Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis

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Erythropoiesis is the process by which nucleated erythroid progenitors proliferate and differentiate to generate, every second, millions of nonnucleated red cells with their unique discoid shape and membrane material properties. Here we examined the time course of appearance of individual membrane protein components during murine erythropoiesis to throw new light on our understanding of the evolution of the unique features of the red cell membrane. We found that the accumulation of all of the major transmembrane and all skeletal proteins of the mature red blood cell, except actin, accrued progressively during terminal erythroid differentiation. At the same time, and in marked contrast, accumulation of various adhesion molecules decreased. In particular, the adhesion molecule, CD44 exhibited a progressive and dramatic decrease from proerythroblast to reticulocyte; this enabled us to devise a new strategy for distinguishing unambiguously between erythroblasts at successive developmental stages. These findings provide unique insights into the genesis of red cell membrane function during erythroblast differentiation and also offer a means of defining stage-specific defects in erythroid maturation in inherited and acquired red cell disorders and in bone marrow failure syndromes.

CD44 | CD71 | erythroblast differentiation | cell adhesion | erythrocyte

Erythropoiesis is the process by which erythroid progenitors proliferate and differentiate into nucleated reticulocytes. Two distinct erythroid progenitors have been functionally defined in colony assays, namely, the early-stage burst-forming unit-erythroid (BFU-E) and the later stage colony-forming unit-erythroid (CFU-E) progenitor (1). The earliest morphologically recognizable erythroblast in hematopoietic tissues is the proerythroblast, which undergoes 3–4 mitoses to produce reticulocytes. Morphologically distinct populations of erythroblasts are produced by each successive mitosis, beginning with proerythroblasts and followed by basophilic, polychromatic, and orthochromatic erythroblasts. Finally, orthochromatic erythroblasts expel their nuclei to generate reticulocytes. This ordered differentiation process is accompanied by decreases in cell size, enhanced chromatin condensation, progressive hemoglobinization, and marked changes in membrane organization.

During recent decades, detailed characterization of the protein composition and structural organization of the mature red cell membrane has led to insights into its function (2–6). A 2-dimensional spectrin-based skeletal network consisting of α- and β-spectrin, short actin filaments, protein 4.1R, ankyrin, protein 4.2, p55, adducin, dematin, tropomyosin, and tropomodulin has been shown to regulate membrane elasticity and stability. Mutations in some of these proteins result in loss of mechanical integrity and hemolytic anemia. The skeletal network is attached to the lipid bilayer through 2 major linkages (7). The first is through ankyrin, which itself forms part of a complex of band 3, glycophorin A, RhAG, CD47, and ICAM-4, while the second involves protein 4.1R, glycophorin C, and protein p55, with associated Duffy, XK, and Rh proteins. The loss of the ankyrin-dependent linkage, because of a mutation in ankyrin or band 3, results in loss of cohesion between the bilayer and the skeletal network, leading to membrane loss by vesiculation. This diminution in surface area reduces red cell life span with consequent anemia. A number of additional transmembrane proteins, including CD44 and Lu, have been characterized, although their structural organization in the membrane has not been fully defined.

Some transmembrane proteins exhibit multiple functions. Band 3 serves as an anion exchanger, while Rh/RhAG are probably gas transporters (8, 9), and Duffy functions as a chemokine receptor (10, 11). Another group of transmembrane proteins, including Lu, CD44, ICAM-4, and integrins α4β1 and α5β1, mediates cell–cell and cell–extracellular matrix interactions. CD47 prevents premature removal from the circulation by its function as a marker of “self” on the outer surface, where it binds to the inhibitory receptor SIRPα (12, 13). Kell protein possess endothin-3 converting enzyme activity (14).

In contrast to our broad understanding of the structure and function of the membrane of the mature red cell, our knowledge of the erythroblast membrane protein composition and organization at different development stages is sparse. Previous studies have given evidence for asynchronous synthesis and assembly of membrane proteins, in particular α- and β-spectrin, ankyrin, and band 3, during erythroid differentiation (15–20). A number of studies revealed decreased levels of expression of adhesion molecules, such as α4/β1 integrin, α5/β1 integrin, and Lu during terminal erythroid differentiation (21, 22). Finally, the transferrin receptor (CD71), which is expressed at high levels in erythroblasts at all stages of development, is absent from the mature cell (23).

On the basis of the extensive information available on the mature red cell membrane, we have been able to define the course of expression of the major transmembrane and skeletal proteins at defined stages of erythropoiesis. Our analysis reveals differences in the rates and time of appearances of these constituents during terminal erythroid differentiation. The results have led us to a greatly improved means, based on levels of CD44 expression, of discriminating between successive developmental stages. This has allowed us to isolate populations of erythroblasts at each stage of development, in a much more homogenous state than achieved in earlier work, dependent on expression levels of the transferrin receptor, CD71. We can now, moreover, obtain reliable information on anomalies of membrane protein assimilation in inherited and acquired red cell disorders and in bone marrow failure syndromes.

Results

Expression of Transmembrane Proteins During Erythropoiesis. Populations of erythroblasts at various developmental stages were ob-
proerythroblasts while the glycosylated 130-kDa component is implying that the unglycosylated 94-kDa component is expressed in band of Kell in proerythroblasts (0 h), the 130-kDa band expressed Deglycosylation did not change the migration of the lower 94-kDa component.

Expression comprised band 3, GPA, Rh, RhAG, and Duffy and the decreased expression or loss of β1 integrin. The third pattern was characterized by positive staining of GPA and DAPI. The early- and late-stage erythroblasts were defined by positive staining of GPA and DAPI. Little change in the surface expression of CD44, which progressively decreased by 30-fold from proerythroblasts to orthochromatic erythroblasts. The mean fluorescence intensity of unstained cells and cells stained with secondary antibody was always <100.

Immunofluorescence Staining of Transmembrane Proteins in Bone Marrow Erythroblasts. We next examined the expression of GPA, band 3, RhAG, CD71, and CD44 in primary mouse bone marrow erythroblasts by immunofluorescence microscopy. Erythroblasts were defined by positive staining of GPA and DAPI. The early- and late-stage erythroblasts were distinguished on the basis of cell size, large cells representing early-stage and small ones late-stage erythroblasts. As shown in Fig. S1, an increase in cell surface expression of GPA, band 3, and RhAG from early- to late-stage erythroblasts is readily apparent. In marked contrast, there is a dramatic decrease in surface expression of CD44. Little change in the surface expression of CD71 was noted between early- and late-stage erythroblasts. These findings are consistent with the results on cultured erythroblasts.

Relationship Between Erythroblast Size and Expression Levels of CD44 and CD71. The above results suggest that CD44 might be a much more reliable surface marker for distinguishing between different stages of erythroid differentiation than CD71, which is currently widely used. To test this supposition, we stained bone marrow cells with both CD44 antibody and an erythroid-specific glycophorin A antibody, TER119 (Fig. 3A). On the basis of TER119 staining intensity, 2 distinct populations, TERlow and TERHi are noted. To further distinguish different erythroblast subpopulations, we used an additional parameter, forward scatter (FSC) intensity, because this is a function of cell size and the size of erythroblasts decreases with maturation. The expression levels of CD44 as a function of FSC for all TER-positive cells are shown in Fig. 3B. Five distinct clusters could be resolved. Histograms of CD44 expression levels in
these 5 gated cell populations (Fig. 3C) show progressive decrease of CD44 surface expression with decreased cell size.

In parallel, we also stained bone marrow cells with TER119 and CD71 and analyzed the data in a similar manner (Fig. 3D–F). On the basis of TER119 staining intensity, 2 distinct populations TERlow and TERHi were once again seen (Fig. 3D). However, when CD71 expression levels were analyzed as a function of FSC for all TER-positive cells (Fig. 3E), there was marked overlap in the histogram profiles of CD71 between the gated clusters I–III, implying similar levels of CD71 expression (Fig. 3F).

**Characterization of Erythroblast Populations, Sorted Using Either CD44 or CD71.** To identify different erythroblast populations, we sorted primary bone marrow erythroid cells on the basis of either CD44 or CD71 expression levels and cell size. Representative images of erythroblast morphology on stained cytopsins of each of the 5 CD44 stained populations outlined in Fig. 3B are shown in Fig. 4A. Cells from region I have morphological characteristics of proerythroblasts, namely large size, very high nucleus/cytoplasm ratio, and intensely basophilic cytoplasm. Cells from region II are smaller in size, with increased nuclear condensation and the morphological characteristics of basophilic erythroblasts. Cells from region III are polychromatic erythroblasts, exhibiting a further decrease in cell size and additional nuclear condensation. Initial sorting of the region IV population showed mixed populations of orthochromatic erythroblasts and immature reticulocytes. In an attempt to determine whether we can better distinguish between the 2 cell types, we
gated region IV into 2 distinct populations on the basis of the expression levels of CD44, termed IV-A (higher CD44 expression, top half of region IV) and IV-B (lower CD44 expression, bottom half of region IV). As shown in Fig. 4A, cells from region IV-A have cellular characteristics of orthochromatic erythroblasts, while cells from region IV-B are nonnucleated reticulocytes. Finally, cells from region V are predominantly mature red cells. To quantify the purity of the various sorted populations, a differential count of erythroblasts at different stages of development was performed by examining 1,000 cells. As shown in Fig. 4C, the various sorted populations contained cells at a defined stage of development ranging from proerythroblasts to reticulocytes with purities ranging from 85 to 90%.

Representative images of erythroblast morphology on stained cytospins of each of the 5 CD71 stained populations outlined in Fig. 3E are shown in Fig. 4B. While, as with CD44, 90% of cells from region I were proerythroblasts, there was large degree of heterogeneity in all other regions (Fig. 4D). The purity of erythroblasts at all later stages of development ranged between 40 and 60% in the different fractions.

To further validate our finding that CD44 is a more effective surface marker for distinguishing erythroblasts at different stages of erythroid differentiation than CD71, we compared the expression levels of CD44 and CD71 on the same cell by dual staining of primary bone marrow cells with both antibodies along with Ter-119. As shown in Fig. 5A and B, gating on 5 distinct forward scatter gates of the dual-stained cells identified erythroblasts with 5 distinct levels of CD44 expression, consistent with our findings with CD44.
staining alone. In marked contrast, there was significant overlap in CD71 expression levels in the same 5 gated populations (Fig. 5C). Moreover, as shown in Fig. 5D, there is a wide range of CD71 expression levels at several maturation stages compared to CD44, confirming that CD71 does not change progressively and distinctly during terminal erythroid differentiation.

Discussion

During terminal erythroid differentiation, proerythroblasts, the earliest morphologically recognizable nucleated erythroid cells in hematopoietic tissues, undergo 3–4 mitoses to generate, in humans, 2 million nonnucleated reticulocytes every second. Extensive re-modeling of reticulocytes, first in the bone marrow and then in circulation, results in the generation of mature circulating red cells with their unique discoid shape and membrane material properties (25, 26). In the present study we examined the temporal changes in accumulation of the different membrane protein components during murine erythropoiesis. We found that accumulation of all major transmembrane and all skeletal proteins of the mature red blood cell, except actin, increased progressively during differentiation from the proerythroblast to the orthochromat erythroblast stage. In marked contrast, accumulation of a series of adhesion molecules was highest in proerythroblasts and decreased during erythroid differentiation with very low-level expression in orthochromat erythroblasts. The most important aspect of the results presented here is that they allow us to separate with greater reliability and precision than has been previously possible the successive stages in erythroid differentiation.

A number of earlier studies in the 1980s and 1990s investigated the synthesis and assembly of membrane proteins during erythropoiesis (15–20, 27). These studies encompassed a limited number of the major membrane proteins, spectrin, ankyrin, 4.1R, and band 3. In chicken erythroblasts transformed with avian erythroblastosis virus or S13 virus, Woods et al. have shown that expression of band 3 occurs later than that of the peripheral proteins, spectrin, ankyrin, and 4.1R (28). Similar results on accumulation of spectrin, ankyrin, 4.1R, and band 3 were also found to hold for murine and human erythropoiesis (15, 18, 29, 30). Southcott et al. studied the order of appearance of a number of proteins that encode human blood group antigens in an in vitro culture system and noted that Kell antigen was the first protein to be expressed (31). Our more comprehensive survey confirms these earlier results and also provides information on a series of other proteins, important in red cell membrane function.

Our data extend the earlier studies in that the protein expression profiling was performed on highly synchronous erythroblast populations. This has permitted us to define the stage-specific expression patterns of a range of proteins of the erythrocyte membrane. We have found inter alia that the accumulation of proteins involved in linking the lipid bilayer to the skeletal protein network (band 3, RhAG, ankyrin, and 4.1R) follows behind that of the components of the membrane skeleton (α- and β-spectrin, adducin, and tropomodulin). Thus, the assembly of a fully functional spectrin-based network, which determines the material properties of the membrane, is a late event in erythropoiesis. In this context, it is interesting to note that the components of the spectrin-based network, α- and β-spectrin, adducin, and tropomodulin are synthesized earlier than the linking proteins, starting at the proerythroblast stage and progressively increasing at later stages of differentiation. An exception to the general pattern is actin, another principal component of the membrane skeleton, the expression of which is highest in proerythroblasts and falls off as terminal erythroid differentiation proceeds. The implication is that actin has additional function in erythroblasts, which it probably exercises in its filamentous state in the cytoplasm, whereas only a small proportion is required to form the short protofilaments of the skeletal lattice.

Erythropoiesis in vivo occurs entirely in erythroid niches, termed “erythroblastic islands,” which are made up of a central macrophage surrounded by developing erythroblasts (32, 33). Adhesive interactions in this specialized structure between the central macrophage and erythroblasts, as well as between erythroblasts and extracellular matrix proteins, play a critical role in regulating terminal erythroid differentiation. A number of proteins expressed on erythroblasts, including β1 integrin, CD44, Lu, and ICAM-4, are responsible for various adhesive interactions (33). Five splice variants of β1 integrin, arising from alternative splicing of the cytoplasmic domain designated, β1A, β1B, β1C-1, β1C-2, and β1D, have previously been identified in various cells (34) and we have shown here that 2 of the 5 known isoforms are expressed during erythroid differentiation. The discovery that the adhesion molecules are most strongly expressed in proerythroblasts and are either expressed at very low levels or not at all in orthochromat erythroblasts implies that adhesive interactions are dynamically regulated during terminal erythroid differentiation.

A major outcome of our results is the rational choice they have allowed us to make of a cell surface marker that would best discriminate between erythroblasts at different stages of matura-

Materials and Methods

Antibodies. Eighteen of the 24 antibodies used for Western blot and immunofluorescence microscopy were generated and characterized in our laboratory. All 18 antibodies were affinity purified and underwent stringent validation for their specificity and 11 of these antibodies have been previously described (7). Anti-TER 119, anti-j-1 integrin, and anti-CD44 are from BD PharMingen. Anti-CD71 is from Invitrogen. For flow cytometry and sorting, the antibodies used are as follows: FITC-conjugated anti-Ter 119, APC-conjugated anti-CD44, PE-conjugated anti-CD71, and APC-Cy7-conjugated CD11b are all from BD PharMingen; FITC-conjugated anti-j-1 integrin is from BioLegend; and monoclonal anti-Kell is from our laboratory.

Erythroblast Cultures. Proerythroblasts were isolated and cultured using methods established by Koury et al. (24, 37). Briefly, 2 weeks after infection with 10^6 spleen focus-forming units of the anemia-inducing strain of Friend leukemia virus (FVA), female CDF-1 mice (Charles River) were killed, and splenic proerythroblasts were purified by velocity sedimentation at unit gravity. Proerythroblasts were cultured at 37 °C in a humidified atmosphere of 5% CO2 at an air concentration of 1×10^6 cells/mL. The culture medium was Iscove’s modified Dulbecco’s medium (MEM) (Gibco) with 30% heat-inactivated FBS (Invitrogen), 1% deionized BSA (Millipore), 100 units/mL penicillin G (ATCC), 100 g/mL streptomycin (ATCC), 0.1 mM α-thioglycerol (Sigma), and 0.2 units/mL human recombinant erythropoietin (EPO) (R&D Systems). Erythroblasts of different stages of maturation were collected at 12, 24, and 44 h.
Western Blot Analysis. Whole-cell lysates of erythroid cells were prepared with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 50 mM Tris-HCl, pH 8.0) in the presence of protease inhibitor cocktails (Sigma). Protein concentration was measured using a DC protein assay kit (Bio-Rad). For N-glycosidase treatment, 40 units cocktails (Sigma). Protein concentration was measured using a DC protein assay

Fig. S1. Immunofluorescence images of surface expression of various transmembrane proteins in early and late stage erythroblasts. Representative images of primary mouse bone marrow early and late stage erythroblasts stained with specific antibodies against GPA, band 3, RhAG, CD44, and CD71 are shown. Note the increased expression of GPA, band 3, and RhAG during differentiation, the decreased expression of CD44, and the similar levels of expression of CD71 in early and late stage erythroblasts.
Table S1. Expression of surface markers during erythropoiesis

<table>
<thead>
<tr>
<th>Proteins</th>
<th>0 h, mean fluorescence</th>
<th>12 h, mean fluorescence</th>
<th>24 h, mean fluorescence</th>
<th>44 h, mean fluorescence</th>
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<tr>
<td>CD71</td>
<td>6,700 ± 100</td>
<td>26,000 ± 730</td>
<td>22,000 ± 450</td>
<td>6,500 ± 180</td>
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<tr>
<td>GPA</td>
<td>1,000 ± 80</td>
<td>1,800 ± 50</td>
<td>3,500 ± 130</td>
<td>3,700 ± 100</td>
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<tr>
<td>Kell</td>
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<td>1,600 ± 30</td>
<td>1,600 ± 40</td>
<td>1,170 ± 30</td>
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<tr>
<td>β1 integrin</td>
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