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 nonnucleated 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 $\alpha4\beta1$ and $\alpha5\beta1$, 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 $\alpha4/\beta1$ integrin, $\alpha5/\beta1$ 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-

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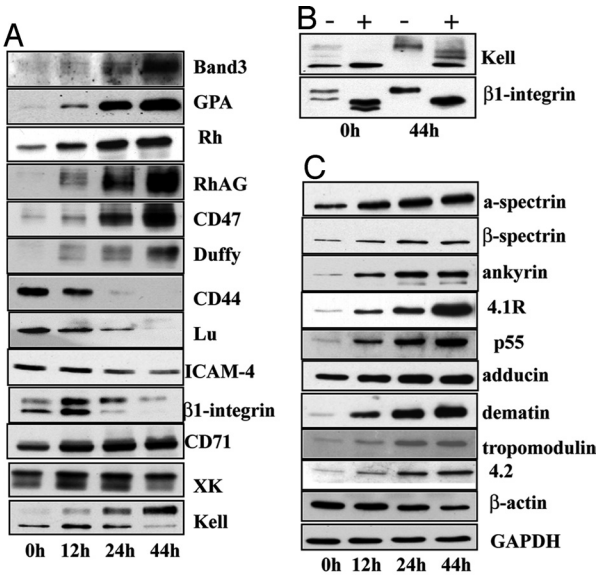

Fig. 1. Immunoblots of membrane proteins of erythroblasts at different stages of differentiation. (*A*) Transmembrane proteins: blots of SDS/PAGEs of total cellular protein prepared from erythroblasts at 0, 12, 24, and 44 h in culture probed with antibodies against the indicated proteins. Note increased expression of band 3, GPA, Rh, RhAG, CD47, and Duffy and the decreased expression or loss of β1 integrin, CD44, Lu, and ICAM-4 during terminal erythroid differentiation. CD71 and XK increased from proerythroblasts to basophilic erythroblasts with no further changes observed at later stage erythroblasts. (*B*) Effect of N-glycosidase treatment on Kell and β1 integrin proteins. 0- or 44-h cells in culture either untreated (−) or treated (+) with N-glycosidase were probed with anti-Kell or anti-β1 integrin antibodies. Note the conversion of the 130-kDa Kell band to the 94-kDa Kell band and the reduced size of both β1 integrin bands following N-glycosidase treatment. (*C*) Skeletal proteins. Blots of SDS/PAGEs of total membrane protein were probed with antibodies against the indicated skeletal proteins. Note the increased expression of all skeletal proteins except for actin during terminal erythroid differentiation. GAPDH was used as loading control for both transmembrane and skeletal proteins.

tained by culturing highly purified proerythroblast populations from mice injected with Friend virus, as previously described by Koury et al. (24). During 44 h in culture in this system, proerythroblasts (0 h) progressively differentiate into basophilic erythroblasts (12 h), polychromatic erythroblasts (24 h), and orthochromatic erythroblasts and reticulocytes (44 h). The expression levels of 13 transmembrane proteins over this period as determined by Western blotting are shown in Fig. 1*A*. Three distinct patterns of protein expression could be distinguished. The first consisted of a low level of expression in proerythroblasts with progressive increase in late-stage erythroblasts. The proteins displaying this pattern of expression comprised band 3, GPA, Rh, RhAG, CD47, and Duffy. The second expression pattern consisted of a high level in proerythroblasts with progressive decrease in late-stage erythroblasts. The adhesion molecules, CD44, Lu, ICAM-4, and β1 integrin adhered to this course of expression. The third pattern was characterized by relatively minor changes in expression during the entire course of erythroid differentiation. The tranferrin receptor (CD71) and XK protein fall into this group.

Two distinct immunoreactive protein bands were observed for Kell and β1 integrin (Fig. 1*A*). To determine if these 2 distinct bands reflect differences in glycosylation or expression of different isoforms, we performed N-glycosidase treatment (Fig. 1*B*). Deglycosylation did not change the migration of the lower 94-kDa band of Kell in proerythroblasts (0 h), the 130-kDa band expressed in orthochromatic erythroblasts (44 h) decreased in apparent size, implying that the unglycosylated 94-kDa component is expressed in proerythroblasts while the glycosylated 130-kDa component is expressed in later stages of erythroid differentiation. Two β1 integrin bands were seen in proerythroblasts (0 h), both of which shifted after N-glycosidase treatment, implying that both are expressed at this stage and are glycosylated. Interestingly, only the glycosylated higher-molecular-weight isoform of β1 integrin is expressed at low levels in orthochromatic erythroblasts (44 h).

Expression of Skeletal Proteins During Erythropoiesis. The expression levels of 10 skeletal proteins during terminal erythroid differentiation determined by Western blotting are shown in Fig. 1*C*. In contrast to the 3 distinct patterns of expression of transmembrane proteins, all skeletal proteins except actin adhered to a single pattern of expression. The expression of α-spectrin, β-spectrin, ankyrin, 4.1R, p55, adducin, dematin, tropomodulin, and 4.2 increased during terminal differentiation, whereas that of actin decreased in late-stage erythroblasts compared to proerythroblasts.

Surface Exposure of Transmembrane Proteins During Erythropoiesis. Because Western blot analysis provides total protein content, we sought to determine the cell surface exposure of a set of transmembrane proteins that included CD71, GPA, Kell, β1 integrin, and CD44, with the aid of flow cytometry [Fig. 2, supporting information (*SI*) Table S1]. The surface expression of CD71 increased 3- to 4-fold from proerythroblasts to basophilic and polychromatophilic erythroblasts. However, late-stage orthochromatic erythroblasts had lower surface levels similar to those on proerythroblasts. GPA exhibited a progressive increase, with 4 times greater abundance in orthrochromatic erythroblasts than in proerythroblasts. No significant change was observed for Kell. As for β1 integrin, in contrast to the results of Western blot analysis, which showed an increase from proerythroblasts to basophilic erythroblasts, followed by a progressive decrease in late stages, surface exposure of β1 integrin is significantly decreased only in orthochromatic erythroblasts. The most dramatic change occurred in 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 CD71was 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. 3*A*). On the basis of TER119 staining intensity, 2 distinct populations, TER^{low} and TER^{Hi} 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. 3*B*. Five distinct clusters could be resolved. Histograms of CD44 expression levels in

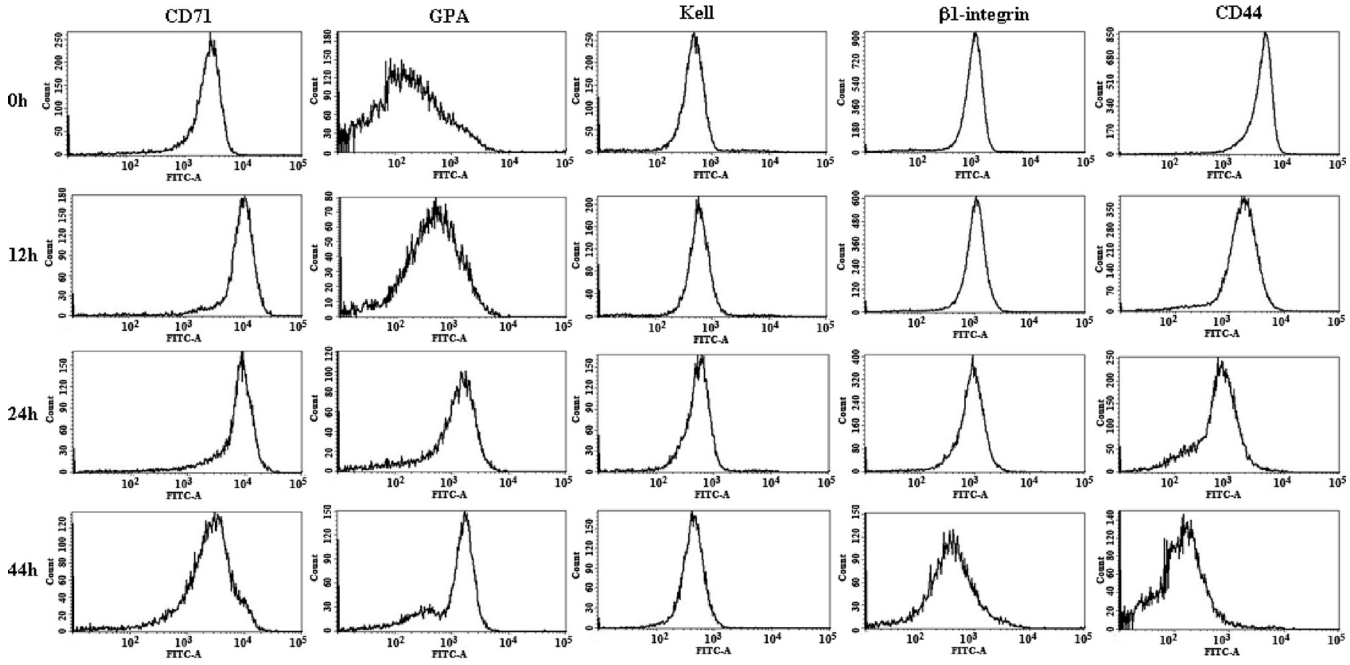

Fig. 2. Flow cytometric analysis of expression of transmembrane proteins CD71, GPA, Kell, β1 integrin, and CD44 at different stages of erythroid differentiation. The ordinate measures the number of cells displaying the fluorescent intensity given by the abscissa. Note increased expression of GPA and decreased expression of CD44 during terminal erythroid differentiation.

these 5 gated cell populations (Fig. 3*C*) 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. 3 *D–F*). On the basis of TER119 staining intensity, 2 distinct populations TER^{low} and TER^{Hi} were once again seen (Fig. 3*D*). However, when CD71 expression levels were analyzed as a function of FSC for all TER-positive cells (Fig. 3*E*), there was marked overlap in the histogram profiles of CD71 between the gated clusters I–III, implying similar levels of CD71 expression (Fig. 3*F*).

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 cytospins of each of the 5 CD44 stained populations outlined in Fig. 3*B* are shown in Fig. 4*A*. 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

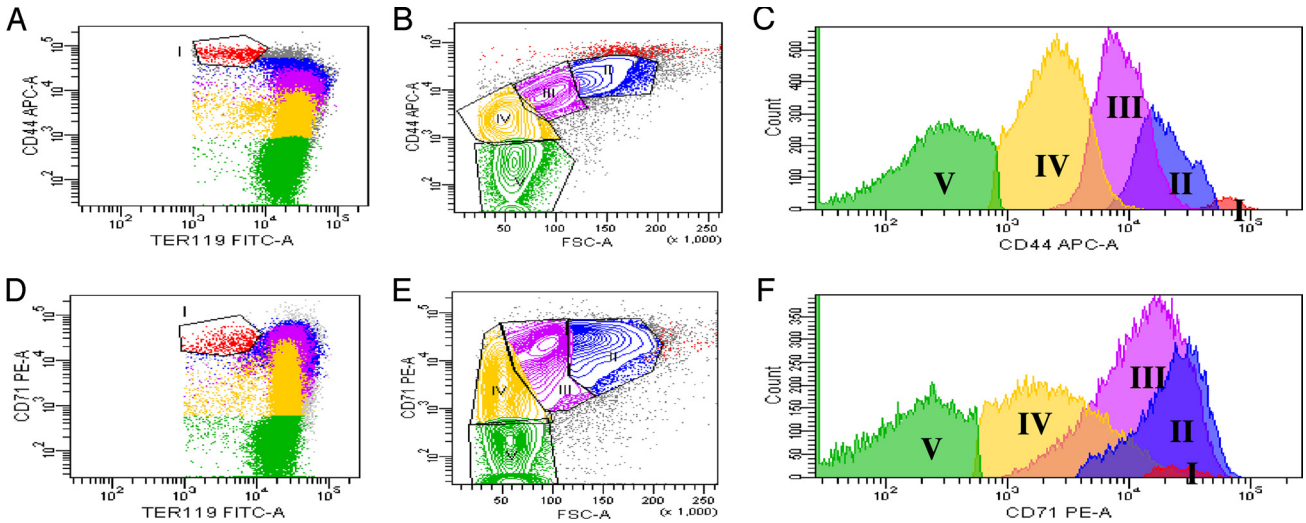

Fig. 3. Flow cytometric analysis of bone marrow cells. (*A–C*) Bone marrow cells labeled with antibodies against TER119 and CD44. (*A*) plot of CD44 versus TER119. (*B*) Plot of CD44 versus FSC of all TER positive cells. Note that 5 distinct clusters can be distinguished. (*C*) The CD44 expression levels in the gated cell population. Note the progressive decrease of CD44 surface expression from region I to region V. (*D–F*) Bone marrow cells labeled with antibodies against TER119 and CD71. Note that cells from regions I to III express similar levels of CD71.

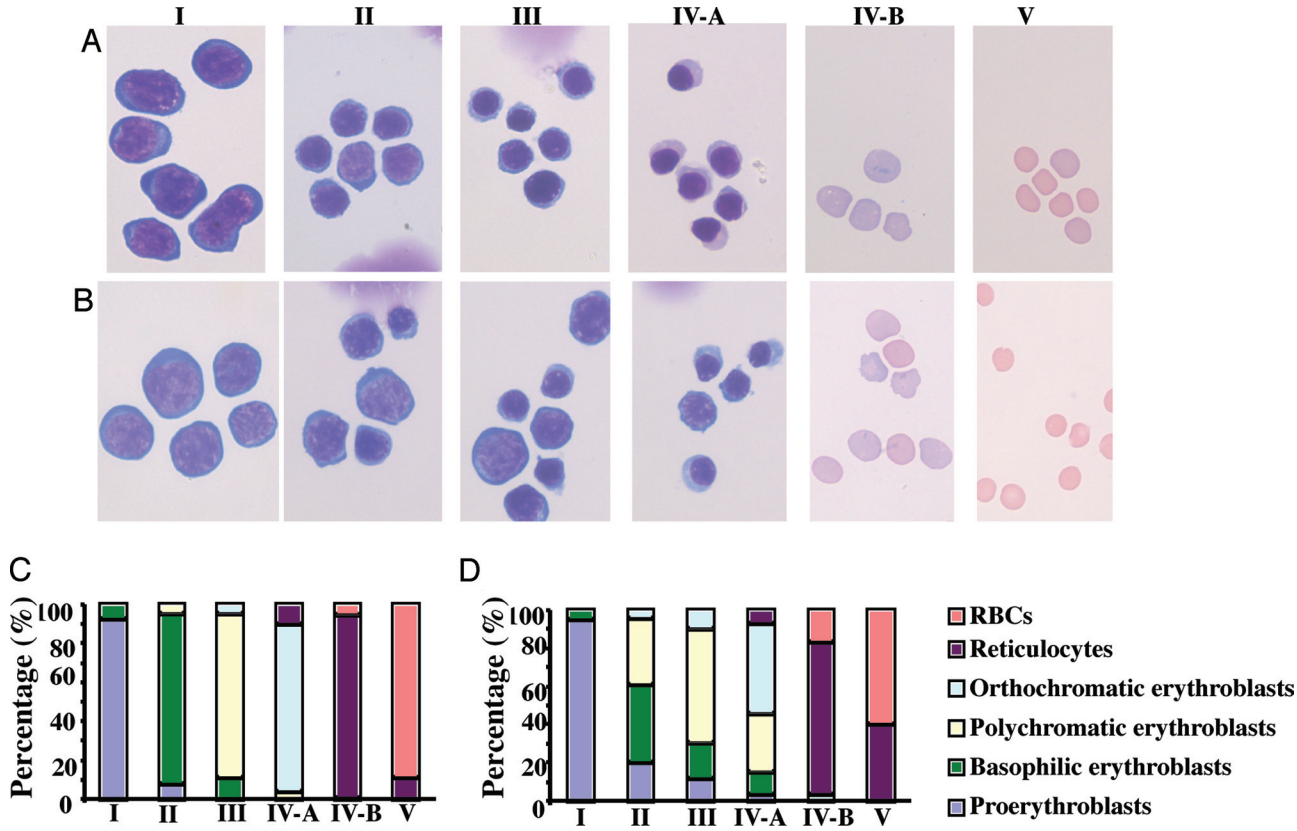

Fig. 4. Isolation of erythroblasts at different stages of maturation by cell sorting using CD44 (or CD71), TER119, and FSC as markers. (*A*) Representative images of erythroblast morphology on stained cytospins from the 5 distinct regions shown in Fig. 3 *A* and *B*. (*B*) Representative images of erythroblast morphology on stained cytospins from the 5 distinct regions shown in Fig. 3 *D* and *E*. (*C*) Quantitation of the purity of erythroblasts at different stages of maturation in various sorted populations using CD44. (*D*) Quantitation of the purity of erythroblasts at different stages of maturation in various sorted populations using CD71.

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. 4*A*, 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. 4*C*, 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. 3*E* are shown in Fig. 4*B*. While, as with CD44, 90% of cells from region I were proerythroblasts, there was large degree of heterogeneity in all other regions (Fig. 4*D*). 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. 5 *A* 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

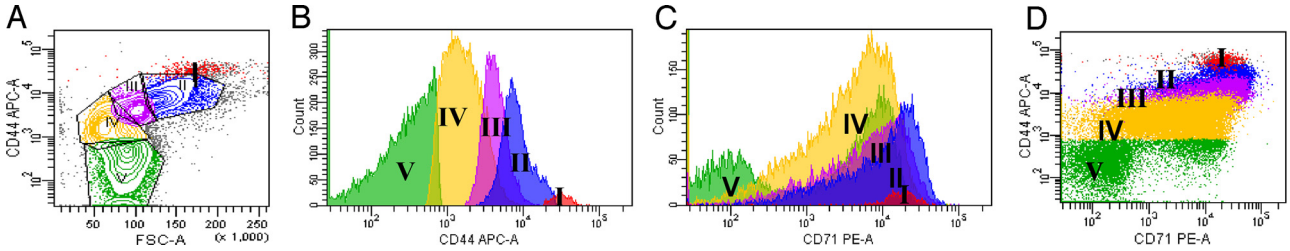

Fig. 5. Comparison of CD44 and CD71 expression in dual-stained bone marrow cells. Primary bone marrow cells were simultaneously stained with Ter-119, CD44, and CD71. (*A*) Plot of CD44 versus FSC of all TER positive cells. Note that 5 distinct clusters can be distinguished. (*B*) The CD44 expression levels in the gated cell population. Note the progressive decrease of CD44 surface expression from region I to region V. (*C*) The CD71 expression levels in the identically gated cell populations. Note the significant overlap of CD71expression. (*D*) Plot of CD44 versus CD71 of all TER positive cells. Note the progressive decrease of CD44 expression but a broad range of CD71 expression from region I to region V.

staining alone. In marked contrast, there was significant overlap in CD71 expression levels in the same 5 gated populations (Fig. 5*C*). Moreover, as shown in Fig. 5*D*, 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 remodeling 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 orthochromatic 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 orthochromatic 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 proerythoblasts and are either expressed at very low levels or not at all in orthochromatic 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 maturation. On the basis of its expression pattern, CD44 was selected because its surface expression decreased by no less than 30-fold in a stepwise manner in passing from the proerythroblast to the orthochromatic erythroblast. The resulting ability to obtain, by cell sorting, highly purified populations of erythroblasts at all stages of maturation from primary bone marrow cells validated the choice of marker. By contrast, CD71, which has been in routine use as a surface marker for this purpose, has proved less effective (35). The reasons are clear, because we have found CD71 expression changes only 4-fold and not in a progressive manner during terminal erythroid differentiation. Our findings with CD71 expression are in agreement with those reported previously by Sawyer and Krantz, using FVA cells (36). This lack of significant decline in CD71 is physiologically relevant because uptake of transferrin-bound iron is needed for heme synthesis at all stages of erythroid differentiation to sustain high levels of hemoglobin synthesis and as such little change in its expression is to be expected.

We suggest that our observations offer the means to gain detailed insights into the genesis of red cell membrane function during erythroblast differentiation and will lead to a deeper understanding of stage-specific defects in erythroid maturation in various inherited and acquired red cell disorders and in bone marrow failure syndromes.

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-Ter119, anti-β1 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-Ter119, APC-conjugated anti-CD44, PE-conjugated anti-CD71, and APC-Cy™ 7-conjugated CD11b are all from BD PharMingen; FITC-conjugated anti-β1-integrin is from BioLegend; and monoclonal anti-Kell is from our laboratory.

Erythroid Cultures. Proerythroblasts were isolated and cultured using methods established by Koury et al. (24, 37). Briefly, 2 weeks after infection with 10^4 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% CO_2 in air at a cell concentration of 1×10^6cells/mL. The culture medium was Iscove's modified Dulbecco's medium (IMDM) (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, 1.0% 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 μg total proteins diluted in 50 μL lysis buffer was digested with or without 5 units N-glycosidase F (NglyF) (Sigma G5166) for 16 h at 37 °C in a water bath. A total of 30 μg of protein was run on a 10% SDS/PAGE gel and transferred to nitrocellulose membrane (Bio-Rad) for 2 h at 60 V. The membrane was blocked for 1 h in PBS containing 5% nonfat dry milk and 0.1% Tween-20 and then probed for 2 h with primary antibody diluted in 5% nonfat milk and 0.1% Tween-20. After several washes, blots were incubated with secondary antibody coupled to HRP (Jackson Labs) diluted in 5% nonfat milk and 0.1% Tween-20, washed, and developed on Kodak BioMax MR film (Sigma), using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Immunofluorescence Microscopy. Bone marrow cells were washed 3 times by centrifugation in PBS/0.5% BSA and allowed to adhere for 1 h to slides coated with Cell-Tak (BD PharMingen). The cells were washed 3 times in PBS, fixed with 4% paraformaldehyde and 0.2% Triton X-100 in PBS at room temperature for 10 min, further permeabilized by 1% Triton X-100 in PBS for 3 min, and then rinsed with PBS. After incubation in 10% donkey serum (Jackson ImmunoResearch) and 10 mg/mL BSA (Sigma) in PBS for 1 h to block nonspecific protein binding, fixed cells were treated with primary antibodies diluted in 10 mg/mL PBS/BSA for 1 h at room temperature. Cells were washed at 5-min intervals 6 times with gentle shaking, incubated for 1 h with secondary antibodies at 1:700 in 10 mg/mL PBS/BSA, washed 3 times in PBS, and then mounted using Vectashield with DAPI (Vector Laboratories). Fluorescence was imaged using a Zeiss Axiovert 135 microscope with a 63×/1.25 oil immersion objective and equipped with a CCD camera.

Flow Cytometric Analysis of Cultured Cells and Primary Bone Marrow Cells. Cultured erythroblasts (1×10^6) were stained with PE-conjugated CD71, APC-conjugated CD44, FITC-conjugated TER119, FITC-conjugated β1 integrin, or unconjugated anti-Kell in PBS/0.5% BSA for 20 min at 4 °C. Then the cells were washed twice in PBS/0.5% BSA. For Kell, the cells were then incubated for 20 min with goat anti-mouse IgG–FITC (Invitrogen) and washed twice in PBS/0.5% BSA and the surface expression of proteins was analyzed. Bone marrow cells were harvested from tibia and femur of 3-month-old mice. For phenotypic analysis by flow cytometry, 2×10^6 cells were resuspended in 80 μL PBS/0.5% BSA. Cells were blocked with rat anti-mouse CD16/CD32 (5 μg/10^6 cells, BD Biosciences no. 553142) for 15 min. Samples were subsequently stained with FITC rat anti-mouse TER-119 (1 μg/10^6 cells, BD Biosciences no. 557915), APC rat anti-mouse CD44 (1 μg/10^6 cells, BD Biosciences no. 559250), and APC-Cy™ 7 rat anti-mouse CD11b (0.3 μg/10^6 cells, BD Biosciences no. 557657) on ice for 20–30 min in the dark. Cells were washed twice with 700 μL PBS/0.5% BSA. Finally, cells were resuspended in 100 μL PBS/0.5% BSA and stained with the viability marker 7-AAD (0.25 μg/10^6 cells, BD Biosciences no. 559925) on ice for 10 min in the dark. Cells were then suspended in 0.4 mL of PBS/0.5%BSA and analyzed within 1 h following staining using BD FACSDiva software on a FACSCantoTM flow cytometer (Becton Dickinson). Unstained cells or cells stained with second antibody only (in the case of Kell staining) were used as a negative control. Mean fluorescent intensity (FLI) was used as a measure of protein surface expression.

Fluorescence-Activated Cell Sorting. To isolate erythroblasts at different stages of maturation by cell sorting, 200×10^6 cells were resuspended in 8 mL PBS/0.5% BSA in a 50-mL tube. Cells were blocked with rat anti-mouse CD16/CD32 (5 μg/10^6 cells, BD Biosciences no. 553142) for 15 min and subsequently stained with FITC rat anti-mouse TER-119 (1 μg/10^6 cells, BD Biosciences no. 557915), APC-Cy™ 7 rat anti-mouse CD11b (0.3 μg/10^6 cells, BD Biosciences no. 557657), and either APC rat anti-mouse CD44 (1 μg/10^6 cells, BD Biosciences no. 559250) or PE rat anti-mouse CD71 (1 μg/10^6 cells, BD Biosciences no. 553267) or both CD44 and CD71 antibodies, and incubated on ice for 20–30 min in the dark. Cells were washed twice with 40 mL PBS/0.5% BSA and resuspended in 10 mL PBS/0.5% BSA and stained with the viability marker 7-AAD (0.25 μg/10^6 cells, BD Biosciences no. 559925) on ice for 10 min in the dark. Sorting was performed on a MOFLO high-speed cell sorter (Beckman-Coulter).

Cytospin Preparation. For determining cell morphology, 100 μL of cell suspension containing 10^5 sorted cells was used to prepare cytospin preparations on coated slides, using the Thermo Scientific Shandon 4 Cytospin. The slides were stained with May–Grunwald (Sigma MG500) solution for 5 min, rinsed in 40 mM Tris buffer (pH 7.2) for 90 sec, and subsequently stained with Giemsa solution (Sigma GS500).

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