RAPID COMMUNICATION

Accelerated Programmed Cell Death (Apoptosis) in Erythroid Precursors of Patients With Severe β-Thalassemia (Cooley's Anemia)

By J. Yuan, E. Angelucci, G. Lucarelli, M. Aljurf, L. M. Snyder, C. R. Kiefer, L. Ma, and S. L. Schrier

The profound and life-threatening anemia in patients with Cooley's anemia is ascribed primarily to intramedullary hemolysis (ineffective erythropoiesis), the cause of which is obscure. Based on prior morphologic data showing nuclear abnormalities, we hypothesized that accelerated apoptosis could occur in these erythroid precursors. The highly successful bone marrow (BM) transplantation program for patients with Cooley's anemia provided us with a unique opportunity to test this hypothesis. We obtained pretransplantation BM aspiration samples from patients undergoing BM transplantation in Pesaro, Italy, and from their allogeneic donors. The erythroid precursors were isolated using ficoll sedimentation and then panning selecting for CD45- cells. Cytospin and Giemsa staining showed that the separation provided greater than 90% erythroblasts. Five million of these erythroblasts were lysed and their DNA was isolated. There were obvious ladder patterns of DNA breakdown products in β-thalassemia major samples, with less occurring in β-thalassemia trait. Normal individuals showed only a slight smear of breakdown of DNA. These results indicate there is enhanced apoptosis in the erythroblasts in the BMs of Cooley's anemia patients. This finding might partially explain why most of these erythroblasts never survive to become mature erythrocytes.

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IN THE SEVERE FORMS of human thalassemia, anemia and its treatment with transfusions cause morbidity and mortality.1-3 Ineffective erythropoiesis in the bone marrow (BM) and peripheral hemolysis of red blood cells (RBCs) leads to anemia.3,4 Peripheral hemolysis of RBCs may contribute to the anemia more in severe α-thalassemia (Hb-H disease),5,6 but intramedullary hemolysis is the major kinetic defect that leads to anemia in β-thalassemia major or Cooley's anemia.3 As shown by Finch et al,3 the plasma iron turnover rate in β-thalassemia major patients, as a measure of the onset of erythropoiesis, can be 10 times that of normal patients. However, the delivery of thalassemic RBCs to the peripheral blood in these patients is much reduced, thereby indicating extraordinary intramedullary erythroid cell death. The cause of this death in the marrow has never been fully identified, but there are morphologic clues showing that erythroid precursors in β-thalassemia major show evidence of α-globin chain deposition,6 cytoplasmic vacuolization,7 and abnormalities of the nuclear membrane.8 Our hypothesis is that unmatched excess α-globin chain accumulation somehow causes these defects and ultimately the death in marrow of about 80% of the affected erythroid precursors. This hypothesis would be consistent with our observations on the important role of specific globin accumulation in the thalassemia.9

The role of programmed cell death (apoptosis) has been shown for many tissues and cell lines.10-12 Apoptosis is a selective process of physiologic cell deletion. Its occurrence plays a major role in the control of normal and abnormal processes.13 Apoptosis has been described in erythroid precursors in vitro systems.14,15 Although there is presently no evidence for programmed cell death in normal erythropoiesis, the late or orthochromic erythroblast with a shrunken pyknotic nucleus being extruded from the cell looks morphologically like apoptosis. In this study, we evaluated the possibility that α-globin chain accumulation could trigger or enhance programmed cell death in erythroid progenitors of patients with β-thalassemia major.

The successful allogeneic BM transplantation program for patients with β-thalassemia major in Pesaro, Italy provided us with a unique opportunity to test the hypothesis that enhanced programmed cell death may contribute to the profound intramedullary hemolysis that occurs in the BM of these patients.

We obtained pretransplantation marrow samples from the β-thalassemia major patients and from their sibling allogeneic donors, who either have the β-thalassemia trait or are normal. Our study consisted of seven β-thalassemia major patients, five β-thalassemia trait donors, and four normal donor bone marrow samples (all shipped on ice from Italy) plus four normal bone marrow samples locally obtained from the Stanford University Hospital Bone Marrow Transplantation Program, according to the protocols approved by the Stanford Institutional Review Board. We also studied two additional "control" subjects. One patient had severe myelodysplastic syndrome with morphologic evidence of ineffective erythropoiesis and megaloblastoid erythroid precursors in marrow. The other patient was a child completing an otherwise uncomplicated induction for acute lymphoblastic leukemia whose marrow aspirate showed brisk nor-
moblastic erythroid hyperplasia (75% of all marrow cells). Hematopoietic progenitors were isolated using ficoll centrifugation to remove mature RBCs and the remaining RBCs were removed by lysis in ammonium chloride solution. It was critical to work with a “pure” population of erythroid precursors, therefore, cells were isolated by panning onto AIS MicroCelлектor plates (Applied Immune Sciences, Menlo Park, CA) and CD45− cells were selected. CD45 is a membrane protein that is present on lymphoid and myeloid lineages but is absent in erythroid precursors. Flasks coated with secondary antibody were used to remove CD45-labeled cells. After the removal of RBCs, the marrow suspension in Cooley’s anemia consists of a mixture of erythroid, myeloid, and lymphoid progenitors (Fig 1A), but our CD45 negative selection process yielded a preparation that contained greater than 95% erythroblasts (Fig 1B). In addition to cytospin and differential counts, these erythroid precursors were also analyzed by laser confocal immunofluorescence microscopy.16 To detect apoptosis, the erythroid precursors were then counted and 5 million of these cells from normal patients, donors, and affected patients were lysed and centrifuged. The DNA from both supernatant and pellet was precipitated. The pellet contains most of the intact chromosomal DNA, whereas the supernatant contains the majority of the DNA breakdown products. The supernatant was used to evaluate apoptosis. After removal of RNA by RNase, the DNA samples from the supernatant were loaded onto an agarose gel and electrophoresed. Examples of the DNA pattern from supernatants are shown in Fig 2. There were obvious “ladder” patterns of DNA breakdown products in β-thalassemia major patients and, to a lesser extent, in β-thalassemia trait donors. Normal individuals had the least amount of this sort of DNA breakdown.

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<th>Table 1. Marrow Erythroid Precursor Differential Count</th>
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Fig 1. Thalassemic BM cells before (A) and after (B) CD45− selection. Ten milliliters of BM was collected from β-thalassemia major patients. The hematopoietic precursors, after the removal of RBCs by ficoll centrifugation and lysis with 0.83 mol/L NH₄Cl, are shown in (A). Ten million of these cells were further treated by reacting with monoclonal anti-CD45 antibodies. After washing with phosphate-buffered saline, these cells were loaded onto goat antimouse lg-coated AIS Microcellectors and incubated at room temperature for 1 hour. The nonadherent CD45− cells are shown in (B).
Neither of the two "controls," one representing intense erythroid hyperplasia and the other a form of dysplastic ineffective erythropoiesis, showed "ladder" pattern formation in their separated erythroid precursors (data not shown). These "ladder" patterns are characteristic of programmed cell death or apoptosis. It has been suggested that endo-
nucleases attack DNA specifically to lead to this orderly breakdown pattern. Thus, there appears to be greatly enhanced apoptosis in the erythroblasts of β-thalassemia major patients and considerably less apoptosis in donors with β-thalassemic trait. This finding could partially explain why most of the erythroblasts in β-thalassemia major never mature. We also harvested CD45+ cells from the respective marrows. The DNA from supernatants of lymphoid and myeloid precursors showed no sign of ladder pattern formation (data not shown).

We next considered the possibility that the β-thalassemic marrow might contain a disproportionate number of the late-stage or orthochromic normoblasts, with pyknotic nuclei thought to be the morphologic indicator of apoptosis. However, the differential counts (200 to 500 cells counted) from one shipment of these marrows samples was as shown in Table I. There are more early- and intermediate-stage erythroid precursors in β-thalassemia major and minor marrow, whereas normal individuals have relatively more late-stage erythroid precursors. Therefore, it is likely that
the apoptosis seen occurs in the early-stage erythroid precursors in severe β-thalassemia.

We then proposed that the α-globin chain deposition somehow resulted in the enhanced apoptosis in the BM of β-thalassemia major patients. If that were the case, we would expect to see α-globin chain deposition in the early stages of erythropoiesis, where we believe apoptosis to occur. We used laser confocal immunofluorescent microscopy, with monoclonal antihuman α-globin as the primary antibody and Texas Red-labeled antimouse IgG as the secondary antibody, and detected the deposition of α-globin chains (Fig 3). The monoclonal antibody

\[ \alpha-1-58782 \] (IgG2b,k) was derived by the fusion of the myeloma cell line FOX-NY with spleen cells from an RBF/On mouse immunized with a conjugate containing a synthetic peptide corresponding to the sequence α17-26. This peptide contains the α-unique sequence gly-ala-his-ala-gly (α17-21), which occurs at the bend of the A and B helices in the native chain. The antibody reacts with α chains, either native or denatured, but does not react with non-α chains. The α-globin chains accumulate progressively as the erythroid cell undergoes progressive differentiation. Although the late-stage precursors have the most α-globin chain accumulation, α-globin chain deposition can be seen as early as the proerythroblast stage. The heterogeneity of α-globin chain deposition in this thalassemic sample is fairly typical. In contrast, in very early stages of normal erythroid progenitors, we saw no such globin chain accumulation (data not shown). This result indicated that the α-globin chain deposition in β-thalassemia major patients occurs early enough to cause the enhanced programmed cell death we observed.

We thus propose that enhanced programmed cell death may partially explain the intramedullary cell death in β-thalassemia major. Abnormal α-globin chain deposition in β-thalassemia occurs early enough in erythropoiesis to cause accelerated apoptosis in the affected stages. The mechanisms by which α-globin chain accumulation could lead to apoptosis is under investigation.

REFERENCES

15. Koury MJ, Bondurant MC: Control of red cell production: The roles of programmed cell death (apoptosis) and erythropoietin. Transfusion 30:8, 1990 (editorial)
Accelerated programmed cell death (apoptosis) in erythroid precursors of patients with severe beta-thalassemia (Cooley's anemia) [see comments]

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