Thalassemic erythrocytes release microparticles loaded with hemichromes by redox activation of p72Syk kinase

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ABSTRACT

High counts of circulating microparticles, originated from the membrane of abnormal erythrocytes, have been associated with increased thrombotic risk in hemolytic disorders. Our studies indicate that in thalassemia intermedia patients the number of circulating microparticles correlates with the capability of the thalassemic erythrocytes to release microparticles. The microparticles are characteristically loaded with hemichromes formed by denatured α-chains. This finding was substantiated by the positive correlation observed in thalassemia intermedia patients between the amount of hemichromes measured in erythrocytes, their capability to release microparticles and the levels of plasma hemichromes. We observed that hemichromes, following their binding to the cytoplasmic domain of band 3, induce the formation of disulfide band 3 dimers that are subsequently phosphorylated by p72Syk kinase. Phosphorylation of oxidized band 3 appears to be relevant for the formation of large hemichromes/band 3 clusters that, in turn, induce local membrane instability and the release of microparticles. Proteomic analysis of microparticles released from thalassemia intermedia erythrocytes indicated that, besides hemichromes and clustered band 3, the microparticles contain a characteristic set of proteins that includes catalase, heat shock protein 70, peroxiredoxin 2 and carbonic anhydrase. High amounts of immunoglobulins and C3 have also been found to be associated with microparticles, accounting for their intense phagocytosis. The effect of p72Syk kinase inhibitors on the release of microparticles from thalassemia intermedia erythrocytes may indicate new perspectives for controlling the release of circulating microparticles in hemolytic anemias.

Introduction

Beta thalassemia intermedia (TI) is caused by a marked imbalance between α- and β-globin chains.[1] This leads to an accumulation of α-globin and damage to the red blood cell (RBC) membrane which causes anemia and necessitates intermittent blood transfusion. TI may result from defective production of β-globin chains due to β-globin gene defects, or from the increased production of α-globin chains, resulting from a triplicate or quadruplicate α-genotype associated with β-thalassemia heterozygosity, the latter situation leading to a milder form of TI.[2-5] The excess free α-chains have been demonstrated to precipitate within the erythroid precursors as hemichromes (HMC), forming large inclusion bodies.[6] In turn HMC alter the membrane clustering band 3 and enhance the deposition of opsonin autologous immunogobulins and C3 fragments.[7,8] Splenectomy, performed to alleviate anemia in TI patients, may result in severe thrombotic episodes[9,10] and may cause a rise of pro-thrombotic circulating microparticles (MP).[11] The composition and pathogenic roles of MP have been extensively studied in various diseases, such as ischemia, diabetes and atherosclerosis, revealing complex pathogenic roles in modulating nitric oxide and prostacyclin production, stimulating cytokine release, inducing tissue factor expression, as well as monocyte chemotaxis and adherence to the endothelium.[12-18]

Recent studies on the mechanisms of redox regulation of RBC membrane stability[19,20] indicate that oxidative stress induces a phosphorylative response that specifically involves two tyrosine residues located in the cytoplasmic domain of band 3.[20] Band 3 is the most abundant RBC membrane protein, and represents one of the major components of the junctional complexes that connect the lipid bilayer to the cytoskeleton. We previously found that the oxidation of two cysteine residues in the band 3 cytoplasmic domain leads to the docking of Syk kinase. RBC appear to possess a mechanism able to recruit Syk kinase to a fraction of less glycosylated band 3 molecules capable of forming disulfide dimers.[20] In turn, the affinity of the phosphorylated band 3 molecules for ankyrin is drastically decreased, their lateral mobility is increased and they have a greater propensity to form large clusters inducing vesiculation.[19] In thalassemias, it has been previously demonstrated that HMC bind to band 3 causing free iron accumulation and free radical production,[7,21] but their role in inducing band 3 phosphorylation and membrane destabilization has never been investigated. In this study we investigated the role of HMC in the release of MP from TI-RBC. The comprehensive study of a group of TI patients with

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different genetic and clinical status provided new insights into the pathogenic role of circulating MP and possible interventions to control their amount in thalassemia.

Methods

Unless otherwise stated, all materials were obtained from Sigma-Aldrich, St. Louis, MO, USA. Additional information about the methods are provided in the *Online Supplementary Data*.

Treatment of red blood cells

Venous blood was drawn from 12 healthy individuals, eight non-splenectomized TI patients and six splenectomized TI patients. All subjects provided written, informed consent before entering the study. The study was approved by the local Ethics Committee and conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki.

Clinical analyses were performed by routine laboratory tests at the Policlinico Hospital (Milan, Italy). Blood anti-coagulated with heparin was stored in citrate-phosphate-dextrose with adenine (CPDA-1) prior to its use. RBC were washed three times with phosphate-buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 8.1mM K_2HPO_4, 1.5mM KH_2PO_4, pH 7.4) containing glucose 5 mM to obtain packed RBC.

To stimulate HMC formation, RBC from healthy donors were suspended at a hematocrit of 30% and incubated with different concentrations (0, 0.25, 0.5, 1, 1.5, 2 mM) of phenylhydrazine at 37 °C for 4 h. To test the antioxidant effect in MP release we incubated 200 μM of 2-mercaptoethanol in the presence of 1 mM phenylhydrazine. When necessary, RBC were pretreated with Syk kinase inhibitors (10 μM Syk inhibitors II and 10 μM Syk inhibitors IV, Calbiochem, Darmstadt, Germany), for 1 h at 37 °C in the dark. Each reaction was terminated by three washes with PBS-glucose. For all protocols described, untreated controls were processed identically except that the stimulant/inducer was omitted from the incubation.

Red blood cell membrane preparation

Standard hypotonic membranes were prepared, as previously described,[20] and stored frozen at -80°C until use. Membrane protein content was quantified using the DC Protein assay (Biorad, Hercules, CA, USA).

Analysis of microparticles

The MP in plasma were analyzed by flow cytometry using a modification of a previously described method:[22] 25 μL of plasma diluted 1:1 with PBS-glucose 5mM were analyzed using anti-CD41 (BD, Franklin Lakes, NJ, USA) and anti-glycophorin-A (Dako, Denmark), both diluted 1:10.

Assay of hemichromes

HMC were quantified by measuring heme absorbance (Abs) using the following equation:

HMC= - 133xAbs577 - 114xAbs630 + 233xAbs560, and expressed as nMoles/mL of solubilized membranes.[23]

Microparticle isolation

To induce MP release *in vitro*, RBC from each volunteer and phenylhydrazine-treated RBC in PBS (30% hematocrit) were incubated as previously described.[24]

Electrophoresis and immunoblotting

Membrane and MP proteins were solubilized in Laemmli buffer[25] under reducing [2% (w/v) dithiothreitol] or non-reducing conditions at a volume ratio of 1:1. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. Western blot analysis was performed using anti-phosphotyrosine (Santa Cruz, CA, USA), anti-band 3 and anti-IgG antibodies.

Mass spectrometry analysis by matrix-assisted laser desorption/ionization – time of flight

Protein bands were excised from gels, and prepared for matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) analysis.[26]

Protein analysis by automated liquid chromatography/mass spectrometry

Gel slices containing the proteins were digested with trypsin. Peptide mixtures were analyzed using microflow capillary liquid chromatography coupled with electrospray quadrupole time of flight tandem mass spectrometry (ESI Q-TOF MS/MS).[26]

Preparation of cells for immunofluorescence

RBC from each donor were fixed and permeabilized as previously described.[27]

Phagocytosis assay of microparticles

The phagocytosis assay and characterization of IgG-mediated uptake were performed using a modification of a previously described method.[28,29]

Measurement of band 3 clusters

The membranes of TI-RBC were prepared and fractionated using Sepharose CL-6B, as previously described.[20]

Results

Assessment of circulating microparticle counts and the amount of microparticles released from erythrocytes in thalassemic patients

The number of circulating MP derived from RBC membranes was measured in a group of TI patients. *Online Supplementary Table S1* shows the genetic and hematologic data of all patients included in our study. It should be noted that the TI patients were genetically heterogeneous. Seventy-five percent of non-splenectomized patients had α gene triplication with heterozygous β thalassemia while all the splenectomized patients were homozygous or compound heterozygous for β globin gene mutations. The latter patients have more severe globin chain imbalance than patients with α chain triplication. Moreover, different β globin mutations have variable effects on β globin synthesis: β codon 39 causes a complete loss of β globin chain synthesis while IVS2-745 and IVS1-6 cause a partial reduction of β globin chain synthesis. Additional differences were present in the series of patients studied, especially related to the degree of iron overload and iron chelation.

Online Supplementary Table S2 shows MP counts and HMC levels in the same patients described in *Online Supplementary Table S1*. In non-splenectomized TI patients we found a moderate increase in plasma MP counts in comparison to those in control subjects, whereas in splenectomized patients we found a marked increase in MP counts. We then estimated the capacity of the RBC obtained from the same patients to release MP. Figure 1A-C shows a representative FACS analysis of MP stained with a fluorescent anti-glycophorin antibody. Figure 1D

demonstrates the positive correlation observed between the number of circulating MP and the amount of MP released from RBC measured in each thalassemic patient (the correlation is significant only in splenectomized patients). We also observed a correlation between the amount of MP released and the concentration of HMC measured in the same TI-RBC (Figure 1E). The finding that the concentration of HMC in patient's plasma correlated with the number of circulating MP (Figure 1F) suggested that HMC may be functionally related to the process of MP release *in vivo*. In parallel with the release of MP, TI-RBC subjected to mechanical stress also showed a measurable amount of hemolysis (1% in control RBC, 2% in splenectomized RBC and 4% in non-splenectomized

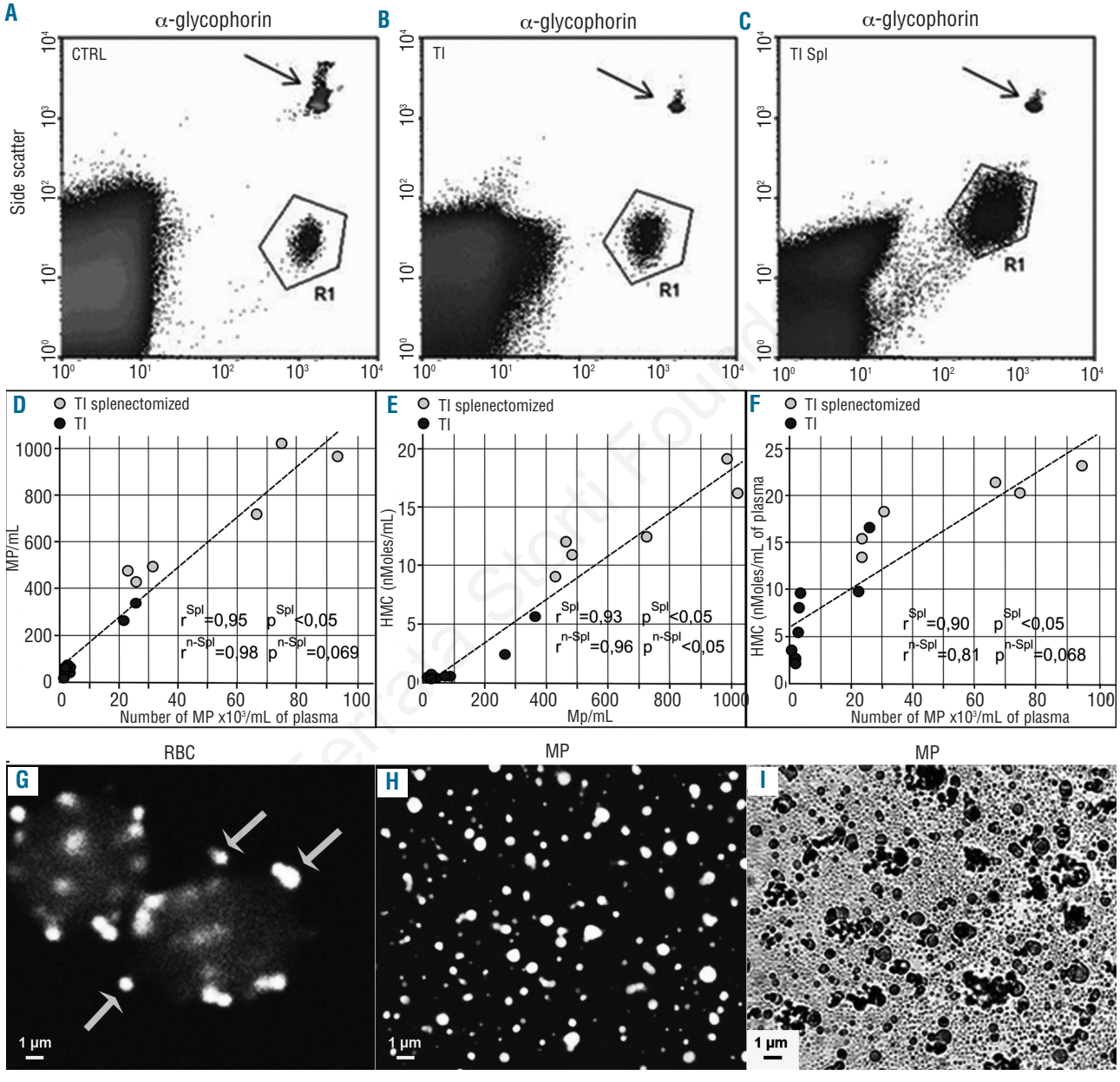

Figure 1. Correlation of MP counts with HMC concentrations from plasma and from RBC of TI patients. (A-C) Representative flow cytometric density plot of MP in plasma samples from a normal healthy subject (CTRL) (A), non-splenectomized TI patient (B) and splenectomized TI patient (C) stained with phycoerythrin-conjugated anti-human CD253a, glycophorin A. RBC MP were identified as glycophorin A-positive events (R1). The number of RBC MP was quantified using the known density of fluorescent CytoCount™ beads (arrows). (D) Correlation between the amount of MP (MP/mL), released from 100 µL of RBC following shear stress at 42°C for 45 min at 1400 rpm, and the number of plasma MP in TI patients (E). Correlation between the amount of HMC in 100 µL of TI-RBC (nMoles/mL), and the number of MP released from 100 µL of TI-RBC (MP/mL). (F) Correlation between the amount of plasma HMC, and the number of plasma MP in TI patients. Each dot corresponds to one patient of *Online Supplementary Table S1*. Correlation coefficient of TI splenectomized (r^{Spl}) and non-splenectomized (r^{n-Spl}) patients with corresponding *P*-value. Confocal images of HMC contained in whole TI-RBC of splenectomized patients (G, bar 1 µm), and in the isolated MP. HMC were visualized by their auto-fluorescence at 488 nm (excitation) / 630–750 nm range (emission) (H, bar 1 µm), or by bright field (I). Arrows show RBC protrusions. Images were acquired using the same magnification with a Leica TCS SP5 X (Leica Microsystems, Germany) confocal microscope equipped with a 6061.4 numerical aperture oil immersion lens.

RBC). As a further indication of the association between circulating MP and HMC, we found that the HMC present in TI plasma were pelleted together with the MP following ultra-centrifugation of plasma. Interestingly, in splenectomized patients we observed a correlation between the number of circulating MP and ferritin levels (r= 077, $P<0.01$): the functional significance of this finding is unclear.

Characterization of microparticles released from thalassemic erythrocytes

Figure 1G shows that red cells from splenectomized TI patients contain clusters of HMC (Heinz bodies) which were clearly evidenced by means of their natural fluorescent emission. Using confocal microscopy it was possible to observe that in TI-RBC from splenectomized patients HMC show a marked tendency to protrude from the membrane surface (see arrows in Figure 1G). Red cells from non-splenectomized patients had fewer HMC inclusions and no protruding formations were observable (*data not shown*). Figure 1H shows MP isolated from TI-RBC of splenectomized patients: the HMC are of a similar size to those observed in TI-RBC (Figure 1G). The larger and variable size of MP isolated from TI patients compared to those isolated from stored red cells[30] could be due to the presence of HCM inclusions. Figure 1I displays the bright field of the same MP shown in Figure 1H, indicating that a large percentage of MP released from TI-RBC of splenectomized patients actually contain HMC. HMC were also visualized in the MP fraction isolated from patients' plasma (*data not shown*).

We have previously demonstrated that oxidation and phosphorylation of band 3 causes uncoupling between the lipid bilayer and the cytoskeleton and thereby induces membrane instability and vesiculation.[19] Moreover phosphorylation of band 3 has already been demonstrated to be increased in thalassemia, although its functional effects have not been investigated. In accordance with a previous report,[20] Figure 2A,B shows that oxidized and clustered band 3 is strongly hyper-phosphorylated in splenectomized TI patients in comparison with the situation in non-splenectomized patients. Figure 2C shows that the amount of MP released from TI-RBC strictly correlates with the level of band 3 tyrosine phosphorylation (correlations are significant in both splenectomised and non-splenectomized patients). Figure 2D,E illustrates that Syk inhibitors cause a significant decrease of band 3 phosphorylation and a parallel reduction of the number of MP released.

Figure 3A (lane 1) shows the characteristic protein profile of the MP released from TI-RBC. When gels were run under non-reducing conditions, a protein aggregate was visible at the top of the gel indicating that most of the proteins present in MP are oxidatively cross-linked (*data not shown*). After sulfhydryl group reduction, we were able to observe resolved protein patterns. Table 1 presents the mass spectrometry-determined identity of the proteins extracted from MP: α–globin and band 3 were the most prominent proteins and a series of proteins including catalase, HSP70, PRX2, stomatin, carbonic anhydrase and IgG were found constantly. The protein identities shown in Table 1 were confirmed by liquid chromatography tandem mass spectrometry. Notably, mass spectrometry analysis of band 3 found in MP revealed that this protein belongs to the less glycosylated fraction described to be present in normal RBC[31] and that it is more susceptible to oxidation and phosphorylation.[20] We also tried to characterize MP isolated from patients' plasma but the high amounts of co-purified plasma proteins present markedly reduced the sensitivity of the detection methods. The presence of MP generated from many cell types poses further methodological limitations to the analysis of circulating MP.[32] In accordance with a study performed in patients with HbE,[33] plasma proteins were the major proteins identified in MP isolated from patients' plasma and we were able to identify only α–globin chains and band 3 as RBC components.

To validate the presence of IgG in MP, we performed a

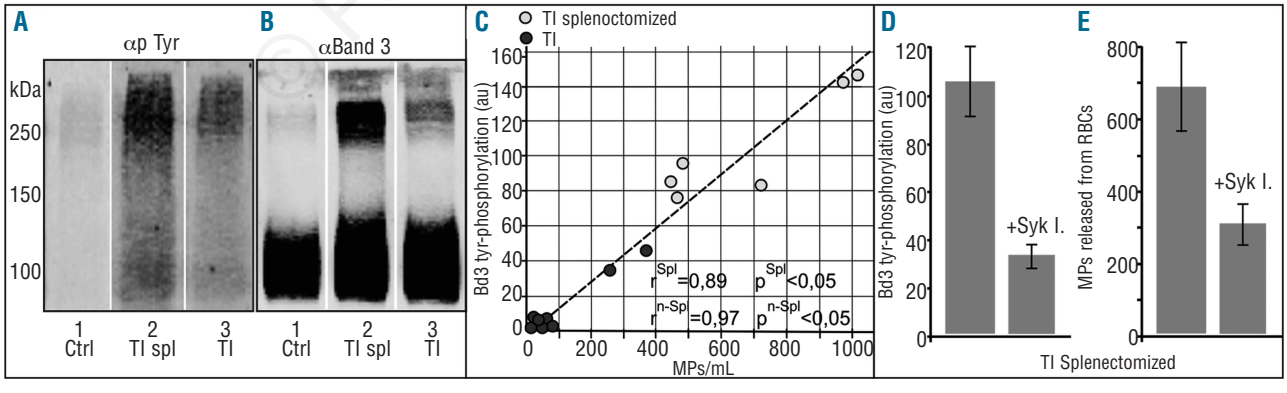

Figure 2. Band 3 tyrosine phosphorylation correlates with MP release from TI-RBC. (A-B) RBC membrane proteins from normal (lane 1), TI splenectomized (lane 2) and TI non-splenectomized (lane 3) subjects. Proteins were separated by 8% SDS-PAGE and analyzed by western blotting using anti-phosphotyrosine (αpTyr) (A) and anti-band 3 (B) antibodies. Images were acquired using a laser IR fluorescence detector (Odyssey, Licor, USA). Results are representative of six to eight separate experiments for each group of patients. (C) Correlation between the amount of phosphorylation of band 3 in tyrosine residues (Bd3 tyr-phosphorylation) in TI-RBC, and the number of MP (MP/mL), released from 100 μL of RBC following shear stress at 42°C for 45 min at 1400 rpm. Each dot corresponds to one patient of *Online Supplementary Table S1*. Correlation coefficient of TI splenectomized (r^{Spl}) and non-splenectomized ($r^{n\text{-}Spl}$) patients with corresponding *P* value. (D) Quantification of band 3 tyrosine phosphorylation levels in TI splenectomized RBC in the presence or absence of Syk inhibitors (Syk I.). (E) Quantification of MP/mL in TI splenectomized RBC in the presence or absence of Syk inhibitors (Syk I.). The analysis was performed using Odyssey V3.0 software, and expressed as arbitrary fluorescence units (au). Values are the average ± SD of three separate experiments.

western blot with anti-IgG antibody (Figure 3B, lane 1) and to demonstrate their superficial exposure, we immuno-precipitated MP using protein G beads. After solubilisation of the bound MP with a non-ionic detergent and extensive washing we evidenced large amounts of oxidized/aggregated band 3 (Figure 3B, lane 2), suggesting that the IgG are exposed on the surface of MP and show specificity for aggregated band 3 similarly to naturally occurring anti-band 3 antibodies.[34] The efficiency of surface-bound IgG to opsonize MP and to induce their phagocytosis was tested by co-incubating MP with human monocytes. Figure 3C shows the rate of MP ingestion indicating a half-life of only 12 minutes. The direct effect of IgG opsonization on MP phagocytosis was tested by blocking the macrophage IgG receptors.

Characterization of the mechanism of microparticle release from red blood cells from patients with thalassemia intermedia

To ascertain the role of HMC in the release of MP, we treated normal RBC with increasing amounts of phenylhydrazine to induce the formation of HMC through the specific oxidation of hemoglobin and then measured: HMC concentrations (Figure 4A), counts of released MP (Figure 4B), levels of band 3 oxidation (Figure 4C), band 3 phosphorylation (Figure 4D), and band 3 clustering (Figure 4E). To exclude a direct oxidative effect of phenylhydrazine on band 3, we treated isolated membranes with phenylhydrazine (Figure 4F). The incubation of RBC with increasing concentrations of phenylhydrazine caused a parallel rise of all parameters measured. Syk kinase inhibition did not affect either HMC formation or band 3 oxidation, but markedly decreased band 3 phosphorylation, band 3 clustering and MP shedding, suggesting a causal relationship between these events. To support the hypothesis that HMC promote the release of MP, exerting an oxidant effect on the RBC membrane, we added 2-mercaptoethanol to RBC incubated with phenylhydrazine. The 2-mercaptoethanol treatment caused a 60% decrease of released MP. As expected phenylhydrazine did not have a direct oxidative effect on isolated membranes, confirming that the observed oxidative action on band 3 following the treatment of whole RBC (Figure 4C) is mediated by the formation of HMC. To further investigate the analogies between RBC loaded with HMC by means of phenylhydrazine treatment and TI-RBC, we examined phenylhydrazine-treated RBC using the same microscopy method used for TI-RBC, observing similar membrane modifications (*data not shown*). The proteomic analysis of MP released from phenylhydrazine-treated RBC showed a

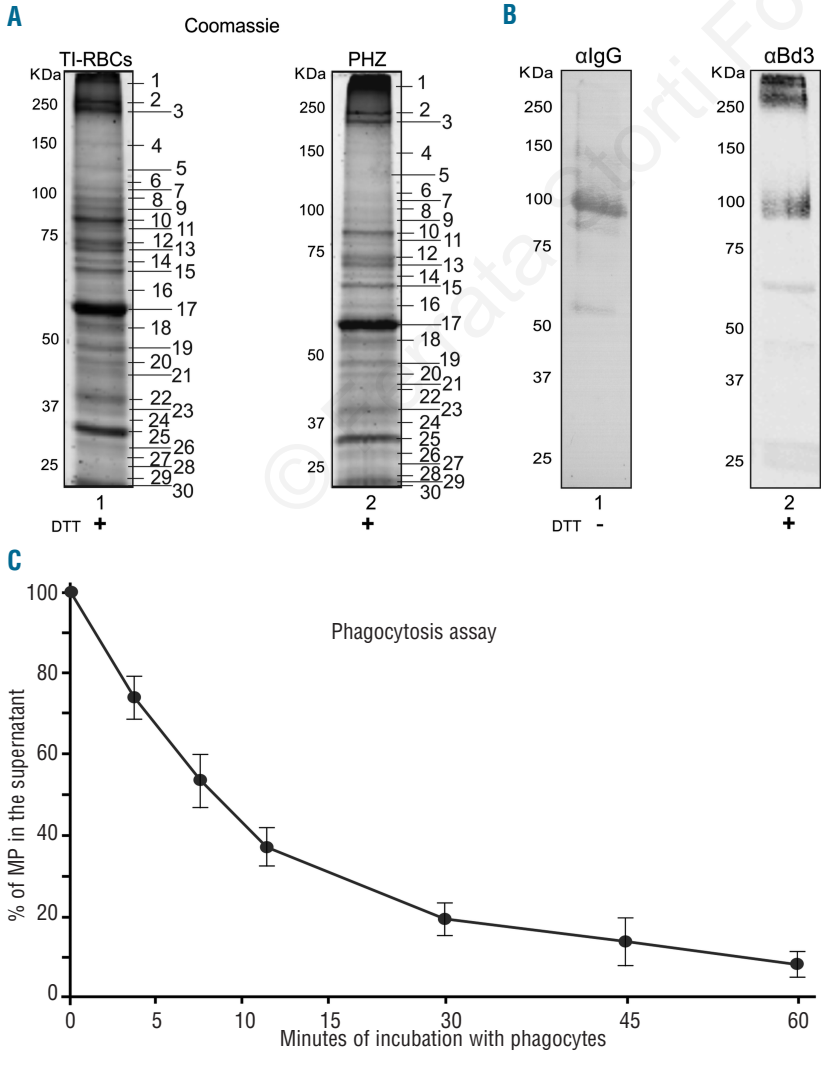

Figure 3. Mass spectrometry analysis of MP from splenectomized TI patients. (A) Gels stained with colloidal blue Coomassie of MP isolated from TI-RBC (lane 1) and from 2 mM phenylhydrazine-treated RBC, after incubation at 42°C for 45 min (lane 2). (B) MP isolated from TI-RBC were analyzed by western blotting using anti-IgG (lane 1), and anti-band 3 (αBd3, lane 2). MP were separated by 8% SDS-PAGE in the presence (panel A, lanes 1, 2; panel B, lane 2) or in the absence (panel B, lane 1) of DTT. Images were acquired using a laser IR fluorescence detector (Odyssey, Licor, USA) or a Panasonic scanner KX-MB2010 (panel B, lane 1). Results are representative of five separate experiments. Band numbers correspond to the numbers of Table 1. (C) Phagocytosis assay using MP isolated from TI-RBC, measured as percentage of MP detected in the supernatant during phagocyte incubation. Results are the average ± SD of three separate experiments.

very similar protein profile to that of MP released from TI-RBC (Figure 3A, lane 2). This similarity was confirmed by mass spectrometry analysis (Table 1).

Figure 5 shows a schematic representation of the HMC-driven mechanism of release of MP.

Discussion

Three main factors are responsible for the clinical sequelae of TI: ineffective erythropoiesis, chronic hemolytic anemia, and iron overload.[35] The degree of ineffective erythropoiesis is the primary determinant in the severity of anemia, however several specific complications are thought to be more frequent in TI than in thalassemia major, such as thrombosis, pulmonary hypertension, extramedullary erythropoietic masses, leg ulcers and cholelithiasis. The rates of almost all complications are significantly higher in splenectomized patients than in non-splenectomized patients.[36] The development of these complications has been attributed to the presence of high platelet counts and/or to an increased number of pathological RBC with potentially thrombogenic, negatively charged membranes. Thrombin generation is significantly higher in splenectomized TI patients than in control subjects or patients who have not undergone splenectomy.[37] Moreover, a study showed significantly higher levels of circulating MP originated from RBC membranes in TI patients than in controls, with this difference being greater in splenectomized patients.[12] Circulating MP can be very noxious in different clinical settings such as acute coronary syndromes, atherosclerosis, post-transfusional acute respiratory distress syndrome, diabetes mellitus, systemic inflammatory disease and disseminated intravascular coagulation.[10,12,36,39,40] MP originating from RBC membranes have also been considered a major cause of premature atherosclerosis described in TI patients.[13] The mechanisms responsible for the generation of MP appear very complex in different cell types[32] and very little is known about thalassemic RBC.

This report describes the first study on the mechanism of generation of MP in thalassemia and the first comprehensive analysis of the composition of MP released from TI-RBC. We found that in TI patients the number of circulating MP was correlated with the capability of TI-RBC to release MP *in vitro*. Moreover, the amount of HMC bound to the membrane of TI-RBC also correlated with the rate of MP released *in vitro*. The demonstration that HMC are actually contained in circulating MP and the consequent correlation between the number of circulating MP and the plasma concentration of HMC suggested that HMC may play a direct role in the mechanism of MP release from TI-RBC.

Following α or β globin oxidation, unstable HMC are formed and bind to the RBC membrane.[41-43] HMC are redox active and release free iron to the membrane,[21] so the generation of free radicals may be responsible for the formation of disulfide bonds between adjacent band 3 cytoplasmic domains.[7] This is conceivable given that the cytoplasmic domain of band 3 represents the major binding site of HMC to the membrane and that it contains two relatively accessible cysteine residues.[44,45] Indeed, we showed that cysteine oxidation involves a distinct fraction of band 3 molecules that has been described to be less glycosylated and more susceptible to oxidation and then phosphorylation by Syk kinase.[20] In turn, phosphorylation of Tyr 8 and 21 residues located at the N-terminal of band 3 determines its uncoupling from the cytoskeleton and markedly increases its lateral mobility leading to an increased propensity to form clusters, to be easily extracted from the membrane and to induce membrane vesiculation.[19,46]

A number of findings indicate that this mechanism may be working in the generation of MP in TI patients: (i) HMC are contained in MP found in patients' plasma or are released from TI-RBC *in vitro*; (ii) the degree of band 3 oxidation and phosphorylation in TI-RBC correlates with the amount of membrane-bound HMC; (iii) in TI-RBC and

Table 1. Proteins identified in MP. MP proteins isolated from TI-RBC and from phenylhydrazine-treated RBC, were analyzed by mass spectrometry (MALDI-TOF). Numbers in the Table correspond to MP protein bands numbered in Figure 3, panel A, lanes 1 and 2. Marked proteins (*) were found only in MP from TI-RBC.

N.	Protein	MW	Score	Coverage
1	Band 3	102	88	12
2	Band 3	102	66	13
3	Band 3	102	78	18
4	Hemoglobin chain α	15	51	6
5	Hemoglobin	16	65	7
6	Band 3	102	72	13
7	Band 3 Hemoglobin	102 16	84 69	15 7
8	Band 3	102	110	16
9	HSP 90	85	119	16
10	Serotransferrin * Hemoglobin	77 16	68 86	12 9
11	Hemoglobin chain α	15	74	8
12	HSP 71	71	70	13
13	HSP 70	70	94	11
14	Hemoglobin	16	52	7
15	Hemoglobin	16	68	7
16	HSP 70	70	57	9
17	Catalase	60	107	14
18	Hemoglobin	16	89	9
19	Selenium binding protein 1 Ig μ chain C region *	52 49	154 71	21 8
20	Alpha enolase * Hemoglobin chain α Ig μ chain C region *	47 15 49	63 83 60	11 8 10
21	Hemoglobin	16	70	6
22	Hemoglobin	16	69	11
23	Hemoglobin	16	86	8
24	Hemoglobin	16	56	6
25	GAPDH	36	109	10
26	Hemoglobin	16	55	6
27	Hemoglobin	16	58	7
28	Carbonic anhydrase Hemoglobin chain α	29 15	86 66	8 7
29	Hemoglobin chain α	15	68	5
30	Peroxiredoxin-2 Hemoglobin chain α	22 15	93 66	7 7

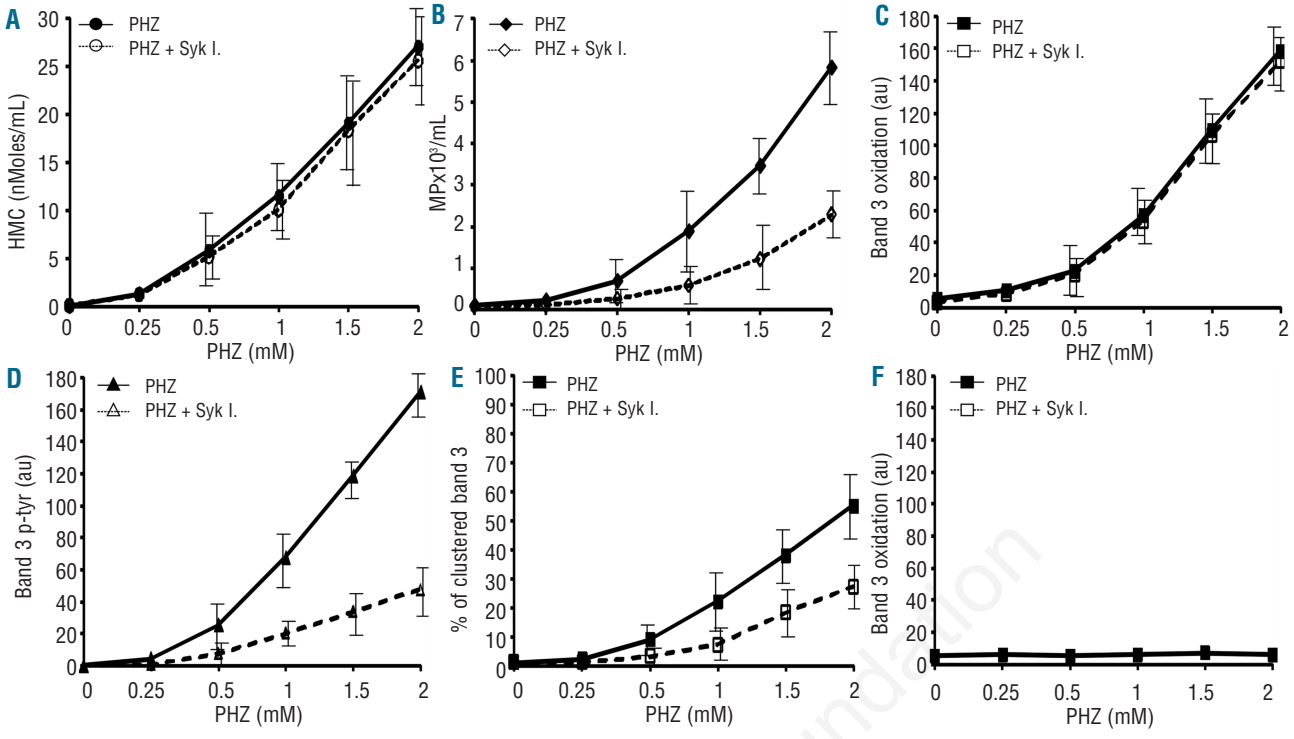

Figure 4. Hemichrome formation, band 3 modifications and MP release following phenylhydrazine (PHZ) treatment. Erythrocytes were treated with different concentrations of PHZ for 4 h, in the presence or absence of Syk inhibitors (Syk I.). (A) HMC contained in RBC (nMoles/mL). (B) MP released from RBC (MP x 10^3/mL). (C) Amount of oxidized band 3 (au). (D) Levels of band 3 tyrosine phosphorylation (au). (E) Percentage of clustered band 3. (F) Amount of oxidized band 3 following isolated membrane incubation with increasing concentrations of PHZ (au). Band 3 oxidation and phosphorylation levels were quantified by acquiring anti-band 3 and anti-phosphotyrosine western blots with an IR fluorescence detection scanner (Odyssey, Licor, USA) and analyzing images with Odyssey V3.0 software. Results are the average ± SD of four separate experiments and values are expressed as arbitrary units (au).

phenylhydrazine-treated RBC there was microscopy evidence that HMC are bound to protruding areas of the membrane that can be easily shed following moderate mechanical stress; (iv) in TI-RBC, Syk inhibitors diminished both band 3 phosphorylation and the rate of MP release; and (v) phenylhydrazine, which induces HMC formation in control RBC without directly oxidizing band 3, caused a Syk kinase-dependent release of MP accompanied by oxidation, phosphorylation and clustering of band 3.

The proteomic analysis of MP released from TI-RBC provided a number of new insights into the composition of the MP and their possible pathogenic roles. Denatured α-globin is the major protein contained in MP, suggesting that a selective sorting mechanism may lead to its elimination in MP. Band 3 and a number of protective enzymes, such as HSP90, HSP70, catalase and peroxiredoxin 2, have been identified in MP indicating that RBC may respond to the localized production of free radicals induced by HMC, recruiting anti-oxidant enzymes and chaperonins. Interestingly, band 3 molecules recruited in MP belong to the less glycosylated fraction[31] that was previously found to be more susceptible to oxidation and phosphorylation. Additional proteins, such as GAPDH, carbonic anhydrase and α enolase, are known to be physiologically linked to band 3 and have been found in MP.

Notably, the proteomic analysis of MP also revealed the presence of two plasma proteins: transferrin and IgG. We have no clue to understanding the binding of transferrin to MP but its significance could be related to the trapping and detoxification of iron, which may be released from HMC.[21] Indeed, TI-RBC membranes have been demonstrated to contain measurable amounts of free iron which has been found to be highly concentrated in the membrane areas containing band 3/HMC aggregates[21] eliciting oxidative damage.

Some of the findings of this study suggest that the HMC contained in circulating MP could be related to the alterations of iron compartmentalization and metabolism found in TI patients: considering the heme content of the HMC present in MP (up to 20 nMoles/mL in splenectomized TI patients), a large amount of iron could be transported in MP. Interestingly, in splenectomized patients we also observed a correlation between the number of circulating MP and ferritin levels (r=077, P<0.01) possibly caused by the iron overload of non-splenic macrophages. Additional studies of iron metabolism and compartmentalization are required to investigate the functional correlations between iron transported in MP and the complex alterations of iron homeostasis in thalassemias.

The presence of IgG binding to MP was strongly expected. Previous work demonstrated that some naturally occurring antibodies have strong affinity for oxidized/aggregated band 3.[47] Anti-band 3 antibodies have been previously described to bind RBC in a variety of hemolytic diseases, in senescent RBC and in malaria-infected RBC, possibly representing a general mechanism for the clearance of abnormal RBC.[34] In the present study

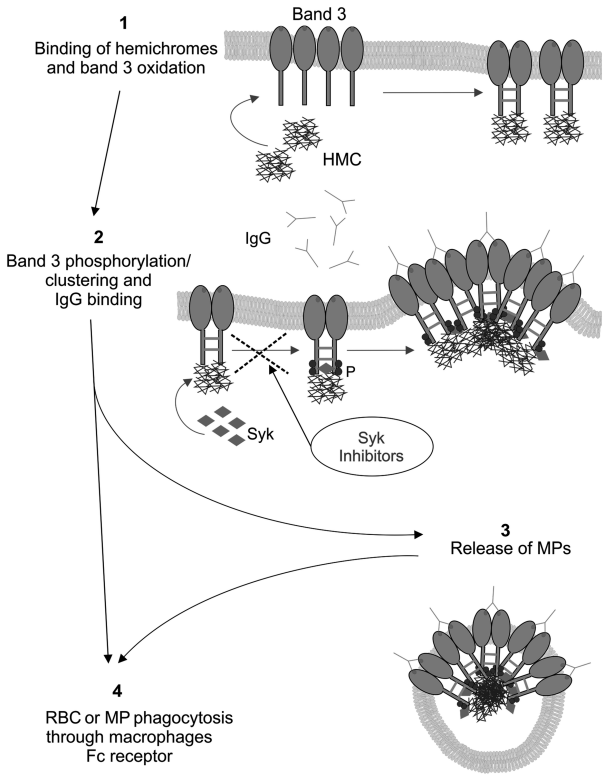

Figure 5. Graphical representation of MP release from RBC. **1.** Hemichromes (HMC) oxidize band 3 and form disulfide bridges between the two Cys residues in the cytoplasmic domain of band 3. **2.** Syk kinase binds oxidized band 3 and phosphorylates (P) Tyr 8 and 21 residues in the cytoplasmic domain of band 3. Tyrosine phosphorylation causes the weakening of band 3 from the cytoskeleton allowing band 3 clustering and binding of naturally occurring IgG (IgG). **3.** Band 3/hemichrome clusters are released in MP. **4.** MP and RBC opsonized by IgG can be phagocytosed by macrophages.

we demonstrated that anti-band 3 antibodies opsonize MP, being responsible for their recognition and engulfment by macrophages. Moreover, IgG binding to band 3/HMC complexes, coupled to the local destabilization of the RBC membrane, may facilitate the pitting of HMC by macrophages. There is already morphological evidence of "pitting" or "culling" of HMC by spleen macrophages.[48,49] On the other hand, elimination of HMC in the bone marrow has been previously hypothesized in β-thalassemia[6] and the loss of HMC through the vesiculation of specific membrane areas may play an important role in this phenomenon.

In conclusion, in depth analysis of MP originating from erythrocytes of TI patients has revealed a number of specific features providing new insights into the mechanisms that underlie their origin and new pharmaceutical perspectives to control their release.

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Authorship and Disclosures

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