivo desensitization of platelets from these patients to thromboxane may constitute a form of regulation that may prevent the propagation of aggregation by this potent inducer, as has been hypothesized in in vitro studies. Our results may also provide a rationale for using antiplatelet drugs in the prophylaxis of thrombotic complications in sickle cell patients. (Arteriosclerosis and Thrombosis 1993;13:421-426)

KEY WORDS • thromboxane • platelets • sickle cell disease • U46619

The primary defect of sickle cell disease (SCD) is the inherited abnormality of hemoglobin structure (hemoglobin S [Hb S]) that leads to a complex pathophysiology including a number of transient or permanent abnormalities of the red blood cell membrane.1 A variety of acute and chronic complications, including vascular occlusion, derive from this molecular disorder. In addition, thrombosis and vasospasm are involved in the pathogenesis of major organ damage, which may occur in this disease as a consequence of severe vascular damage and/or vascular intimal hyperplasia.2 Similarly, patients with the thalassemia syndrome demonstrate increased platelet activation as reflected by a shortened platelet life span,3 a hypercoagulable state,4 and increased β-thromboglobulin levels.5 Recently, we presented evidence for an enhanced production of the proaggregatory thromboxane (Tx) A2 in thalassemia intermedia in vivo as reflected by an increased excretion of its urinary metabolites.6 Measurement of the urinary metabolites of TxA2 and prostaglandin (PG) I2 can constitute unique markers of platelet activation and/or interaction between platelets and endothelial vascular cells. Such analysis reflects the in vivo production of these mediators and has the advantage of circumventing the artifactual synthesis of eicosanoids that occurs during blood or tissue sampling.7'8 The labile arachidonic acid metabolite TxA2 and its prostaglandin endoperoxide precursor PGH2 are formed on platelet activation; they produce a change in platelet shape, secretion, and aggregation. These effects are mediated by a receptor9 that has been recently purified10 and cloned.11 In vitro studies of the regulation of the platelet receptor using stable analogues of PGH2/TxA2, such as U46619, have shown that distinct receptor subtypes may exist on the cell membrane.12-14 One of these receptors controls stimulation of phospholipase C, increase of intracellular Ca2+, and activation of protein kinase C. These processes are known to act synergistically to stimulate platelet activation, secretion, and aggregation. The formation of TxA2 represents an important point in the propagation of platelet activation in that this event signals the recruitment of other platelets to the site of aggregation. The TxA2 receptor can undergo specific desensitization12-14 indicating that platelet activation is a tightly regulated process. Such regulation has been hypothesized to represent a natural control mechanism for the extension of platelet activation (and/or aggregation) in vivo.
indicate significant inhibition.

macrophages was determined by the toxicity of the media to 200 nglmL lipopolysaccharide and either octimibate or BMY murine peritoneal macrophages. Cells were incubated with inhibition of tumor necrosis factor (TNF) production by FIGURE 10. Mononuclear Cell Adhesion to Endothelium and Monocyte Chemotaxis

Cell counts of mononuclear cells on the luminal surface of the aortic arch indicated that octimibate and BMY 42393 reduced the number of leukocytes attached to the endothelium in vivo. Both prostacyclin agonists inhibited the chemotactic response of human monocytes to fMLP in vitro. Prostacyclin had a similar effect on monocyte chemotaxis, although it had no effect on monocyte adhesion to endothelial monolayers in vitro.

Taken together these findings suggest that octimibate and BMY 42393 inhibited mononuclear cell adhesion and/or chemotaxis to the artery wall and, in turn, diminished monocyte diapedesis into the aorta. This notion partially explains the reduced number of intimal macrophage–foam cells accumulating in vivo.

Cholesteryl Ester Accumulation in Macrophages

Modification of LDL in the artery wall and subsequent uptake by the macrophage scavenger receptor lead to the formation of foam cells and, ultimately, fatty and the area of the fatty streak also decreased in the BMY 42393 group, despite persistent elevation of plasma lipids and normal blood pressure, suggesting that the prostacyclin agonists may modulate atherosclerosis through additional mechanisms.

Mononuclear Cell Adhesion to Endothelium and Monocyte Chemotaxis

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Therefore, octimibate and BMY 42393 directly impeded the functions of activated monocyte–macrophages, such as adhesion, chemotaxis, scavenger receptor activity, and cholesteryl ester accumulation. In addition, prostacyclin agonists suppressed the production of mediators by macrophages that may promote the atherogenic process, i.e., the cytokine TNF and macrophage-derived growth factor
serotonin reuptake, since preliminary experiments showed no difference in the presence or absence of this compound. An aliquot of the supernatant was used for determination of the \[^{[3]C}\]serotonin release. Release was expressed as the percentage of total incorporated \[^{[3]C}\]serotonin.

Phosphorylation of platelet 47-kD protein (p47), a protein kinase C substrate, was assessed as described.\(^{20}\) PRP was centrifuged and suspended in 1/10th the initial volume of plasma. After a 1-hour incubation at 37°C with 1 mCi/mL of \[^{[32}P\]orthophosphate (370 MBq/mL, Amersham), platelets were diluted with washing buffer (1 volume of PRP per 100 volumes of buffer). After centrifugation, they were adjusted to a final concentration of 2x10\(^{10}\) cells/L. Platelets were stimulated for 20 seconds (thrombin) or 3 minutes (U46619) at 37°C. The reaction was stopped by addition of 2% sodium dodecyl sulfate (final concentration); the proteins were reduced by adding 5% \(\beta\)-mercaptoethanol and subjected to 13% polyacrylamide gel electrophoresis.\(^{21}\) A phosphorylated protein (p47) appeared after cell stimulation at 47 kD as detected with protein standards. The extent of phosphorylation in the 47-kD protein was assessed by laser densitometry (Ultrascan XL, LKB, Bromma, Sweden) after autoradiography. In some cases, blood was also collected without anticoagulant and immediately incubated at 37°C for 1 hour according to the procedure of Patrono et al.\(^{21}\) All cells were removed from the serum centrifugation and kept frozen until analysis of TxB\(_2\) by EIA.\(^{18}\)

**Statistical Analysis**

Comparison of the different parameters between patients and control subjects was performed by an unpaired two-tailed \(t\) test (T-ease, Institute for Scientific Information Software, Philadelphia, Pa.). Comparison of the different parameters before and after 7 days of low-dose aspirin therapy was done by a two-tailed \(t\) test for paired data.

**Results**

Determination of the urinary excretion of TxA\(_2\) and PGI\(_2\) metabolites showed a significantly increased excretion of 11-dehydro-TxB\(_2\) and dinor-6-keto-PGF\(_{1\alpha}\) in samples from all 49 patients with SCD compared with the 33 healthy individuals (1,227±191 versus 299±20 pg/mg creatinine, respectively, mean±SEM, \(p<0.0002\)). A difference was also shown with 2,3-dinor-6-keto-PGF\(_{1\alpha}\) (298±46 versus 150±11 pg/mg creatinine, respectively, \(p=0.0005\)) (Table 1). Similar results were found in the nine compound heterozygous subjects (1,099±291 and 449±199 pg/mg creatinine for 11-dehydro-TxB\(_2\) and 2,3-dinor-6-keto-PGF\(_{1\alpha}\), respectively, \(p=0.002\) and 0.005 versus controls). However, patients in vaso-occlusive crisis (\(n=15\)) had increased urinary levels of 11-dehydro-TxB\(_2\) compared with the group in remission (\(n=43\)): 1,836±536 compared with 963±109 pg/mg creatinine \((p<0.02)\). However, no difference was found in the same patients for 2,3-dinor-6-keto-PGF\(_{1\alpha}\) (481±88 pg/mg versus 354±56 pg/mg creatinine). In a smaller group of samples (\(n=25\)), we also analyzed urinary dinor-TxB\(_2\), another platelet-derived urinary metabolite; the increase of this compound (patients versus control subjects: 860±108 versus 206±19 pg/mg creatinine, \(p<0.0001\)) paralleled that of 11-dehydro-TxB\(_2\) (899±139 pg/mg creatinine), indicating that the elevation observed was not due to a change in the metabolism of TxA\(_2\) in these patients.

To verify that the elevated levels of urinary 11-dehydro-TxB\(_2\) reflected an increase in platelet production in vivo, we administered low-dose aspirin (50 mg/day) for 7 days to four of the patients with SCD. We measured urinary excretion of the metabolites before and after treatment. Aspirin caused a 75% reduction of the urinary 11-dehydro-TxB\(_2\) level \((p<0.04)\), with a 95% reduction in serum TxB\(_2\) from clotted blood. In contrast, the production of 2,3-dinor-6-keto-PGF\(_{1\alpha}\), reflecting vascular production of prostacyclin (PGI\(_2\)), was only reduced by 30% \((p<0.11)\).

We investigated more carefully 14 patients from the large group from whom urinary samples and blood were collected on the same day to quantify their excretion of urinary metabolites and to test platelet function. Analysis of urinary metabolites showed that the excretion of 11-dehydro-TxB\(_2\) and dinor-6-keto-PGF\(_{1\alpha}\) was increased compared with control subjects, with a respective mean±SEM of 769±102 and 363±67 pg/mg creatinine, \(p<0.0001\), for the two metabolites (Figure 1). Five patients were studied in a state of vaso-occlusive crisis, and urinary metabolites were compared with those obtained when the patients were in remission. The series was too small to find a significant difference between the patients in crisis compared with those in remission, although the mean was different (3,138 versus 892 pg 11-dehydro-TxB\(_2\) per milligram of creatinine and 662 versus 335 mg dinor-6-keto-PGF\(_{1\alpha}\) per milligram of creatinine).

Platelets from the small series of patients and from control donors were stimulated by the PGI\(_2\)/TxA\(_2\), mimetic U46619. The cells from SCD patients exposed to 0.25 and 1 \(\mu\)M U46619 were 30% less responsive \((p<0.05)\) than control as measured by the aggregation response (Figure 2A); a 40% \((p<0.05)\) decrease in the liberation of \[^{[3]C}\]serotonin was also observed. Thrombin (0.1 unit/mL)-stimulated aggregation and release were not significantly decreased in SCD patients compared with control subjects (Figure 2B). Impairment of platelet responses (aggregation and release) was observed over a large range of concentrations of U46619 (0.125–2 \(\mu\)M).
Phosphorylation of a 47-kD protein in control and SCD patient platelets was assessed as an index of protein kinase C activation. U46619 (1 μM) and thrombin (0.1 unit/mL) both stimulated 32P incorporation into a 47-kD protein in control platelets. In platelets from SCD patients, stimulation of phosphorylation by U46619 was attenuated (Figure 3); however, thrombin-stimulated 47-kD-protein phosphorylation was not significantly changed. The cumulative response of the results obtained with the 47-kD protein are shown in Figure 4. In the patients' platelets, stimulation of phosphorylation was attenuated to 20% of the value of control platelets ($p < 0.05$). Thrombin stimulation of the 47-kD protein was not significantly changed compared with platelets from normal subjects (less than 2%).

Discussion

We have shown that patients with SCD, even in a steady state, have increased production of TX in vivo as assessed by enhanced excretion of urinary TX metabolites. These data suggest that an indirect consequence of the red blood cell abnormality is the activation of platelets that may participate in some of the thrombotic complications of the disease.4–5 These results are consistent with our recent finding of increased urinary TX production in β-thalassemic patients, another type of hemoglobin disorder.6 Control subjects were taken from healthy individuals in the department whose sex and age matched those of the SCD patients. Because we could not test any control subjects whose geographic origins were similar to those of our patients, we cannot exclude the possibility of racial difference. However, such a remote possibility has never been reported, and only dietary intake has been shown to be the major cause for variation in the excretion of the urinary metabolites.22 The finding that patients in vaso-occlusive crises had higher values than during the chronic phase of the disease (Table 1) argues in favor that elevated levels are indeed a consequence of SCD.

We can only speculate on the possible mechanisms accounting for the in vivo activation of platelets. It seems reasonable that a likely inducer could be ADP released from lysed erythrocytes. Although ADP is a weak inducer of TX formation in platelets,22 the extent to which it could be liberated from abnormal red blood cells (either by a metabolically active process or because of cell lysis) may modify this effect. Recent reports have shown that in vitro, normal red blood cells can alter platelet recruitment, reactivity, and activation, including the production of TX.24,25 In addition, a portion of the ADP found in the supernatant of the platelet–erythrocyte mixtures originates from the erythrocytes, suggesting a two-way interaction between these cells and platelets.25 These in vitro findings may be relevant to the thrombotic complications of SCD. Additional mechanisms linked to the abnormal red blood cell membrane could also play a direct role in the hypercoagulable state of these patients.1

Aspirin treatment of patients resulted in a selective inhibition of TXA2 production as assessed by TxB2 in serum and its metabolites in urine while sparing vascular prostacyclin formation to a certain degree. These data are similar to previous reports8,21 and are consistent with increased production of the urinary 11-dehydro-TxB2, reflecting in vivo platelet formation of TXA2.

The marked increase in TX production in these patients suggests that SCD could be used as a model to study some of the ex vivo reactivity of these platelets to an in vitro challenge to TXA2 by using the stable mimetic U46619. Recent studies have characterized the existence of at least two different forms of PGH2/TXA2 receptor(s) on the surface of human platelets, one leading to platelet shape change,12,26 and the other to activation (phosphorylation and release) and final aggregation.28 The pathway(s) responsible for activation/aggregation can be desensitized, probably via a specific G-protein coupled to this receptor; indeed, desensitized platelets responded normally to thrombin. Our results are consistent with such a concept: we observed decreased aggregation and [3H]serotonin release of platelets from patients with SCD in response to U46619 even at the high concentration of 1 μM compared with control subjects, but all responses were normal when thrombin was used. Although samples stimulated with a low concentration of thrombin (0.1 unit/mL) seem to present a slightly diminished response (although not significant), 0.2 unit thrombin per milliliter easily overcame this tendency. Similarly, we found impaired p47 protein phosphorylation (a substrate for protein kinase C activation) in response to U46619, whereas the response to thrombin was normal. Although the data presented here lack the magnitude of desensitization...
observed in in vitro work, one should keep in mind that 1) SCD patients may contain heterogenous platelet populations because of the high turnover of new platelets and 2) the micromolar concentration of U46619 used in in vitro studies clearly exceeds those that might be generated in vivo during thrombotic episodes. Consequently, we cannot establish with certainty that there is a direct correlation between impairment of platelet function and enhanced formation of TX in vivo, as assessed by the measurement of urinary metabolites. It is likely that these two events are linked. However, several reasons may explain these apparent discrepancies. First, 11-dehydro-TxB₂ or dinor-TxB₂ represents only a small part of the TxA₂ that is generated (i.e., 6.8% and 5.3%, respectively); measurement of these compounds cannot discriminate a systemic reaction from activation localized to only specific areas. Second, it is hard to speculate on the minimal (or maximal) desensitization threshold in vivo that can significantly affect platelet function(s) at the TX receptor in chronic situations. Finally, there is a large discrepancy between the kinetics of TX generation (minutes) and metabolism (few hours) and the half-life of platelets (days). Additionally, while desensitization has been demonstrated to last for the life span of the platelets (several days), platelet activation by other stimuli (during in vivo stimulation) may lead to desensitization by cross-reacting pathways; earlier results have shown partial desensitization of the TX receptor to other inducers such as platelet-activating factor.

The present results confirm that increased activation of platelets occurs in vivo in β-thalassemic patients. Although measurement of the TX metabolites clearly demonstrates an in vivo activation of platelets, it does not reflect the precise extent of platelet activation and/or TX production over a chronic period such as is likely to occur in such patients. Such results support the relevance of in vitro work designed to study the influence of normal red blood cells on platelet activation. It is tempting to link these results (i.e., increase of TX production) to the regulation of the PGH₂/TxA₂ receptor established in vitro. As assessed in this work, the selective impairment of platelet responses to U46619 in the ex vivo tests performed on the cells from these patients reproduces the in vitro observations. Additional work should be performed before these two
events can be linked unequivocally; such work is presently being conducted. The therapeutic implications of these results clearly point out the potential advantage of using antiplatelet therapy in these patients. However, there could still be some problems specifically linked to the "activated state" of red blood cells, since in vitro work has also suggested that antiplatelet drugs such as aspirin could have a reduced therapeutic benefit in the presence of normal red blood cells. The validity of such hypotheses needs to be tested in clinical trials.

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