**Absence of Erythroblast Macrophage Protein (Emp) Leads to Failure of Erythroblast Nuclear Extrusion**

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Received for publication, April 5, 2006, and in revised form, May 12, 2006 Published, JBC Papers in Press, May 16, 2006, DOI 10.1074/jbc.M603226200

In mammals, the functional unit for definitive erythropoiesis is the erythroblastic island, a multicellular structure composed of a central macrophage surrounded by developing erythroblasts. Erythroblast-macrophage interactions play a central role in the terminal maturation of erythroblasts, including enucleation. One possible mediator of this cell-cell interaction is the protein Emp (erythroblast macrophage protein). We used targeted gene inactivation to define the function of Emp during hematopoiesis. Emp null embryos die perinatally and show profound alterations in the hematopoietic system. A dramatic increase in the number of nucleated, immature erythrocytes is seen in the peripheral blood of Emp null fetuses. In the fetal liver virtually no erythroid islands are observed, and the number of F4/80-positive macrophages is substantially reduced. These present lack cytoplasmic projections and are unable to interact with erythroblasts. Interestingly, wild type macrophages can bind Emp-deficient erythroblasts, but these erythroblasts do not extrude their nuclei, suggesting that Emp impacts enucleation in a cell autonomous fashion. Previous studies have implicated the actin cytoskeleton and its reorganization in both erythroblast enucleation as well as in macrophage development. We demonstrate that Emp associates with F-actin and that this interaction is important in the normal distribution of F-actin in both erythroblasts and macrophages. Thus, Emp appears to be required for erythroblast enucleation and in the development of the mature macrophages. The availability of an Emp null model provides a unique experimental system to study the enucleation process and to evaluate the function of macrophages in definitive erythropoiesis.

In mammals, erythropoiesis occurs in the bone marrow or fetal liver in distinct anatomic units called erythroblastic islands, which consist of a central macrophage surrounded by a ring of developing erythroblasts (1, 2). The association of erythroblasts with macrophages plays a central role in the formation of erythroblastic islands. Scanning electron microscopy of these islands reveals the typical macrophage topography and cytoplasmic extensions. These extensions surround peripheral erythroid cells providing intimate membrane contact between the macrophage and developing erythroblasts (3). In addition to the interactions between macrophages and erythroblasts, the formation and integrity of erythroblastic island structure involves multiple adhesive interactions between adjacent erythroblasts and between cells of the island and the extracellular matrix (4–14). Cell-cell interactions must determine specificity because only erythroblasts and a few lymphocytes (reported recently in Ref. 15) are found associated with macrophages in erythroblastic islands.

The molecular interaction(s) that support formation of the erythroblastic islands have only been partially defined. Previously, we identified Emp (erythroblast macrophage protein) in both erythroblasts and macrophages in in vitro cultures of human peripheral blood erythroid progenitors (16). These cultures support the formation of erythroblastic islands that are similar morphologically to those reported in vivo in the bone marrow. In the presence of anti-Emp antibody in cultures, erythroblastic islands were not formed, and terminal maturation of erythroblasts was impaired. Based on these in vitro studies, we proposed that Emp mediates the association of erythroblasts with macrophages and that this attachment promotes terminal maturation of erythroblast cells. In addition to the Emp-induced association between erythroblasts and macrophages (16, 17), another molecular interaction reported between these two cell types is the very late antigen-4 (or \( \alpha_4\beta_1 \) integrin)-VCAM-1 interaction (18). The interaction between erythroblast very late antigen-4 and macrophage VCAM-1 plays a critical role in maintaining island integrity because antibodies to either molecule disrupt island structure (18). Treatment of mice with very late antigen-4 antibodies in utero specifically induces anemia (19). Two other macrophage receptors, which could be involved in adhesion to erythroid cells, include erythroblast receptor, EbR, (20) and a lectin-like sheep erythrocyte receptor, SER (21–23). Neither of these receptors nor their corresponding ligands have been fully characterized.

The central macrophages of the erythroblastic islands are thought to induce several key biologic processes in the erythroid precursors including proliferation and differentiation, prevention of apoptosis, and promotion of erythroblast enucle-
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The role of macrophages in enucleation has been suggested for a long time (24, 25), but only recently gene targeting studies have shown conclusively that DNase II in macrophages is responsible for destroying the nuclear DNA expelled from erythroblasts (26, 27). Also, MEK kinase I activity appears to be required for macrophage-mediated nuclear DNA destruction (28). The importance of central macrophages in inducing attachment to immature erythroblasts was illustrated by recent studies of the mouse retinoblastoma (Rb) gene, which demonstrated that Rb-deficient embryos carry profound abnormalities in fetal liver macrophages (FLMs) that prevent physical interactions with erythroblasts (29).

In the present study, we utilized gene targeting to evaluate the *in vivo* function of Emp. Emp null mice are nonviable and exhibit differentiation defects in both erythroid and macrophage lineages. Our studies reveal that Emp is involved in erythroblast enucleation and macrophage development and may exert its effects through interaction with the actin cytoskeleton. The availability of the Emp null model provides a novel experimental system to study the erythroid cell enucleation process.

**EXPERIMENTAL PROCEDURES**

An Insertional Mutation in Emp—Mouse embryonic stem (ES) cells containing a gene trap insertion in Emp (cell line XGT02) were obtained from BayGenomics (San Francisco) (30). Insertion of the gene trap vector (containing coding sequence for β-geo, a β-galactosidase-neomycin hybrid) into Emp was verified by direct sequencing of cDNA obtained by 5’ rapid amplification of cDNA ends (31). ES cells carrying the Emp mutation were injected into mouse blastocysts, and chimeric offspring were crossed with C57BL/6J mice to create heterozygous (Emp+/−) mice. Offspring were genotyped by PCR of genomic DNA with primers specific for the wild type Emp allele (Emp forward, EMP-F, 5’-TTGCTGCTGCTGAGAAT-3’; Emp reverse, R31, 5’-TGATGATGCCCTTTGATGCTCTCTGAG-3’) and for the mutant allele carrying the gene trap insertion (Emp forward, as above; vector reverse, 5’-AGTTAGGGGCTTGGGTAGAG-3’), and confirmed by Southern hybridization using a 632-bp DNA probe from the wild type gene.

Wild type and heterozygous embryos were indistinguishable morphologically as well as in all the parameters tested. Therefore, throughout this study, only wild type and homozygous mutant data are shown.

Histological Analysis and Immunohistochemistry—Freshly collected embryos from timed pregnancies were analyzed. For blood smears, peripheral blood cells collected from fresh embryos were cyto spun and stained with Wright-Giemsa. Freshly collected livers from timed pregnancies were either mixed to make cyto spin preparations or were fixed in 10% neutral formalin, embedded in paraffin, and cut at 5-μm serial sections. Both the cytopsins and sections were stained with Wright-Giemsa or processed for immunohistochemistry using biotin-labeled F4/80 antibodies (Caltag Laboratories, Burlingame, CA) or fluorescein isothiocyanate-conjugated ER-MP12 antibodies (BD Pharmingen). A Vectastain kit (Vector Laboratories, Burlingame, CA) was used to detect biotin-labeled primary antibodies.

Isolation of Native Erythroblastic Islands and Reconstitution of Heterologous Erythroblastic Islands—Clusters composed of FLMs and erythroblasts were isolated essentially as described previously (29). Briefly, livers from individual E15.5 embryos were placed in prewarmed 0.05% collagenase (Sigma) and 0.02% DNase (Sigma) in RPMI and digested for 1 h at 37 °C with gentle rotation. After tissue dissociation, the cells were washed three times, suspended in 100 μl of complete medium (RPMI containing 10% fetal bovine serum), and allowed to attach to glass coverslips for 20 min at 37 °C in 5% CO2. Complete medium was then added to cover the cells, and incubation was continued for an additional 4 h. Thereafter, the coverslips were gently dipped in RPMI to remove nonadherent cells from adherent macrophages with attached erythroid cells (“native” islands). To strip erythroblasts from macrophages, the cells were incubated in phosphate-buffered saline lacking calcium and magnesium for 10 min and washed five times in phosphate-buffered saline to remove erythroblasts. The resulting “stripped” macrophages were allowed to respread in complete medium for 2 h before the addition of fresh erythroblasts to obtain “reconstituted” islands. Collagenase-digested fetal livers from wild type or mutant E14.5 embryos were used as the source of erythroblasts. The cells were plated in 100-mm dishes for 4 h at 37 °C to remove the attached macrophages. Nonadherent erythroid cells were recovered by flushing and washed in phosphate-buffered saline. 106 cells in 50 μl of medium were incubated for 20 min with stripped macrophages derived from individual embryos. Finally, the reconstituted islands were dipped in RPMI to remove the unbound cells before being processed for double F4/80-TER119 (Caltag Laboratories) immunofluorescence using standard procedures. The number of erythroblasts attached to macrophages was determined by counting the total number of cells present in each island visualized using a Leica Confocal fluorescence microscope (Tufts University, Center for Neuroscience Research).

To analyze erythroid differentiation and enucleation after association with FLMs, wild type FLMs were stripped and reconstituted with 106+ or −/− erythroblasts, and the reconstituted, heterologous erythroblastic islands were cultured on glass coverslips for 12 h in medium containing 30% fetal bovine serum, 1 unit/ml recombinant erythropoietin (Epo), and 1000 units/ml human recombinant colony-stimulating factor 1 (CSF-1). The cells were fixed and stained for F4/80 and TER119. The presence or absence of nuclei was established by staining with Hoechst (Bisbenzimide Trihydrochloride, H 33258, Calbiochem) for 1 min at room temperature by using a Leica Confocal fluorescence microscope (Tufts University, Center for Neuroscience Research).

Cell Culture—Primary mouse embryonic fibroblasts were prepared from individual E14.5 embryos using standard protocols (32). The cells were maintained at 37 °C in 5% CO2 with Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum, penicillin/streptomycin, and 2 mM glutamine.

Primary cultures of fetal liver-derived macrophage progenitors were generated from E15.5 wild type and Emp null embryos and cultured in RPMI medium supplemented with 10% fetal calf serum plus 5% Pokeweed media as a source of CSF-1 (StemCell Technologies), 2 mM glutamine, and 50 units/ml penicillin-streptomycin. After 7 days of culture, the
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Emp Mutation Is Lethal at Birth—To produce Emp mutant mice, we used an ES cell line carrying an insertional mutation in intron 1 of the Emp gene (BayGenomics cell line XG702) (Fig. 1A). Correct targeting of the Emp allele in ES cells was confirmed by Southern blot hybridization using a probe from the wild type gene (Fig. 1B). Because the position of the probe overlaps the insertion site, it is expected to hybridize with new BamHI fragments at the beginning and end of the insert. As shown in Fig. 1B, wild type ES cells (+/+ ) show the expected 12.6-kb BamHI fragment, whereas the targeted ES cells (+/−) yield three fragments of 12.6, 7.3, and 6.0 kb, respectively. The 7.3- and 6.0-kb fragments originate from internal BamHI sites within the β-geo gene. One of the positive ES clones was injected into mouse blastocysts to generate chimeric mice, which were mated to generate heterozygous mice. Heterozygotes were intercrossed, and the offspring were genotyped by PCR of genomic DNA with primers specific for the wild type Emp allele, EMP-F, and R31 and for the mutant allele (EMP-F/Geo-R), D, Western blot analysis of mouse embryonic fibroblasts using anti-Emp antibody. Two samples each from +/+ , +/− , and −/− embryos are shown in B, C, and D.

TABLE 1
Genotypes of viable progeny derived from heterozygous parents

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<tr>
<th>Stages</th>
<th>Total</th>
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<th>−/−</th>
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</tr>
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<td>4</td>
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</tr>
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<td>4</td>
<td>3</td>
</tr>
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<td>86</td>
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RESULTS

Emp Null Embryos Exhibit Defective Erythropoiesis—In the mouse embryo, primitive erythropoiesis is initiated in the yolk sac at E7.5. At E9.5, erythropoiesis is initiated in the fetal liver (33, 34), and yolk sac erythropoiesis gradually declines; by...
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Figure 2. A, comparative morphology of wild type (+/+ ) and Emp null (−/−) embryos at E15.5 dissected free of the yolk sacs. B, peripheral blood cells collected from E15.5 +/+ and −/− embryos were cytospun and stained with Wright-Giemsa. C and D, livers isolated from +/+ and −/− E15.5 embryos were either minced to make cytospin preparations or fixed in 10% neutral formalin, embedded in paraffin, and cut as 5-μm serial sections. Both the cytospins ( C) and sections ( D) were stained with Wright-Giemsa. Note the large number of more mature erythroblasts and enucleated erythrocytes in wild type cytospins and sections compared with the null fetal livers.

Table 2

<table>
<thead>
<tr>
<th>Stages</th>
<th>Wild type*</th>
<th>Emp (−/−)</th>
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<tr>
<td></td>
<td>Nucleated</td>
<td>Enucleated</td>
</tr>
<tr>
<td>E15.5</td>
<td>108 ± 15</td>
<td>998 ± 20</td>
</tr>
<tr>
<td>E16.5</td>
<td>55 ± 7</td>
<td>793 ± 14</td>
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* Each value is mean number of cells/5 μl of peripheral blood ± S.D. Two or three independent embryos were examined at each stage. Peripheral blood cells collected from each embryo were counted by hemacytometer, and 50,000 cells were used to make each cytospin. The number of nucleated and enucleated erythrocytes were assessed from Giemsa-stained cytospins (at least 10 different fields were counted from each cytospin). Wild type (+/+ ) and heterozygous (+/−) embryos gave indistinguishable results and were therefore combined.

∼E14.5, 90% of the erythrocytes in circulation are of fetal liver origin (35, 36). The development of definitive erythropoiesis in the fetal liver proceeds through two distinct phases. In the first (E11–13), erythroid maturation proceeds in the absence of hematopoiesis, we examined embryos at E12.5–16.5. Homozygous Emp (−/−) embryos appeared normal until E12.5. However, at E14.5 and beyond, homozygous Emp (−/−) embryos became small and pale as compared with their littermates (Fig. 2A).

At E14.5, wild type embryos contained only 25% nucleated erythrocytes as compared with 65–70% in the mutant embryos. As development proceeded in the wild type embryos, this percentage decreased to 8% at E15.5 and 5% at E16.5. In contrast, the mutant embryos showed virtually no change in the proportion of nucleated erythrocytes (data not shown). To determine whether the increased proportion of nucleated cells in mutant embryos is due to a continuous production or a failure of maturation, we determined the absolute number of nucleated and enucleated cells in the fetal blood. As shown in Table 2, mutant embryos contained significantly increased number of circulating nucleated erythrocytes as compared with the wild type embryos, suggesting a defect in terminal maturation and enucleation of nucleated precursor cells in the absence of Emp.

Because fetal liver is the primary site of definitive erythropoiesis, we examined Giemsa-stained fetal liver cytospins from E15.5 +/+ and −/− embryos. Wild type fetal livers showed a large number of nucleated erythroblast precursors as well as enucleated erythrocytes. In contrast, the mutant livers contained predominantly large immature erythroblasts with very few enucleated erythrocytes (Fig. 2C). Although nucleated erythrocytes were found in both wild type and mutant embryos, their relative proportions were very different; less mature forms (proerythroblasts) predominated in −/− embryos, whereas more mature forms (polychromatophilic/orthochromatic and enucleated erythrocytes) were most common in +/+ embryos. Similarly, upon examination of the paraffin-embedded liver sections, we found few mature erythrocytes in the sinusoids of homozygotes, in contrast to those of either wild type or heterozygotes, where abundant enucleated red blood cells were observed (Fig. 2D).

Emp Null Fetal Liver Macrophages Show Immature Morphology and Are Unable to Form Erythroblastic Islands—FLMs are indispensable for definitive erythropoiesis (26). Hence we examined the effect of Emp deficiency on FLM development. The expression of CSF-1 receptor was determined by Western blot analysis (Fig. 3A). Densitometric quantitation showed that the mutant livers contained ∼40–50% of the wild type CSF-1 receptor in three separate experiments, suggesting a reduction in the number of macrophages in mutant livers. Immunohistochemical staining of fetal liver cytospins (Fig. 3B) or paraffin-embedded sections (Fig. 3C) with F4/80 antibody, a marker of mature macrophages (37, 38), revealed numerous F4/80-positive cells throughout the liver of normal embryos. In mutant livers, however, the number of F4/80-positive macrophages was reduced to 25–30% of the normal values. Furthermore, morphologically, Emp null FLMs were generally round and small and appeared to lack the cytoplasmic extensions typically seen in wild type FLMs, suggesting an immature morphology. However, ER-MP12, an antigen expressed by immature macrophage precursors, was similar in wild type and Emp (−/−) fetal livers.
Absence of Emp causes a defect in the formation of erythroblastic islands in the fetal liver. Native erythroblastic islands isolated from wild type and Emp (−/−) fetal livers were double-immunostained with F4/80 and phycoerythrin-conjugated TER 119 antibodies (red). F4/80 antigen was detected with Alexa Fluor 488-conjugated (green) goat anti-rabbit IgG. The scale bar is 20.0 μm.

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The results presented above show that Emp null erythroblasts retain the ability to adhere to normal macrophages. To determine whether this physical contact enables erythroblasts to undergo terminal maturation, reconstituted heterologous erythroblastic islands consisting of normal FLMs and wild type or Emp null erythroblasts were cultured in the presence of erythropoietin and CSF-1. These culture conditions have been previously shown to allow survival and FLM-mediated enucleation of bound wild type erythroblasts (29). Indeed, under these conditions cultures containing wild type erythroblasts/wild type FLM, n = 4) but were unable to attach to the Emp null macrophages (0.5 ± 0.3 erythroblast/null FLM, n = 4). The values reported above, mean ± S.D., were obtained by counting at least 20 islands each in four different experiments. These results suggest that small and immature Emp null macrophages fail to establish significant contacts with erythroblasts.

Reconstitution experiments were performed to determine whether impaired erythroblastic island formation in the absence of Emp is due to an abnormality in macrophages, erythroblasts, or both. Native erythroblastic islands obtained from wild type and Emp null embryos (as described above) were incubated in a solution lacking calcium and magnesium to remove erythroblasts leaving intact the adherent macrophages ("stripped" macrophages). These stripped macrophages were then challenged with heterologous freshly isolated erythroblasts from E14.5 wild type or Emp null fetal livers to obtain "reconstituted islands." As shown in Fig. 5A, erythroblasts from either wild type or Emp null backgrounds attached to wild type macrophages with similar efficiency (8 ± 1.5 erythroblasts/wild type FLM, n = 4) but were unable to attach to the Emp null macrophages (0.5 ± 0.3 erythroblast/null FLM, n = 4). The results presented above show that Emp null erythroblasts retain the ability to adhere to normal macrophages. To determine whether this physical contact enables erythroblasts to undergo terminal maturation, reconstituted heterologous erythroblastic islands consisting of normal FLMs and wild type or Emp null erythroblasts were cultured in the presence of erythropoietin and CSF-1. These culture conditions have been previously shown to allow survival and FLM-mediated enucleation of bound wild type erythroblasts (29). Indeed, under these conditions cultures containing wild type erythroblasts/wild type macrophages showed the presence of several enucleated cells as indicated by TER 119-positive and Hoechst negative labeling (Fig. 5B, arrows). In contrast, a marked reduction in the number of enucleated cells was seen in cultures containing Emp null erythroblasts bound to wild type macrophages under identical conditions (Fig. 5B). Quantitation of the number of enucleated cells showed that ~40% (35 ± 3, n = 3) wild type erythroblasts were enucleated compared with ~10% (9 ± 2, n = 3) Emp null erythroblasts. Thus, physical interaction with wild type macrophages does not rescue the enucleation defect of Emp null erythroblasts.
Actin Distribution Is Altered in Emp-deficient Erythroblasts and Macrophages—Based on previous studies, which showed that F-actin is concentrated at the base of the extruding nuclei in enucleating erythroblasts (39), we hypothesized that Emp might, in association with F-actin, aid in the expulsion of the nucleus. As an initial attempt to test this hypothesis, we determined the distribution and localization pattern of F-actin in wild type and Emp null erythroblasts. Primary erythroblasts were isolated from E15.5 wild type and mutant embryos as described under “Experimental Procedures.” Immunofluorescence labeling with Alexa Fluor 647-labeled phalloidin showed that actin localized to the plasma membrane and to numerous actin bundles throughout the cytoplasm in the wild type erythroblasts. In striking contrast, actin bundles were not apparent in Emp null erythroblasts. Instead, actin staining was largely confined to the plasma membrane (Fig. 6A). To rule out a possibility that the strikingly different actin staining pattern is due to maturational differences between the normal and mutant erythroid cells, we examined a large number of cells at all stages of maturation; cells of varying maturity (judged by their size) are shown in Fig. 6A. Our results indicate that the actin staining pattern is clearly different in normal and mutant erythroid cells regardless of their stage of maturation. Double-labeling of wild type erythroblasts showed that Emp colocalized with both the cortical and cytoplasmic F-actin in vivo. Furthermore, in enucleating wild type erythroblasts, Emp staining colocalized with F-actin aggregates seen in the region between the extruding nucleus and incipient reticulocyte (Fig. 6B). Of note, a significant amount of Emp was seen segregating with the nucleus, whereas actin remained with the reticulocyte. Segregation of Emp with the extruding nucleus is consistent with a recent report by Lee et al. (40). To determine whether endogenous Emp and actin interact in erythroblasts, primary erythroblasts isolated from wild type E15.5 embryos were used in coimmunoprecipitation assays with anti-Emp or anti-actin antibodies. Normal rabbit IgG served as a negative control. Immunoblot staining using anti-Emp or anti-actin revealed coprecipitated polypeptides. As shown in Fig. 6C, anti-Emp antibody efficiently immunoprecipitated actin, and anti-actin antibody immunoprecipitated Emp, whereas control IgG did not precipitate any immunoreactive band. These results suggest that endogenous Emp associates with F-actin and perhaps regulates actin organization in erythroblasts.

Next, because the actin cytoskeleton and its reorganization is an important aspect of macrophage development (41), we hypothesized that loss of Emp may perturb the actin cytoskeleton, which in turn negatively regulates formation of macrophage filopodia. To test this hypothesis, we cultured primary macrophages from the livers of wild type and mutant embryos. Coimmunoprecipitation/Western blot experiments confirmed the existence of Emp and actin in a complex in vivo (data not shown). To determine the organization of the actin cytoskeleton, we labeled macrophages with rhodamine-conjugated phalloidin (Fig. 6D). Wild type macrophages, which were largely spread out and elongated, showed many long, thick actin filaments. In contrast, mutant macrophages were small and imma-

**FIGURE 5. Emp-deficient erythroblasts but not Emp-deficient FLMs reconstitute erythroblastic islands. A,** reconstituted, homologous (wild type (WT) macrophages/wild type erythroblasts or mutant macrophages/mutant erythroblasts) and heterologous (wild type macrophages/mutant erythroblasts or mutant macrophages/wild type erythroblasts) erythroblastic islands were immunostained with F4/80 (green) and phycoerythrin-TER119 (red) antibodies as described in the legend to Fig. 4. The scale bar is 20.0 μm. B, reconstituted erythroblastic islands of indicated genotypes were cultured for 12 h and stained for F4/80 (green), TER119 (red), and Hoechst (blue). The arrows indicate enucleated cells. Mφ, macrophage.
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Our studies reveal that Emp null erythroblasts attach to wild type macrophages in reconstituted heterologous erythroblastic islands cultured under conditions conducive to erythroid maturation and enucleation. However, we found that enucleation was defective despite their ability to attach and produce erythroblastic islands, consistent with the notion that enucleation of erythroid precursors occurs cell autonomously (26, 44). Enucleation, the terminal step in the maturation of mammalian erythroid cells, has been studied widely, but the precise mechanism still remains elusive. A number of studies have shown a significant increase in the number of nucleated erythroid cells in E14.5–E16.5 mutant embryos. By E16.5, in the wild type embryos, greater than 95% of the circulating cells are definitive erythrocytes (42, 43). Based on morphology, the circulating cells in the mutant embryos (E15.5/16.5) also appear to be of definitive origin, suggesting an ineffective maturation of nucleated precursor cells in the absence of Emp. Furthermore, macrophage development is significantly altered. Emp null macrophages are small and round and lack cytoplasmic projections typically present in wild type macrophages, suggesting immature morphology.

The generation of an Emp-deficient in vivo model provided a unique opportunity to test our hypothesis that Emp is involved in the formation of erythroblastic islands. Using an in vitro coculture technique, we show that the number of erythroblastic islands in homozygous null fetal livers is dramatically reduced compared with wild type fetal livers. Furthermore, reconstitution of heterologous erythroblastic islands showed that although the wild type macrophages attach to both wild type and Emp null erythroblasts, mutant macrophages are unable to bind either type of erythroblasts. These results suggest that the formation of erythroblastic islands requires the presence of Emp in macrophages but not in erythroblasts. This observation is surprising in light of our previous notion that Emp might be involved in homophilic interactions between homotypic and heterotypic cells (16). Because Emp has been detected on the surface of normal erythroblasts and macrophages (data not shown), it is possible that Emp might, in fact, be involved in homophilic binding interaction, but in the absence of erythroid Emp, the expression of one or more of the other known proteins involved in erythroblastic island formation is up-regulated. Another possibility is that the erythroid Emp has a function other than cell attachment (see below). These possibilities will be explored in future studies.

FIGURE 6. Actin distribution is altered in Emp-deficient erythroblasts and macrophages. A, wild type and mutant erythroblasts were immunostained with Alexa 647-conjugated phalloidin (red) and anti-Emp antibody that was counter stained with Alexa 488-conjugated (green) goat anti-rabbit IgG. B, wild type erythroblast undergoing enucleation is shown after staining with Phalloidin, Emp, and Hoechst. DIC, differential interference contrast. C, immunoprecipitates from wild type primary erythroblasts with anti-actin or anti-Emp antibodies or with normal rabbit immunoglobulin (NRIg) were analyzed by Western blot for Emp (upper panel) and actin (lower panel). The same antibodies were used to detect proteins from total cellular lysates. The migration of specific Emp and actin bands is indicated by arrows. The scale bar is 200 μm. D, cultured primary mouse macrophages (wild type and mutant) were fixed in paraformaldehyde and stained with rhodamine-conjugated phalloidin. The nuclei were stained with Hoechst dye (Bisbenzimide Trihydrochloride, H 33258; Calbiochem). All of the images were magnified identically after being photographed through a 40× objective.
proposed that enucleation may be a process similar to cytokinesis (45–49) and have suggested that F-actin is present in the form of a ring in the constriction, similar to the cleavage furrow of mitotic cells (50, 51). Using splenic erythroblasts from mice infected with the anemia-inducing strain of Friend virus, Koury et al. (39) showed that F-actin is concentrated between the extruding nucleus and incipient reticulocyte in enucleating erythroblasts. Furthermore, these authors showed that the disruption of F-actin bundles with cytochalasin D treatment inhibited enucleation. Interestingly, our recent studies using transfected mammalian cells showed that Emp is localized in the contractile ring during cytokinesis and that it exists in a complex with actin in vivo (52). Therefore, we investigated whether Emp interacts with actin in erythroid cells and thereby participates in the enucleation process. Immunofluorescent labeling of wild type and mutant erythroblasts with phalloidin detected a striking difference in the localization pattern of F-actin: examination of more than 500 cells each for normal and mutant samples (each sample containing cells at all stages of maturation) showed that in the wild type erythroblasts, actin staining, which completely colocalized with Emp staining, was present throughout the cytoplasm as well as on the plasma membrane. In the mutant erythroblasts, however, actin staining was detected largely near the plasma membrane. Almost no cytoplasmic actin was detected in mutant cells, suggesting that in the absence of Emp, the actin distribution is impaired in erythroblasts.

The colocalization of Emp with F-actin in erythroblasts may suggest a role of Emp in enucleation. Consistent with this notion is the observation that Emp colocalizes with concentrated F-actin bundles that are detected in wild type erythroblasts undergoing enucleation. Coimmunoprecipitation experiments further substantiated the apparent close association of endogenous Emp and actin. As shown in Fig. 6B, although a large portion of Emp segregates with the extruding nucleus, significant amount remains with the incipient reticulocyte where it colocalizes with phalloidin staining. Thus, in addition to being involved in nuclear expulsion, Emp-actin association may function to regulate the actin cytoskeleton in reticulocytes. Our studies, however, do not address whether Emp binds directly to the cytoplasmic actin and whether it regulates actin bundling. Furthermore, it is not known how Emp, a transmembrane protein, segregates with the nucleus. In light of a recent study suggesting the role of cytoskeletal connectivity in protein sorting during erythroblast enucleation (40), future studies will address whether the degree of the linkage of Emp to the underlying skeleton changes during erythroid differentiation, because this linkage might control its sorting pattern. The potential role of Emp in regulating actin cytoskeleton has implications in macrophage development as well. Emp-deficient macrophages, which display condensed, less organized actin filaments, are unable to efficiently develop long cytoplasmic extensions. Detailed characterization of Emp null macrophages in terms of their capacity to migrate, invade, and phagocytose, all of which are actin-based cellular events, is the focus of ongoing research. The results presented here show that the phenotype of Emp null FLMs is similar to that of Rb null FLMs. Both show immature morphology and are unable to produce erythroblastic islands. However, there are contrasting differences between the erythroblasts from these two backgrounds: Rb null erythroblasts undergo enucleation efficiently after attachment with wild type macrophages, but the Emp null erythroblasts do not.

Together, our studies have provided evidence that Emp is required for the formation of erythroblastic islands as well as for the normal differentiation of both erythroid and macrophage cell lineages in vivo. Although the precise mechanisms remain to be elucidated, our studies implicate the involvement of Emp in the expulsion of the erythroid nucleus and in the development of macrophage filopodia, both perhaps via actin association.

Acknowledgments—We thank Dr. Kenneth M. Rosen for invaluable scientific discussions throughout this study and Donna-Marie Mironchuk for the art work.

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Absence of Erythroblast Macrophage Protein (Emp) Leads to Failure of Erythroblast Nuclear Extrusion

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doi: 10.1074/jbc.M603226200 originally published online May 16, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M603226200

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