Defective erythroid maturation in gelsolin mutant mice

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Online Supplementary Design and Methods

Generation of gelsolin null mice on a BALB/c congenic strain

Mice with a C57BL/6 outbred background[1] homozygous for the mutation were crossed with mice of BALB/c inbred background. Among the F1 animals, mice heterozygous for the mutation were selected by genotyping. These F1 animals were crossed with mice of BALB/c inbred background to produce F2 progeny, among which only mice heterozygous for the mutation were used for the next generation. The same cycle was repeated until F10 mice were obtained. Heterozygous F10 mice were crossed to produce mice homozygous for the mutation, with a genetic background very close to the BALB/c inbred background. For timed pregnancies, BALB/c *Gsn* heterozygous mice were intercrossed and mated overnight and females examined for a vaginal plug in the morning. Noon of the day of vaginal plug appearance was considered day 0.5 *post-coitum* (E 0.5). Pregnant mice were killed by cervical dislocation, and embryos were dissected from uteri at different time points of pregnancy. For genotyping, DNA was prepared from tail snips and analyzed with polymerase chain reaction (PCR) reactions for the presence of the targeted alleles, as previously described.[1] All experiments/treatments in mice were approved by the Italian Ministry of Health and followed procedures aimed at minimizing animal stress and pain, in accordance with European Union guidelines.

Histology

Embryos collected from timed pregnancies were analyzed. They were fixed in fresh 4% buffered paraformaldehyde (Sigma-Aldrich Corp., St. Louis, MO, USA), embedded in paraffin, and sectioned into 5 μm slices before staining. Freshly collected spleens were fixed with 4% (w/v) paraformaldehyde, cryoprotected in 30% sucrose, embedded in OCT (Sigma-Aldrich Corp.), frozen and sectioned at 10 μm before staining.

For hematoxylin/ eosin and Wright-Giemsa staining (Sigma Aldrich Corp.), samples were stained with standard histological procedures. O-dianosidine (Sigma Aldrich Corp.) staining was according to the manufacturer's protocol. Images were taken with a Leica DMI 6000 microscope.

Antibodies and dyes

Primary antibodies and antisera were obtained and used as follows: rabbit antisera against mouse Gsn (1:400 dilution);[1] allophycocyanin (APC) anti-mouse CD117 (c-Kit) and fluorescein isothiocyanate (FITC) anti-mouse Ter119 (both from BD Biosciences, Rockville, MD, USA), anti-mouse βH1 globin (a generous gift from Prof. J. Palis[2]). Secondary antibodies were obtained and used as follows: anti-mouse or anti-rabbit conjugated, respectively, with Alexa Fluor 488 and Alexa Fluor 546 (1:800 dilution; Molecular Probes, Eugene, OR, USA). Fluorescent dyes were as follows: Alexa Fluor 488–phalloidin (1:3000 dilution; Molecular Probes), 4,6-diamidine-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich.).

Immunocytochemistry and immunofluorescence analysis

Samples were fixed for 15 min in 4% paraformaldehyde, extensively rinsed and permeabilized for 5 min at room temperature in phosphate-buffered saline (PBS) containing 10% normal goat serum and 0.2% Triton X-100. After rinsing, samples were incubated for 90 min with the appropriate primary antibodies or antisera in PBS containing 10% normal goat serum, 0.2% Triton X-100. After washing, cells were then incubated with the appropriate secondary antibodies and cell nuclei were counterstained with DAPI (Sigma-Aldrich, 50 μg/mL in PBS) for 15 min at room temperature. Samples were rinsed several times with PBS, once with distilled water and mounted in Fluorsave (Calbiochem, La Jolla, CA, USA). FACS sorted cells were cytospun and stained as described above. Samples were examined using a Leica DMI 6000 epifluorescence microscope.

βH1 globin staining was performed according to Kingsley *et al.*[2] Briefly, blood was collected in PBS, centrifuged at 1000xg for 5 min and then the cells were resuspended in PBS with the addition of heparin (12.5 mg/mL) and 4% BSA. Cytospins were fixed in cold ethanol for 5 min and air dried. Cells were permeabilized with PBS/0.5%TritonX-100, blocked with PBS/1.5% goat serum, and incubated with anti βH1 antibody (a gift from Prof. J. Palis). A biotinylated secondary antibody was used and visualized by using a streptavidin-horseradish peroxidase DAB substrate kit for peroxidase (Vector Laboratories, Burlingame, CA, USA; cat. SK-4100). More than 100 cells were scored for three independent fields.

Fetal liver cell purification and sorting

Freshly extracted mouse E13.5 fetal livers cells were disaggregated to single cells by gentle pipetting in PBS containing 2mM EDTA and 0.5% bovine serum albumin (BSA). Cells were washed and incubated with the following labeled antibodies: APC anti-mouse CD117 (c-Kit) and FITC anti-mouse Ter119. The cells were sorted using a MoFlo (DAKO-Cytomation, Carpinteria, CA, USA) cell sorter. The purity of the obtained cell populations was >95%.

For embryonic total blood collection, intact embryos were transferred to dishes containing heparinized PBS solution (12.5 g/mL heparin). Each embryo was allowed to exsanguinate from severed umbilical, vitelline, and jugular vessels, and all blood cells in the dish were collected and counted, as described elsewhere.[2]

Each slide was prepared from 10^5 cells by cytospin centrifugation (400 rpm for 3 min) and slides were either air-dried or fixed for 5 min in ice-cold methanol.

Quantitative reverse transcriptase polymerase chain reaction analysis

Total RNA was purified from each cell population (10^5 cells) with TRI Reagent (Applied Biosystem, Carlsbad, CA USA; AM9738). Before cDNA synthesis, RNA was treated with RQ1 DNase (Promega, Madison, WI, USA) for 30 min at 37°C. cDNA was prepared using the High Capacity cDNA reverse Transcription Kit (Applied Biosystem cat n. 4368814) and then appropriately diluted for amplification. Negative control reactions (without reverse transcriptase) gave no signal. Real time analysis was performed using ABI Prism 7500 (Applied Biosystems). Specific PCR product accumulation was monitored by SYBR Green dye fluorescence in a 25-μL reaction volume. Dissociation curves confirmed the homogeneity of PCR products.

Primers used: Gsn: FW 5'AGATCTGGCGTGTGGAGAAG; Rev. 5' CACAGTAAAGATGGCAGCAG CD44: FW 5' TACCCACCATGGACCAAATG; Rev 5' CGTGACGAGGATATATACTCC; HPRT: FW 5' TCCTCCTCAGACCGCTTTT; Rev 5' CCTGGTTCATCATCGCTAATC; GpA: FW 5' Rev 5' AACCCAAATCAGCATTCAGC; Rev: 5' TAATCCCTGCCATCACGCCC.

Hanging drop culture[3]

E13.5 fetal livers were collected and disaggregated by pipetting in DMEM 10% FCS/1% Pen/Strep stock solution (10,000 U/mL penicillin/10,000 U/mL streptomycin). Cells were centrifuged for 5 min at 2,000 rpm and suspended in hanging drop medium (DMEM supplemented with 20% FCS, 0.1% β–mercaptoethanol, 200 mM hemin, 1% Pen/Strep from the stock solution, 2 U/mL erythropoietin, 5 mg/mL insulin) at a concentration of 5×10^4 cells for each 20 μL drop. Drops were pipetted onto the inner side of the lid of a 2 cm diameter dish and the lid was gently inverted and placed onto the dish containing 1.5 mL of PBS to create a humidified chamber, When indicated, Cytochalasin D (Sigma-Aldrich, C-8273) and Jasplakinolide (Sigma-Aldrich, C-5231) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D-2650) and added to the hanging drop medium at the final concentration of 50 nM. As a control, a culture was established containing DMSO at the final concentration equivalent to that used to dissolve drugs. Cells were harvested 24 h and 48 h after seeding, washed in PBS and cytospun for further staining. Quadruplicates - four drops- were analyzed for each condition.

Phenylhydrazine treatment

Age-matched mice were weighed and injected intraperitoneally for two consecutive days with 15 mg/Kg body weight of phenylhydrazine (PHZ, Sigma-Aldrich, P-6926). Two days after the second PHZ administration, animals were sacrificed and hematologic parameters and spleen morphology were analyzed.

Flow cytometry

Cells from blood or fetal liver or spleen were collected in DMEM, centrifuged and suspended in PBS with 0.5% BSA and stained with FITC-conjugated anti-CD71 and with phycoerythrin (PE)-conjugated anti-Ter119. Samples were acquired using FACS-Calibur (BD Bioscience) flow cytometer. Data were analyzed with Flow Jo software (Tree Star, Ashland, OR, USA).

Hematologic and biochemical analysis

Mice were bled into EDTA-containing tubes and the hematologic analysis was performed using a hemocytometer (Sysmex KX-21N Hematology Analyzer). Reticulocytes were scored by counting them on blood smears stained with new methylene blue (Sigma-Aldrich, R-4132), according to the manufacturer's protocol.

Osmotic fragility test

The osmotic fragility test was performed on fresh blood drawn from tail veins of age-matched mice (6 months old), $Gsn^{-/-}$ (n=3) and wt (n=3) mice. Blood was diluted to a 1% hematocrit in a series of hypotonic solutions with NaCl content starting from 160 mM and incubated for 30 min at 37°C. After centrifugation (5 min at 5,000 rpm) the concentration of proteins released in the supernatant was measured by the Bradford protein assay (BioRad, Hercules, CA, USA; cat. 500-0006). The osmotic fragility curve is obtained by plotting the measured absorbance at 595 nm for each solution against NaCl concentrations. Duplicates for each NaCl concentration point were read.

References

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2. Kingsley PD, Malik J, Fantauzzo KA, Palis J. Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. Blood. 2004;1:104(1):19-25
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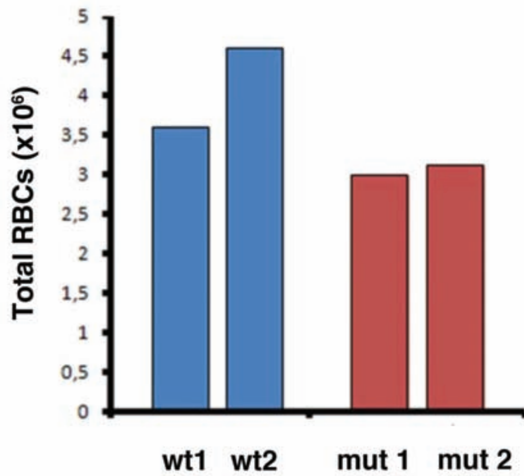
Online Supplementary Table S1. Timed matings of gelsolin heterozygous null mice. Timed matings revealed a normal proportion of homozygous mutant embryos until day 13.5. Thereafter the recovery of viable null mutant embryos decreased steadily with a significant first reduction of null mutant embryos observed by day 13.5. Rare homozygous mutant mice were found post-partum. $^{**}P < 0.0005$; $^{*}P < 0.001$. The P value calculated for all mutants at all time points is < 0.0005.

Embryonic stage	Litters	Total Embryos	+/+ (25%)	+/- (50%)	-/- (25%)	Resorbed Embryos
E 11.5	6	52	14	27	11	1
E 12.5	6	32	11	14	7	13
E 13.5	6	45	16	25	4 *	10
E 14.5	4	25	8	8	9	7
E 15.5	4	22	5	12	** 5	7
E 16.5	6	34	14	14	6	13
E 17.5	6	50	15	27	8	4
E 18.5	6	47	14	28	8	2
P0	9	40	17	21	2 **	



Online Supplementary Figure S1. Erythroid differentiation is impaired in $Gsn^{-/-}$ embryos. Total number of red blood cells in wt (n=2) and $Gsn^{-/-}$ (n=2) embryos.