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

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Supplementary data

Analysis of MPs in plasma

Analysis of MPs in plasma was performed using a FACS Calibur flow cytometer (Becton Dikinson Biosciences, San Jose, CA). The RBCs were excited with 488 nm light from a 15 mW argon-ion laser, and logarithmic green and red fluorescence of FITC and PE were measured through 530/30 nm and 585/42 bandpass filters, respectively. Data from at least 30,000 events were acquired and analyzed with CELLQuestTM software (Becton Dikinson Biosciences, San Jose, CA). MPs from RBCs were identified by their fluorescence (Glycophorin-A positive cells) and quantified with CytoCountTM beads (DakoCytomation, Glostrup, Denmark). MPs were localized within the platelet region (R1) and were distinguished from platelets (CD41-FITC positive) by their glycophorin-A positive/CD41 negative response. The number of CytoCountTM beads was counted and the absolute numbers of MPs were then calculated by the following formula: as(No. of Glyco-A positive events/No. of beads collected) x CytoCountTM conc. x dilution factor. 25 µl of plasma were incubated with 20 µl of PBS-G (PBS containing 2 mM glucose) containing 3 µl of PE-conjugated anti-human CD253a, Glycophorin A, Clone JC159 (DakoCytomation, code No. R7078) monoclonal antibody, alone or with 5 µl of FITC-conjugated anti-human CD41, Platelet Glycoprotein IIb, Clone 5B12 (DakoCytomation, code No. F7088) monoclonal antibody for 30 min at room temperature in the dark to identify MPs and platelets. After staining, the MPs were then diluted in 400 µl PBS-G-paraformaldehyde 1% and finally put into flow cytometer tubes preloaded with a known density of fluorescent CytoCountTM beads (DakoCytomation, code No. S2366) and analyzed for quantification.

Microparticle (MP) isolation

To induce MP release *in vitro*, RBCs from each volunteer and PHZ treated RBCs in PBS (30% hematocrit) were incubated at 42°C for 45 minutes under stirring at 1400 rpm in a microtube and the supernatants were collected and centrifuged at 25,000xg for 10 min at 4°C to eliminate spontaneously formed red cell ghosts. Supernatants, after addition of phosphatase inhibitors, were centrifuged for 3 hours at 100,000 x g on an ultracentrifuge (Beckman) at 4°C to isolate MPs.

Electrophoresis and immunoblotting

Membrane and MP proteins were solubilized in Laemmli buffer under reducing [2% (w/v) DTT (dithiothreitol)] or non-reducing conditions at a volume ratio of 1:1. SDS/PAGE was conducted by heating the samples for 5 min at 95 °C and loading on an 8% gel for protein staining by colloidal Coomassie Blue. For Western blot analysis, proteins, separated by 8 % of SDS-Page, were transferred to nitrocellulose filters and probed with anti-band 3 antibody diluted 1:50000, anti-phosphotyrosine (Santa Cruz Biotechnology, CA), diluted 1:2000. Secondary antibodies, anti-mouse diluted 1:50000, conjugated to infrared fluorescent dyes excitable at 700/800 nm (IRDye 700/800CW, Li-COR-USA) were then used to visualize the desired antigens using an 700/800 nm laser scanner (Odyssey). Quantitative densitometry analysis was performed using Odyssey V3.0 software. For anti-IgG western blot, nitrocellulose membranes were immunostained

with anti-human IgG antibody, alkaline phosphatase coniugated, diluted 1:10000, and detected using a chromogenic substrate BCIP /NBT Blue. Images were acquired using a Panasonic scanner KX-MB2010.

Mass Spectrometry analysis by MALDI-TOF

Protein bands were excised from gels, and prepared for MALDI-TOF analysis. MS analysis was performed with a MALDI-TOF micro MX (Micromass, Manchester, UK) according to the tuning procedures recommended by the manufacturer. Peak lists generated with Proteinlynx Data Preparation, were sent to MASCOT PMF search (http://www.matrixscience.com) using a Swiss-Prot database (release 50.0, May 30, 2006).Only protein identifications with significant MASCOT scores ($p<0.05$) were considered as relevant for our analysis.

Protein analysis by automated LC-MS/MS.

Gel slices containing the proteins were digested with trypsin as described in the previous section. Peptide mixtures were analyzed using microflow capillary liquid chromatography coupled with electrospray quadrupole time of flight tandem mass spectrometry (ESI Q-TOF MS/MS). Mass data collected during an RP-LC-MS/MS analysis were processed and converted into a Pkl file to be submitted to the automated database searching "Mascot, MS/MS Ions Search" (MatrixScience UK; www.matrixscience.com). Search parameters were: parent tolerance 0.6 Da, fragment tolerance 0.3 Da, tryptic specifity allowing for up to 1 missed cleavage, database SWISSPROT . Only protein identifications with significant Mascot scores ($p< 0.05$) were taken in consideration.

Preparation of cells for immunofluorescence

RBCs from each donor were fixed and permeabilized as previously described[27]. RBCs and MPs were suspended in PBS and allowed to attach to cover slips coated with polylysine, and the cover slips were mounted by using Aqua-Mount (Lerner Laboratories, New Haven, CT). The auto-fluorescence of hemichromes was visualized by exciting at 488 nm and observing their emission in the 630–750 nm range. Samples were imaged with a Leica TCS SP5 X (Leica Microsystems, Germany) confocal microscope equipped with a 6061.4 numerical aperture oil immersion lens.

Phagocytosis assay of MPs.

Phagocytosis assay and characterization of IgG mediated uptake was performed using a modification of a previously described method[28,29,]. MPs isolated from TI-patients plasma have been incubated in suspension with human monocytes, the number of phagocytized MPs has been quantified measuring the amount of ingested heme by a chemiluminescence technique.

Determination of serum iron

Iron was measured by a standard colorimetric method by Beckman, Synchron LX20 .

Tables

Table S1, Clinical data. Normal (Ctrl), non splenectomized TI (TI) and splenectomized TI (TI Spl) subjects analyzed in the present study, listed with genetic and clinical data. **Table S2**, **Experimental data.** Normal (Ctrl), non splenectomized TI (TI) and splenectomized TI (TI Spl) subjects analyzed in the present study, listed with experimental data: hemichromes levels (HMC) and microparticle counts (MPs) in plasma of TI patients.

(S1)

Code	**Ctrl**	**TI 1**	**TI 2**	**TI 3**	**TI 4**	**TI 5**	**TI 6**	**TI 7**	**TI 8**	**TI Spl 1**	**TI Spl 2**	**TI Spl 3**	**TI Spl 4**	**TI Spl 5**	**TI spl 6**
Genotype alpha	αα/αα	ααα/αα	ααα/ααα	αα/αα	ααα/ααα	ααα/αα	ααα/αα	ααα/αα	αα/αα	αα/αα	αα/αα	αα/αα	αα/αα	αα/αα	αα/αα
Genotype Beta	ββ/ββ	cod39/N	IVS1-1/N	cod39/IVS1-6	IVS2-745	cod39/N	cod39/N	cod39/N	cod39/HbD-Los Angeles	Beta thal/Hb S	cod39/-87	IVS1-1/IVS1-6	IVS1-6/IVS1-6	cod39/IVS1-6	IVS-1/IVS1
Transfusions	No	No	No	No	No	No	No	No	No	No	No	Yes	No	Irregular	No
Hb g/dl	14	9,1	9,8	9,4	12,7	12,8	10,3	10,8	10,4	8,7	9,4	7,9	9,1	6,3	9,4
Average	**14,5**	**9,1**								**9,8**					
RBCs $10^6/m^3$	5,2	4,5	4,82	5,06	6,67	6,34	5,02	5,44	5,35	4,17	3,82	3,05	3,96	2,78	3,72
Hct %	35	29,6	32,7	27,3	38,1	38,4	31,8	34,1	30,9	27	29,1	24,1	30,8	19	29,3
MCV	89,8	65,8	67,8	54	57,1	60,6	63,3	62,7	57,8	64,7	76,2	79	77,8	68,3	78,8
RDW	44,6	16,8	23,4	31,9	17	17,5	17,3	21,1	18	22,5	23,5	24,2	27	24,4	24,5
MCH	27,9	20,2	20,3	18,6	19	20,2	20,5	19,9	19,4	20,9	24,6	25,9	23	22,7	25,3
MCHC	41,4	30,7	30	34,4	33,3	33,3	32,4	31,7	33,6	32,2	32,3	32,8	29,5	33,2	32,1
Reticulocytes (%)	0,5 (1,5)	0,071 (1,57)	0,102 (2,11)	0,12 (2,44)	0,11 (1,62)	0,14 (2,18)	0,08 (1,6)	0,14 (2,53)	0,17 (3,16)	0,213 (5,1)	0,51 (13,44)	0,396 (12,98)	0,285 (7,21)	0,17 (6,02)	0,34 (9,02)
LDH U/l	390	395	511	726	433	342	382	730	435	465	281	298	569	378	405
Bilirubin (directly) mg/dl	0,8 (0,17)	0,92 (0,4)	2,63 (0,92)	2,66 (0,47)	4,57 (0,43)	4,64 (0,68)	2,43 (0,69)	2,49 (0,66)	1,88 (0,63)	1,78 (0,61)	1,85 (0,61)	1,04 (0,31)	1,58 (0,46)	2,7 (0,5)	3,3 (0,31)
Haptoglobin mg/dl	92	25	<20	<20	<20	24	<20	<20	<20	<20	82	52	<20	<20	<20
Serum iron µg/dl	81	103	244	117	146	86	119	219	108	97	146	205	252	252	265
Ferritin ng/ml	144	294	675	238	254	206	529	1067	379	148	742	970	2208	401	335
transferrin mg/dl	320	204	226	144	250	237	231	228	213	233	210	163	209	207	230
Platelets n/m^3	241	145	146	160	175	169	156	109	188	674	348	716	719	608	1070

(S2)

Code	**Ctrl**	**TI 1**	**TI 2**	**TI 3**	**TI 4**	**TI 5**	**TI 6**	**TI 7**	**TI 8**	**TI Spl 1**	**TI Spl 2**	**TI Spl 3**	**TI Spl 4**	**TI Spl 5**	**TI spl 6**
HMC nMoles/ml of Plasma	0,2	9,9	3,8	2,4	16,2	8,7	5,3	2,7	9,8	13	18,6	20	23	21,1	15,1
Average	**0,5+ 0,2**	**7,4 + 4,7**								**18,5 + 3,8**					
MPs/ml of Plasma	673,5	2698	646	1481	26033	2022	1911	1125	20871	21399	30703	74989	93640	66265	23194
Average	**914**	**7098,3 + 10205,8**								**51698,3 + 30610,7**					