Defective Assembly of Membrane Proteins in Erythroid Precursors of β-Thalassemic Mice

By J. Yuan, E. Rubin, M. Aljurf, L. Ma, and S.L. Schrier

β-Thalassemic mice provide a useful model for studying the pathophysiology of human β-thalassemia in that one can perform experiments that are difficult to perform in humans. The ease of access to β-thalassemic mouse marrow provided the opportunity to explore the cause of the ineffective erythropoiesis that characterizes severe β-thalassemia in mouse and man. We hypothesized that the accumulation of excess α-globin might interfere with the normal assembly of red blood cell (RBC) membrane proteins, thus contributing to the severe intramedullary lysis. Femoral marrow was obtained from normal and β-thalassemic mice, and RBC precursors were purified (>90%) by panning and harvesting CD45 cells. The assembly of RBC membrane proteins was assessed by observing immunofluorescence patterns obtained on fixed permeabilized precursors using rabbit polyclonal antibodies directed against human spectrin, and band 4.1, and murine band 3. The distribution of the proteins was shown with a fluorescein-tagged goat antirabbit antibody. In contrast to normal mice, about 30% of intermediate and late stage erythroblasts in β-thalassemic mice appear abnormal. Neither spectrin nor band 4.1 formed crisp rim fluorescence in these erythroid precursors of thalassemic mice, whereas assembly of band 3 appeared normal. Therefore, the assembly of membrane skeletal proteins is abnormal in murine β-thalassemic erythroid precursors perhaps because of the deposition of unmatched α-globin chains.

© 1994 by The American Society of Hematology.

The Thalassemias are a group of inherited disorders in which α- or β-globin chain synthesis is impaired. Thalassemic individuals have anemia because of a combination of ineffective erythropoiesis and removal of abnormal red blood cells (RBCs) from the peripheral blood, and both are thought to be secondary to accumulation of excess unmatched globin chains. The mechanism(s) by which globin chain accumulation leads to these events is not completely understood. In β-thalassemia major (Cooley’s Anemia), the major kinetic defect is ineffective erythropoiesis.

Mice, homozygous for a deletion of the β-major globin gene, have a clinical syndrome very similar to human β-thalassemia intermedia. Therefore, they provide a useful experimental model for the study of globin chain regulation and the consequences of accumulation of excess α-globin chains.

These homozygous β-thalassemic mice have a microcytic, hypochromic anemia, dysmorphic RBCs, a shortened RBC lifespan, and erythroid hyperplasia in the bone marrow (BM) and spleen, which can be an organ of erythropoiesis in the mouse. Although the steady-state values of RBC production, calculated from the survival curves, show only a two-fold increase in ineffective erythropoiesis, the increase in overall erythropoiesis in BM and in the spleen in β-thalassemic mice is impressive. Most dramatically, splenic colony-forming unit erythroid are increased about 300-fold. The discrepancy between this large onset of erythropoiesis and the modest two-fold increase in effective erythropoiesis points to extensive intramedullary and intrasplenic death of erythroid precursors, ie, ineffective erythropoiesis. This again is very similar to the situation in human β-thalassemia major or intermedia.

In three previous studies on peripheral RBCs of severely affected β-thalassemic mice, we noted abnormal membrane material properties including increased rigidity and decreased stability, both directly related to the α-globin content of ghosts and skeletons. Disease severity was also directly related to the α-globin content of ghosts and skeletons and to the extent of oxidation of band 4.1. These studies indicated that abnormal membrane function and particularly abnormal skeletal function may be important in the pathophysiology of murine as well as human β-thalassemia. Excess insoluble α-globin chains do accumulate in the murine thalassemic model, accounting for up to 35% of the membrane proteins in the adult murine RBCs. These considerations lead to our hypothesis that the accumulation of excess α-globin chains would begin to occur in erythroid precursors and, thus, might interfere with the normal assembly of membrane proteins. The excess globin chain deposition might also cause postassembly membrane distortion. Erythroid precursors with defective membranes could be subjected to macrophage attack or could be unable to withstand otherwise normal events in erythropoiesis. Irrespective of the mechanism(s), abnormal membrane assembly or postassembly membrane disruption could contribute to the ineffective erythropoiesis of the thalassemic erythroid precursors. Therefore, experiments were designed to identify the events that occur during membrane protein assembly or postassembly in erythroid precursors in β-thalassemic mice.

MATERIALS AND METHODS

Normal mice (C57BL/6 strain) and thalassemic mice (Hbbb^a-1/ Hbbb^a-1 strain) were bred at the Lawrence Berkeley Laboratory (Berkeley, CA). BM cells were obtained from the femur. Spleen
cells were prepared by gentle needle aspiration of the minced spleen using phosphate buffered saline (PBS) and a 21-gauge needle.

Preparation of the Antibody-Immobilized MicroCELLector Flask

Antirat Ig (Boehringer Mannheim, Indianapolis, IN)-coated MicroCELLectors (surface activated; Applied Immune Sciences, Menlo Park, CA) T-25 Cell Culture Flasks were prepared according to protocol. Briefly, 10 mL of a goat antirat antibody solution (30 μg/mL in PBS and 1 mmol/L EDTA) was filter sterilized through a 0.2-μm filter. Using a sterile pipet, 5 mL of antibody solution was added onto the binding surface of T-25 flasks. The flasks were rocked to completely wet the binding surface. The flasks were then placed on a level surface with the binding surface side down and incubated at room temperature for 3 hours. After incubation, excess antibody solution was removed using a sterile pipet, and 10 mL of PBS was immediately added to the flask. The cap was replaced, and the flasks were shaken vigorously for 10 seconds, maintaining PBS coverage of the binding surface. The PBS was aspirated, and the washing step was repeated twice more. Then, blocking of nonspecific sites on the flask was performed by slowly adding 5 mL of PBS containing 0.2% bovine serum albumin (BSA) onto the binding surface. After 10 seconds of rocking while maintaining fluid coverage of the binding surface, the flask was incubated at room temperature for 30 minutes with the flask surface down. After incubation, the 0.2% BSA in PBS was aspirated, and the flask was washed again 3 times with PBS after the above procedures. The last wash was retained in the flask until it was ready for use.

Isolation of Erythroid Precursors

To isolate a pure population of erythroid precursors, cells from BM or spleen were washed 3 times in PBS. The cell count was determined by hemacytometer, and viability was estimated by trypan blue staining. A total of 1 x 10^7 of these hematopoietic precursor cells was collected and reacted with a rat monoclonal antismouse CD45 antibody (Boehringer Mannheim). CD45 is a membrane protein that is present on lymphoid and myeloid lineages but not on erythroid precursors.19 Negative selection with CD45 will leave erythroid precursors only. After washing twice with PBS and once with PBS containing 1 mmol/L EDTA, 1 x 10^7 cells were resuspended in a total volume of 4 mL of PBS containing 1 mmol/L EDTA and 0.1% mouse serum to reduce nonspecific binding and incubated for 15 minutes at room temperature. Immediately before adding the cell suspension, the PBS solution was completely removed from the MicroCELLector T-25 flask. The cell suspension was repipetted in and out several times to create a homogenous suspension that was slowly added to the T-25 flask. The flask was incubated on a flat surface for 1 hour at room temperature, making sure that the entire binding surface was covered. At the end of the incubation, the MicroCELLector was gently rocked for 10 seconds to resuspend the nonadherent CD45− cells that were then collected using a sterile pipet. Standard morphologic examination of Wright-Giemsa–stained cytoospin preparations identified the classical appearance of proerythroblasts and basophilic, polychromatophilic, and orthochromic normoblasts. To show that the few unidentified mononuclear cells were not lymphocytes, cells before and after CD45− selection were reacted with a mixture of rat monoclonal antibodies to murine CD4, CD8, and a B-cell surface marker (kindly provided by Dr Irving Weissman and Dr Liao Xingsheng, Stanford Medical School, Stanford, CA), in a 1:100 dilution in PBS with 5% nonfat milk.

Cell Fixation and Immunofluorescence

The erythroid precursors were fixed with 0.2% paraformaldehyde in PBS, pH 7.4, for 30 minutes at room temperature. After washing twice with PBS, 20 μL of the resuspended cells were placed on an Alcian blue-coated coverslip. The cells were allowed to dry and were then permeabilized with 0.5% Triton in PBS (1 minute at room temperature). They were then washed 3 times with PBS and kept in a wet chamber. To block nonspecific binding, 5% nonfat dry milk in PBS was added, and the cells were incubated for 15 minutes. Rabbit polyclonal antibodies were generated against human spectrin and human band 4.1.16 They were diluted: 1:50 in 5% nonfat dry milk in PBS and were shown to react with murine spectrin and band 4.1 both in immunofluorescence and immunoblots. Rabbit polyclonal antibody to murine band 3 (kindly provided by Jiri Palek, Tufts University School of Medicine, Boston, MA) was also diluted 1:50 in PBS with 5% nonfat milk. In separate experiments, these antibodies were added, and the cells were incubated for 1 hour in the wet chamber at room temperature. The cells on the coverslips were then washed 3 times with PBS, and then Texas-Red–labeled goat antirabbit secondary antibody (1:40 dilution in PBS with 5% nonfat milk) was added and incubated for another 30 minutes. The cells were again washed 3 times in PBS, and the coverslips with fixed cells were mounted onto a slide with antifade mounting fluid.

Evaluation of the Shape, Contour, and Membrane of Erythroid Precursors by Phospholipid Fluorescence and Nomarski Microscopy

Because there was a concern that thalassemic erythroid precursors might have abnormal membrane wrinkling or bizarre shapes that could interfere with the planned studies of membrane protein assembly, the following experiments were performed. Normal murine and thalassemic erythroid precursors were labeled with N-(7-nitro-2-1,3-benoxadiazol-4-yl) diacyl-phosphatidylcholine (NBD-PC) or NBD-phosphatidylethanolamine (NBD-PE). NBD-PC or NBD-PE were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). The precursor cells were fixed with 0.2% paraformaldehyde in PBS for 1 hour at room temperature. After washing twice in PBS, 20 μL of cells was layered onto Alcian blue-coated coverslip for 10 minutes. The excess solution was removed from the coverslip, which was allowed to dry. The cells were then incubated with 20 μL of NBD-PC or NBD-PE (1:100 dilution in PBS) for 1 hour at room temperature. The cells were washed 3 times in PBS, and fluorescence of phospholipids incorporated into the membrane bilayer was detected in the laser confocal microscope. In addition, Nomarski microscopy was performed to visualize the curvature, shape, and contour of the erythroid precursor.

Confocal Scanning Optical Microscopy

An Odyssey Real Time Laser Confocal Microscope (Noran Instruments, Middleton, WI) was used in this study.18,19 Cells were illuminated with 514-nm light from the instrument’s argon laser through a Nikon DIAPHOT-TMD inverted microscope using a 60×/1.4 numerical aperture Neuflor objective (Nikon, New York, NY). Light passing through the aperture was filtered by a 570-nm long-pass filter before detection by the instrument’s photomultiplier tube. The photocurrent coming from the photomultiplier was passed through a 38-kHz low-pass (four pole) RC filter (model 3200; Krohn-Hite Corp, Avon, MA) to reduce pixel noise through temporal integration before the signal was stored in the frame buffer of the microscope’s host computer (IBM Compatible, Los Altos, CA). Then, noninvasive optical sections through labeled cells at 0.5-μm cuts were obtained. A set of these optical sections collected at several focal levels through a cell was then processed digitally for display as a computer reconstruction. The microscope also has a program for Nomarski microscopy.
RESULTS

Isolation of Erythroid Precursors

After cytospin and Giemsa staining, the erythroid precursors obtained before and after CD45 separation are shown in Fig 1. Normal mouse BM consists of a mixture of erythroid, myeloid, and lymphoid precursors (Fig 1A), but the CD45⁻ selection process yielded a preparation that contained greater than 90% erythroblasts (Fig 1B). The immunofluorescent studies using a mixture of rat monoclonal antibodies to murine CD4, CD8, and a B-cell surface marker showed that the CD45⁻ population contained fewer than 1% lymphoid cells compared with approximately 30% lymphoid cells before CD45 selection (data not shown). These erythroid precursors were further analyzed by laser confocal fluorescence microscopy¹⁸,¹⁹ and Nomarski scanning.

Detection of Shapes of the Erythroid Precursors

The fluorescence of the incorporated NBD-PC was used to show the shape and contour of the erythroid precursor membranes (Fig 2). The surface of the erythroid precursors was evaluated by Nomarski microscopy (Fig 2). We could detect no differences between normal and thalassemic ery-
ABNORMAL MEMBRANE ASSEMBLY IN β THALASSEMIA

The overall shape, surface, and outline of erythroid precursors were assessed by Nomarski microscopy. The smoothness and contour of the membrane was detected by incorporating fluorescent NBD-PE into the membrane phospholipid bilayer and by recording the fluorescence in the laser confocal microscope.

Fig 2. The overall shape, surface, and outline of erythroid precursors were assessed by Nomarski microscopy. The smoothness and contour of the membrane was detected by incorporating fluorescent NBD-PE into the membrane phospholipid bilayer and by recording the fluorescence in the laser confocal microscope.

Erythroid Membrane Protein Assembly as Related to Stage of Maturation

Normal mice. During erythroid differentiation, the precursors undergo a sequential and graded decrease in size. We used this size measurement, made by the confocal microscope, to identify the stages of erythroid differentiation. The incorporation of spectrin is shown in Fig 3, where the number indicates the diameter in microns of the cell as an index of the stage of erythroid differentiation. As erythroid differentiation progresses, spectrin gradually appears on the membrane where it participates in the formation of the skeleton. There may be in the normal late erythroblast (d = 7.2-μ), some residual cytosolic spectrin. The appearance of band 4.1 is believed to occur relatively late in erythroid maturation (Fig 4). The major integral protein, band 3, appears somewhat after spectrin is first identified and is mostly localized to the membrane (Fig 5).

β-Thalassemic mice. When the same studies were per-
formed on β-thalassemic mice, abnormal assembly of spectrin (Fig 3) was observed in about 30% of the erythroid precursors. The abnormalities consisted of cytosolic clumping of spectrin, variable and irregular rim fluorescence, and erratic progression of rim fluorescence as the precursors mature. The actual amount of spectrin per cell roughly estimated by the degree of fluorescence was approximately the same in normal and thalassemic mouse erythroid precursors. Similarly, immunofluorescent staining of band 4.1 (Fig 4) showed abnormal assembly and incorporation of band 4.1 into thalassemic erythroid precursor membranes. However, the assembly of band 3 in erythroid precursors in β-thalassemic mice appeared generally normal (Fig 5), although an area of uneven clumping was observed in a few thalassemic erythroid precursors. Thus, two proteins of the cytoskeletal network are poorly incorporated and distributed in the membranes of about 30% of β-thalassemic erythroid precursors, whereas the integral protein, band 3, shows more normal incorporation.

DISCUSSION

The purpose of this study was to explore the hypothesis that abnormalities of the assembly or postassembly of erythroid membrane proteins could contribute to the ineffective erythropoiesis that partly explains the severe anemia in one form of murine β-thalassemia. We used methods that allowed us to purify erythroid precursors from normal and thalassemic mouse BM. The fact that these are very early stage erythroid precursors that have just begun to synthesize and assemble their characteristic membrane proteins means that the usual immunologic identification of erythroid cells by antibodies (eg, to glycophorin A) will not be useful. However, standard morphology plus use of a panel of monoclonal antibodies to murine lymphocyte markers showed very little reactivity with these separated CD45- cells. These erythroid precursors were then analyzed by laser confocal fluorescent microscopy, a powerful new tool for cellular and developmental biology. Its chief advantage is the ability to generate noninvasive optical sections of fluorescent-labeled specimens with a virtual absence of out-of-focus blur. These images may subsequently be digitally enhanced and photographically recorded. It is particularly useful for analysis of the three dimensional organization of cellular macromolecules. Using these methods, our studies on normal murine erythroid precursors are consistent with prior data. We confirmed that spectrin is synthesized early in erythroid...
development, appearing initially in the cytosol with membrane-associated spectrin increasing as the precursors mature. Band-4.1 synthesis is known to occur relatively late in erythroid differentiation, and much of the band 4.1 is associated with the membrane. As reported, band 3 begins to appear just after spectrin synthesis is identified and is predominantly localized to the plasma membrane.

We then studied membrane assembly of erythroid precursors in β-thalassemic mice. A total of 30% of these erythroid precursors show abnormalities in distribution of spectrin and band 4.1 (Figs 3 and 4) into the developing murine membrane skeletal lattice. In contrast, the assembly of band 3, the major integral protein, was essentially normal in thalassemic precursors (Fig 5). We have performed similar studies on mouse spleen cells, because in thalassemic mice the spleen is a prominent erythropoietic organ and similar abnormalities of spectrin and band 4.1 distribution were also observed (data not shown).

We suggest that those precursors showing abnormal patterns of membrane assembly probably have excessive α-globin chain deposition that disrupts the assembly of the skeletal lattice. The other possibility is that normally synthesized and assembled spectrin and band 4.1 appear disrupted because the thalassemic lesion produces abnormal membrane invaginations and protuberances. Because of a lack of specific monoclonal antibody-to-mouse α-globin chains, we are unable, at this time, to prove that the cells which have the most accumulation of the unpaired α-globin chains are most disordered with regard to skeletal protein assembly. We suspect that these abnormalities of the membrane cytoskeletal proteins may contribute to intramedullary death of these β-thalassemic erythroid precursors or, alternatively, may render these cells a target for removal by macrophages.

REFERENCES

From www.bloodjournal.org by guest on October 22, 2017. For personal use only.
Defective assembly of membrane proteins in erythroid precursors of beta-thalassemic mice

J Yuan, E Rubin, M Aljurf, L Ma and SL Schrier