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CHANGING PATTERN OF THE SUBCELLULAR DISTRIBUTION OF ERYTHROBLAST MACROPHAGE PROTEIN (EMP) DURING MACROPHAGE DIFFERENTIATION*

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Abstract

Erythroblast macrophage protein (Emp), mediates the attachment of erythroid cells to macrophages, and is required for normal differentiation of both cell lineages. In erythroid cells Emp is believed to be involved in nuclear extrusion however, its role in macrophage differentiation is unknown. Information on the changes in the expression level and subcellular distribution of Emp in differentiating macrophages is essential for understanding the function of Emp. Macrophages of varying maturity were examined by immunofluorescence microscopy and biochemical methods. Our data shows that Emp is expressed in all stages of maturation, but its localization pattern changes dramatically during maturation: in immature macrophages, a substantial fraction of Emp is associated with the nuclear matrix, whereas in more mature cells, Emp is expressed largely at cell surface. Pulse-chase experiments show that nascent Emp migrates intracellularly from the cytoplasm to the plasma membrane more efficiently in mature macrophages than in immature cells. Incubation of erythroid cells with macrophages in culture show that erythroid cells attach to mature macrophages but not to immature macrophage precursors. Together, our data shows that the temporal and spatial expression of Emp correlates with its role in erythroblastic island formation, and suggests that Emp may be involved in multiple cellular functions.

Keywords

Erythroblast-macrophage protein; Erythroblast; Macrophage; Erythroblastic islands

INTRODUCTION

Erythroblast macrophage protein (Emp) mediates the attachment of erythroblasts to macrophages within erythroblastic islands [1,2] that are formed during mammalian erythropoiesis in the fetal liver and the bone marrow. Erythroblastic islands are consisted of a central macrophage surrounded by erythroblasts of varying maturity [3,4,5]. Extensive cell:cell interactions, both erythroblast-macrophage and erythroblast:erythroblast occur within these islands. Although a variety of cell adhesion molecules are expressed in both erythroblasts and macrophages, only a few that support formation of the erythroblastic islands have been defined.

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First identified adhesive interaction within islands involves vascular cell adhesion molecule-1 (VCAM-1) on macrophages and $\alpha_4\beta_1$ integrin on erythroblasts [6]. A second adhesive interaction characterized entails erythroid intercellular adhesion molecule-4 (ICAM-4) binding to macrophage av integrin [7]. Recent gene targeting studies have clearly documented the role of ICAM-4 in erythroblastic island formation *in vivo* [8]. The final adhesive interaction involves Emp that we earlier identified as a mediator of this cell-cell interaction, and showed that this interaction promotes differentiation and enucleation of erythroid cells [1,2].

To explore the *in vivo* function of Emp, we recently generated an animal model lacking a functional endogenous Emp using homologous recombination in ES cells. We showed that Emp null fetuses die perinatally, have significantly reduced numbers of erythroblastic islands, and exhibit defects of terminal erythroid maturation [9]. Emp null mutation impacts erythroid cell enucleation in a cell autonomous fashion. Furthermore, in the absence of Emp, terminal differentiation of fetal liver macrophages is severely compromised: Emp null macrophages are small, immature, and lack cytoplasmic projections, suggesting that Emp plays an important role in macrophage differentiation *in vivo*. This latter finding is a somewhat unexpected but very exciting outcome of the Emp gene disruption and has thus led to considerable interest in defining the role of Emp in monocyte/macrophage differentiation and maturation.

The distribution pattern of a protein within the cell can provide important clues to its function. In erythroid cells, Emp is localized at the plasma membrane as well as intracellular where it co-localizes with actin bundles [9]. In enucleating cells, Emp staining co-localizes with F-actin aggregates seen in the region between the extruding nucleus and incipient reticulocyte, and eventually segregates with the exiting nucleus [9]. Segregation of Emp with the extruding nucleus has also been shown previously by Lee *et al* [10]. Our unpublished studies have shown that the expression of Emp in erythroid cells declines during terminal maturation suggesting that the expression of Emp is developmentally regulated. Emp is expressed in erythroid precursors but not mature erythrocytes, consistent with the fact that mature red blood cells do not adhere. However, no information is available on the expression of Emp in macrophages. In hematopoietic tissues, central macrophages of erythroblastic islands are resident macrophages that have differentiated from monocyte precursors. Hence, bone marrow and the fetal liver contain monocytes/macrophages at various stages of differentiation and phenotype. In erythroblastic islands, erythroid cells of varying maturity are found but no information is available on the maturation stage of the central macrophages.

Here, we have characterized the subcellular distribution of Emp in differentiating primary mouse fetal liver macrophages using immunofluorescence microscopy and biochemical methods. Collectively, our data shows that the localization pattern of Emp changes dramatically during maturation and that it correlates with its role in erythroblastic island formation. Furthermore, localization of Emp in the nucleus as well as the plasma membrane suggests that its functions may not be limited to cell attachment.

MATERIALS AND METHODS

Antibodies

Anti-EEA1 (early endosome antigen 1) antibodies were from Transduction Laboratories (San Diego, CA), anti-LAMP1 (lysosomal associated membrane protein 1) and anti-SC-35 antibodies were from BD Biosciences (San Jose, CA). Secondary antibodies conjugated to Alexa 488 or 594 were from Molecular Probes (Eugene, OR).

Cell culture

Primary cultures of fetal liver-derived macrophage progenitors were generated from E15.5 wild-type embryos of C57BL/6J breeding pairs. Fetal livers were collected in IMDM containing 10% FCS, minced, flushed through a 70 μm sieve, and resuspended in RPMIglutamine medium containing 10% heat-inactivated FCS, 5% Pokeweed medium as a source of colony stimulating factor-1, CSF-1 (StemCell technologies), 2 mM glutamine, and 50 U/ml penstrep. Cells were seeded in chambers (Lab-Tek 8 well, ~500 μl/well of 1 X 10^6 cells/ml suspension, or ~4 X 10^6/plate in 35-mm petri dishes), and cultured at 37°C, 5% CO_2. After 4 days, adherent cells were washed twice with Hank's Balanced Salt Solution, and medium was renewed each day.

Cellular fractionation

Mouse fetal liver macrophages grown in 10cm dishes for 4 days or 8 days were washed twice with PBS (10 mM sodium phosphate, pH 7.4, and 150 mM NaCl), then scraped in this buffer. The cells were pelleted from this suspension by centrifugation at 500g for 2 min, then lysed in hypotonic buffer (20 mM Tris-HCl, pH 7.4, protease inhibitors cocktail) (2 ml/dish) and passed through a 21-gauge needle for 20 times. The lysate was centrifuged at 200g for 10 min to pellet nuclei. The supernatant was centrifuged at 125,000g at 4°C for 30 min to isolate total membranes. An aliquot from each fraction was mixed with SDS sample buffer, heated at 100° C for 5 min, and analyzed by SDS/PAGE and Western blotting.

Preparation of Nuclear Matrix Proteins

Isolation of nuclear matrix was performed essentially as described previously [11]. Macrophages were washed 3 times in PBS, swollen in RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) for 30 min on ice, and sheared in RSB containing 0.3% NP-40 by passing through a 21-gauge needle for about 20 times until nuclei were free of cytoplasm by microscopic examination. Nuclei were pelleted through a 0.25 M sucrose in RSB cushion for 10 min at 1,500g at 4°C. Typically, >95% nuclei were recovered. Sequential extraction procedures were performed using buffers containing protease inhibitor cocktail (Roche). Briefly, nuclei were successively extracted in CSK buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100), extraction buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 250 mM ammonium sulphate, 3 mM MgCl2, 1 mM EGTA, and 0.5% Triton X-100), digestion buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100) with DNase I and RNase A, and re-extracted with 250 mM Ammonium Sulphate. For fractionation of nuclear matrix for in situ microscopy, subconfluent cells growing in chamber slides (Lab-Tek) were rinsed in PBS and sequentially processed as described above.

Immunofluorescence microscopy

Cells on day 4, 6, and 8 were fixed in 4% PFA, stained with anti-Emp antibody and counter-stained with Alexa-488-conjugated (green) goat anti-rabbit IgG. Nuclei were stained with Hoechst (Bisbenzimide Trihydrochloride, H 33258, Calbiochem) for 1 minute at room temperature by using a final concentration of 1 μg/ml in PBS. For live cell labeling, macrophages were stained without prior fixation. Fetal liver macrophages collected on day 4, 6, and 8 were incubated with anti-Emp antibody for 1 hour at 37°C and then moved to ice and incubation was continued for another 2 hours. Non-specific sites were blocked with normal goat serum, and the cells were then fixed with 1% PFA, followed by incubation with Alexa-488 fluor-conjugated goat-anti rabbit IgG.

For nuclear matrix preparation, cells were permeabilized with 0.5 % Triton X-100 in CSK buffer for 3 min at RT, washed with PBS, and fixed in paraformaldehyde. Cells were then

incubated with DNase I and extracted with 0.25 M ammonium sulphate [12]. After incubation in 10% goat serum to block nonspecific protein binding, fixed cells were incubated overnight at 4°C with primary antibodies diluted in the blocking buffer, washed, and then incubated with secondary antibodies (Alexa-488-conjugated (green) goat anti-rabbit IgG or Alexa-594-conjugated (red) goat anti-mouse IgG secondary antibody) diluted 1:100 in blocking buffer.

Biotin labeling

Cell surface proteins were covalently labeled using a membrane-impermeant biotinylation reagent (NHS-SS-Biotin, Pierce). All steps were performed at 4°C. Primary cultured macrophages on day 4, 6, and 8 were washed 3 times in PBS and then incubated with 1 mg/ml sulfo-NHS-SS-Biotin in PBS for 30 min on ice. Unreacted biotinylation reagent was quenched by incubating the sample with TBA (25mM Tris pH 8.0, 137mM NaCl, 5mM KCl, 2.3 mM $CaCl_2$, 0.5mM $MgCl_2$ and 1mM Na_2HPO_4) for 5 min on ice. Biotin labeled cells were then washed 3 times with PBS and solubilized in lysis buffer (20 mM Tris-HCl, pH 7.5, 125 mM NaCl, 10% glycerol, 1% NP-40) containing protease inhibitor cocktail. Cell lysates were centrifuged at 14,000 x g for 10 min at 4°C and a sample was taken from the supernatant (this sample represents the total cellular Emp). Neutravidin-agarose beads (Pierce) were added to the remaining supernatant (50 μl packed beads/500 μl lysate) and left to tumble at 4°C for 2 h. Beads were collected by centrifugation and washed 3 times in lysis buffer. The protein was extracted from the beads by heating at 95°C with SDS-PAGE sample buffer (this sample represents the surface Emp pool). Equivalent volumes of the 2 samples at each stage of maturation were analyzed by SDS/PAGE and Western blotting.

Metabolic labeling

Mouse fetal liver macrophages cultured for 4, 6, and 8 days were washed and preincubated in methionine-free medium for 60 min and labeled with [^{35}S] methionine (50 μCi/ml) (Perkin Elmer) at 37°C for 30 min. Cells were collected, washed and resuspended in complete medium containing 1 μg/ml cold methionine for different time points (0–2.5h). Harvested cells were subfractionated into plasma membrane, cytoplasm and nuclear fractions. Each fraction was then immunoprecipitated with anti-Emp antibody as described previously [9], and analyzed by SDS-PAGE and autoradiography.

Polyacrylamide gel electrophoresis and Western blotting

Proteins were separated by SDSpolyacrylamide gel electrophoresis according to the buffer system of Laemmli [13]. Western blotting was performed essentially as described by Towbin et al. [14]. The immunoreactive bands were detected by an enhanced chemiluminescence (ECL) system (Pierce).

RESULTS

Localization pattern of Emp in macrophages is developmentally regulated

To characterize the distribution pattern of Emp, we immunostained mouse fetal liver macrophages cultured for 4, 6, and 8 days representing cells of varying maturity with affinity purified polyclonal rabbit antihuman Emp antibody. As shown in Fig. 1A, Emp was detected in macrophages at all stages of maturation, and was detected both at the plasma membrane and intracellular. Interestingly, the distribution of Emp changed dramatically during maturation. In immature cells (day 4), the majority of Emp labeling was detected intracellular near the nucleus with very little staining on the membrane. With maturation, as the cells became large and displayed membrane ruffles and numerous filopodia-like projections, Emp staining was detected more on the plasma membrane than intracellular. Intense Emp staining was detected all along these projections.

Next, we used a biochemical approach to determine the partitioning of Emp between different cellular fractions. Immature (day 4) and mature (day 8) macrophages were lysed and fractionated to obtain the nuclear, cytosolic, and total membranous fractions, which were then subjected to SDS/Western blot analysis (Fig. 1B). Consistent with the above immunofluorescence data, we found that only one-fourth of cellular Emp was present in the membranous fraction of immature macrophages compared to almost three-fourths in mature macrophages (Fig. 1C).

Emp is localized on the cell surface

To verify that the membrane-associated Emp is exposed on the cell surface, we performed live cell staining of non-permeabilized cells without prior fixation. As expected, the Emp antibody brightly stained cells along the periphery with no intracellular staining. Consistent with the above result, Emp staining was more intense in day 6 and day 8 cells as compared to that on the day 4 cells (Fig. 2A). Cytoplasmic actin was not detected under identical conditions (data not shown). To quantify the relative distribution of endogenous Emp between surface and internal pools at different stages of maturation, we used a membrane impermeant biotinylation reagent to label surface proteins, and compared the fraction of total Emp that is accessible to the biotinylation reagent with the fraction that is inaccessible in primary cultured fetal liver macrophages on day 4, 6, and 8. As shown in Fig. 2B, the amount of surface Emp increased progressively with maturation. No Emp was detected in the surface fraction in the absence of biotinylation (data not shown). Quantification of the blots showed that on day 4, day 6, and day 8, approx. 5%, 20%, and 60–70%, respectively of total cellular Emp was present at the cell surface (Fig. 2C).

Erythroid cells bind to mature macrophages but not to immature macrophage precursors

Based on the above cell surface expression studies, we hypothesized that erythroblasts might attach more efficiently to mature macrophages than to immature macrophages. To test this hypothesis, we isolated erythroblasts from normal mouse fetal livers as described previously [9]. Equal number of erythroblasts were added to cultures of day 4, 6 and 8 macrophages, and grown in complete culture medium containing 2 units/ml human recombinant erythropoietin (Epo) at 37°C. After 6–12h, floating cells were removed and the adherent cells were washed three times with PBS. Cells adhering to culture dishes were fixed and stained with Wright-Giemsa without detaching from the dishes, and were examined by bright-field microscopy. As shown in Fig. 3, erythroblasts specifically attached to mature macrophages. Virtually no attachment was observed with immature day 4 cells. These results are consistent with the proposed cell attachment role of Emp.

Intracellular localization of Emp

Data shown in Figure 1 suggests that Emp is present in the nucleus and the plasma membrane. To investigate nuclear localization, free nuclei isolated from cultured macrophages were subjected to sequential extractions to obtain the insoluble nuclear matrix proteins and the soluble proteins. Western blot analysis using anti-Emp antibodies showed that Emp was present exclusively in the nuclear matrix (data not shown). This result is consistent with our previous observations in Emp-transfected HEK cells [12]. Likewise, immunofluorescence microscopy showed that Emp was present throughout the nuclear matrix in a non-homogeneous manner representing a strongly stained speckled pattern (Fig. 4A). Since splicing proteins accumulate in typical nuclear speckle domains [15], we sought to determine whether Emp foci colocalize with splicing proteins. A monoclonal antibody against the nonsmall nuclear ribonucleoprotein particle (snRNP) splicing factor SC-35 was used to detect nuclear speckle domains enriched with splicing factors. Double-label immunofluorescence microscopy showed a partial co-localization of Emp and SC-35 signals (Fig. 4A).

In addition to the nucleus and plasma membrane, some Emp appears to be present in the cytoplasm in a diffuse/punctate pattern. Since Western blot analysis of the post-membranous fraction (cytosol in Fig. 1) did not show any Emp, we hypothesized that this staining might represent Emp contained in intracellular compartments, which are likely to sediment with the membranous fraction. To investigate the intracellular compartments that contain Emp, we utilized confocal microscopy after double-labeling the macrophages with polyclonal antibodies to Emp and monoclonal antibodies to EEA1 and LAMP1, protein markers specific for early endosomes and lysosomes, respectively [16]. As shown in Fig. 4B, extensive colocalization of Emp was detected with EEA1 in punctate intracellular structures, indicating Emp localization in early endosomes. A small fraction of intracellular Emp was also seen colocalizing with a subset of intracellular vesicles containing LAMP1, suggesting that a fraction of Emp is localized in lysosomes.

Distribution of nascent Emp

To examine the intracellular distribution and trafficking of newly synthesized Emp in immature and mature macrophages, we immunoprecipitated Emp from [^{35}S]methionine metabolically labeled cells in vitro. Nascent [^{35}S]-labeled cytoplasmic Emp was present very early (<0.5 h) postlabeling and remained at 0.5 h postchase in both immature and mature macrophages. At 2.5 h after chasing with cold methionine however, labeled Emp remained in immature but decreased in mature macrophages (Fig. 5). Plasma membrane Emp also appeared very early (<0.5 h) and gradually increased from 0.5 to 2.5h postchase. Nuclear Emp was detectable initially (<0.5 h) but increased to maximum at 0.5h and remained at 2.5h postchasing with cold methionine. Quantitation by densitometry showed that the relative amounts of newly synthesized Emp in the membrane and nuclear fractions were different in immature vs. mature macrophages. These results suggest that nascent Emp migrated intracellularly from the cytoplasm/ER to the nucleus as well as to the plasma membrane in both immature and mature macrophages. But, the rate of migration to the nucleus is faster in immature cells, and to the plasma membrane is faster in mature cells.

DISCUSSION

Earlier studies suggested that Emp is a cell attachment protein that is involved in erythroblastic island formation. Recent gene targeting studies showed that in addition to its role in erythroblastic island formation, Emp is also involved in terminal differentiation of both the erythroid and macrophage cell lineages. In erythroid cells, Emp is required for attachment of immature precursors to macrophages [2], and for the expulsion of nuclei in enucleating cells [9]. This latter function is believed to involve association of Emp with F-actin in enucleating cells, and eventual segregation of Emp with the exiting nucleus. Partitioning of Emp to the plasma membrane surrounding the extruded nucleus thus provides the nucleus with a macrophage binding partner, thereby facilitating phagocytosis by macrophages [17]. In macrophages, Emp plays a crucial role in differentiation and ability to participate in erythroblastic island formation as evident by the fact that Emp-null macrophages are small, immature and unable to bind erythroid cells [9]. Thus, Emp appears to have multiple cellular functions. In this regard, information on the expression and localization of Emp in macrophages is essential. Therefore, in the present study, we have characterized temporal changes in the expression and subcellular localization of Emp during macrophage differentiation.

Immunolocalization of Emp in cultured mouse fetal liver macrophages showed that it is present at multiple sites, and that the relative distribution of Emp changes dramatically during differentiation. In immature macrophage precursors, intense Emp staining was detected with the nucleus whereas in more mature macrophages, staining was more intense on the plasma membrane than in the interior of the cell. Emp was detected all along the numerous filopodia.

Consistent with this observation, biochemical quantitation showed approximately 3-fold more Emp in the membranous fraction of mature macrophages than that of immature macrophages. Live cell labeling and biotin labeling of surface proteins confirmed that the membrane-associated Emp is exposed on the cell surface, and that the amount of surface Emp increases with maturation. Consistent with the function of Emp in mediating cell attachment, erythroblasts attached to mature but not immature macrophages.

Characterization of intracellular Emp showed that it is localized in distinct compartments. A major fraction of intracellular Emp is localized in the nucleus. Consistent with our previous studies [12], we found that the nuclear pool of Emp is present in the nuclear matrix where it colocalizes with nuclear actin (data not shown), and the nuclear matrix marker, the spliceosome assembly factor SC-35. In light of a recent report [18], suggesting that nuclear actin is involved in the transcriptional activation of CSF-1 gene, a key factor for differentiation, growth and survival of monocytes and macrophages, detection of Emp in the nuclear matrix together with actin is of particular interest and may suggest contribution of Emp to modulation of cell growth and differentiation. In addition to the nucleus, a substantial amount of Emp is localized in early endosomes, and a small fraction in lysosomes. The presence of Emp in the endosomes and lysosmes may represent newly synthesized protein that is in the transportation pathway to the cell surface rather than the constitutive recycling of Emp. Pulse-chase experiments showed that the newly synthesized Emp assembled in the membrane is stable in both immature and mature macrophages. On the other hand, the cytosolic pool representing endosomal/lysosomal fraction accumulates in immature but decreases in mature macrophages within 2.5hr post chase, suggesting that the newly synthesized Emp is transported to the cell surface more efficiently in mature than in immature macrophages.

Overall, the present data characterize distribution of Emp in macrophages during differentiation and demonstrate that Emp undergoes dynamic rearrangements within the cellular environment. The presence of Emp at the cell surface is consistent with its role in erythroblastic island formation, whereas its presence in the nuclear matrix may be of interest in elucidating function of Emp in growth and differentiation of monocytes and macrophages. There are several examples in the literature for localization of membrane receptors at multiple sites within the cellular environment. A few of these include tissue factor (TF), vascular endothelial growth factor receptor-2, VEGFR-2 (or KDR), and CD40, which are all localized at the plasma membrane as well as intracellular [16,19,20]. Trafficking of TF and KDR are regulated by binding of these receptors with their corresponding ligands, clotting factor VIIa and VEGF, respectively. Our studies documenting that maturation of macrophages increases Emp expression at the cell surface with a corresponding decrease of intracellular Emp suggest that similar mechanisms may exist for Emp. Such possibilities will be explored in future studies.

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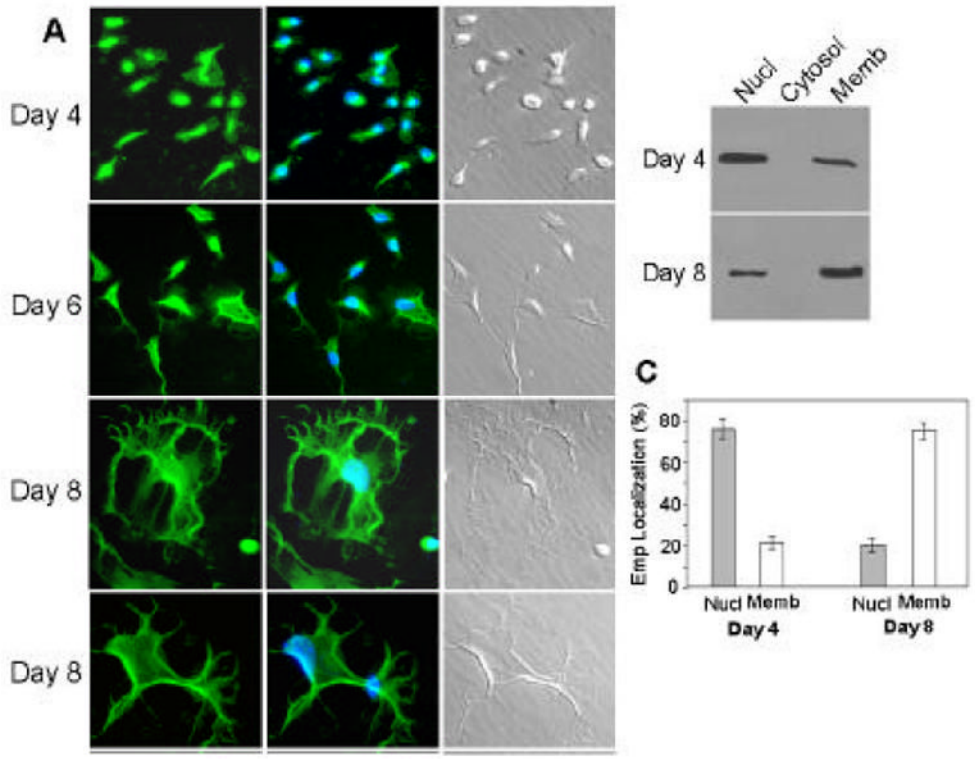

Figure 1.

Cellular distribution of Emp in macrophages. (A) Mouse fetal liver macrophages cultured for 4, 6, and 8 days were fixed in PFA, permeabilized, stained with anti-Emp antibody, and counter stained with Alexa-488-conjugated (green) goat anti-rabbit IgG. Nuclei were stained with Hoechst dye. All images were magnified identically after being photographed through a 40X objective. Left column represents Emp staining; middle column, merged images; the right column, phase contrast. (B) Cellular fractionation. Macrophages cultured for 4 and 8 days were subjected to cellular fractionation as described in Experimental Procedures. Nuclear, cytosolic, and total membranous fractions obtained from an equal number of cells were examined by 12% SDS-PAGE/Western blot analysis using anti-Emp antibody. (C) Densitometric quantification of the relative nuclear and membranous pools of Emp (mean ± S.D; n=3).

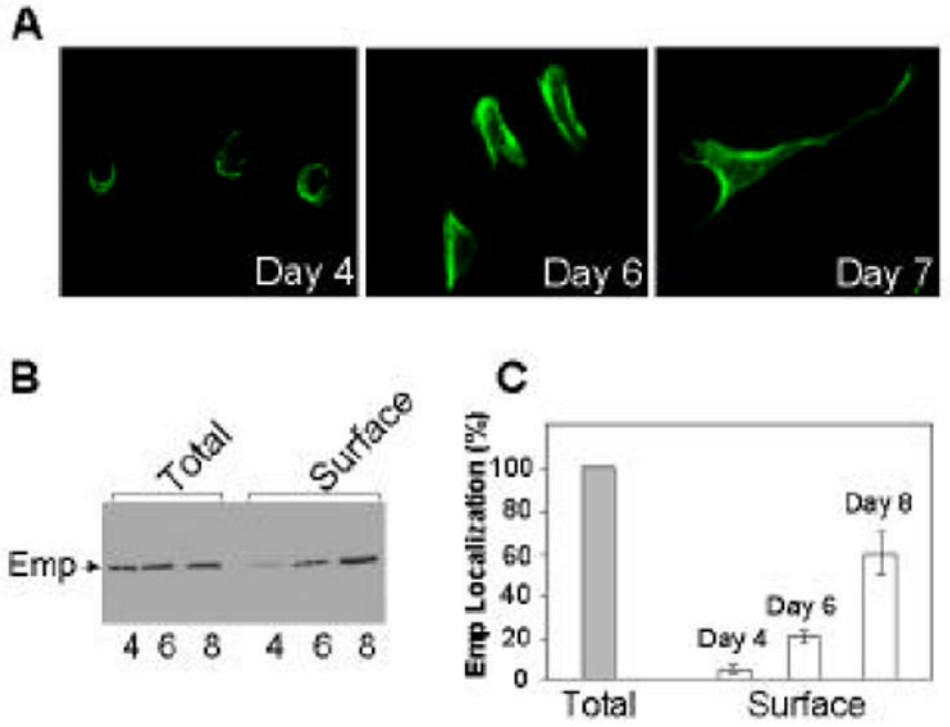

Figure 2.

Emp is localized on the cell surface. (A) Live cell staining. Macrophages cultured for 4, 6 or 8 days were labeled with anti-Emp antibody without prior fixation, and detected with Alexa-488 flour-conjugated goat-anti rabbit IgG. Cytoplasmic actin and no primary antibody controls did not show staining under identical conditions. (B) Biotin labeling. Surface Emp was labeled with the membrane-impermeant biotinylation reagent sulfo-NHS-SS-Biotin. Biotinylated surface Emp was collected by binding to neutravidin-agarose. Aliquots of the total cell lysate and surface fraction were analyzed by Western blotting with anti-Emp antibody. Note that 50% of the total cell lysate at each stage was loaded on the gel. (C) Densitometric quantification of the relative total and surface pools of Emp (mean ± S.D; n=3).

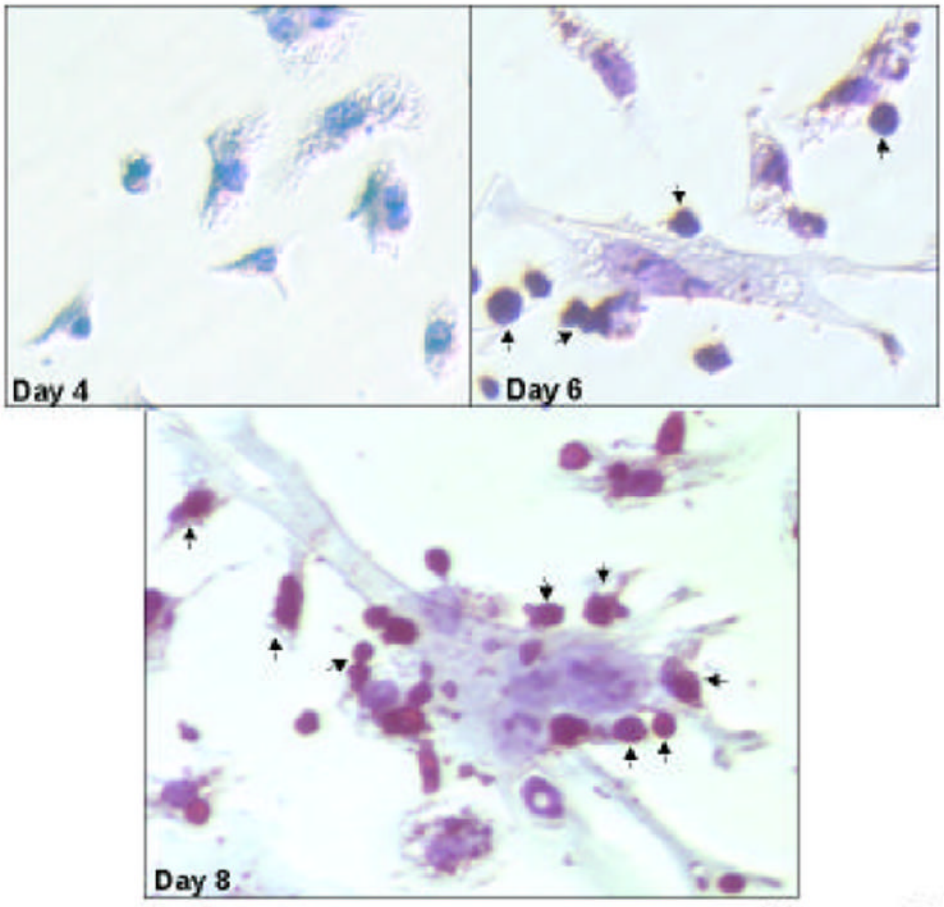

Figure 3.

Attachment of erythroblasts to macrophages. An equal number of erythroblasts were cultured with 4, 6, or 8 day macrophages for 12 hours in medium containing Epo. Subsequently, floating cells were removed and the adherent cells were washed two times to remove any trapped non-adherent cells. Cells adhering on the petri dishes were fixed and stained with Wright-Giemsa without detaching from the dishes and examined by bright-field microscopy. Magnification: 40X. Arrows indicate erythroblasts attached to cultured macrophages.

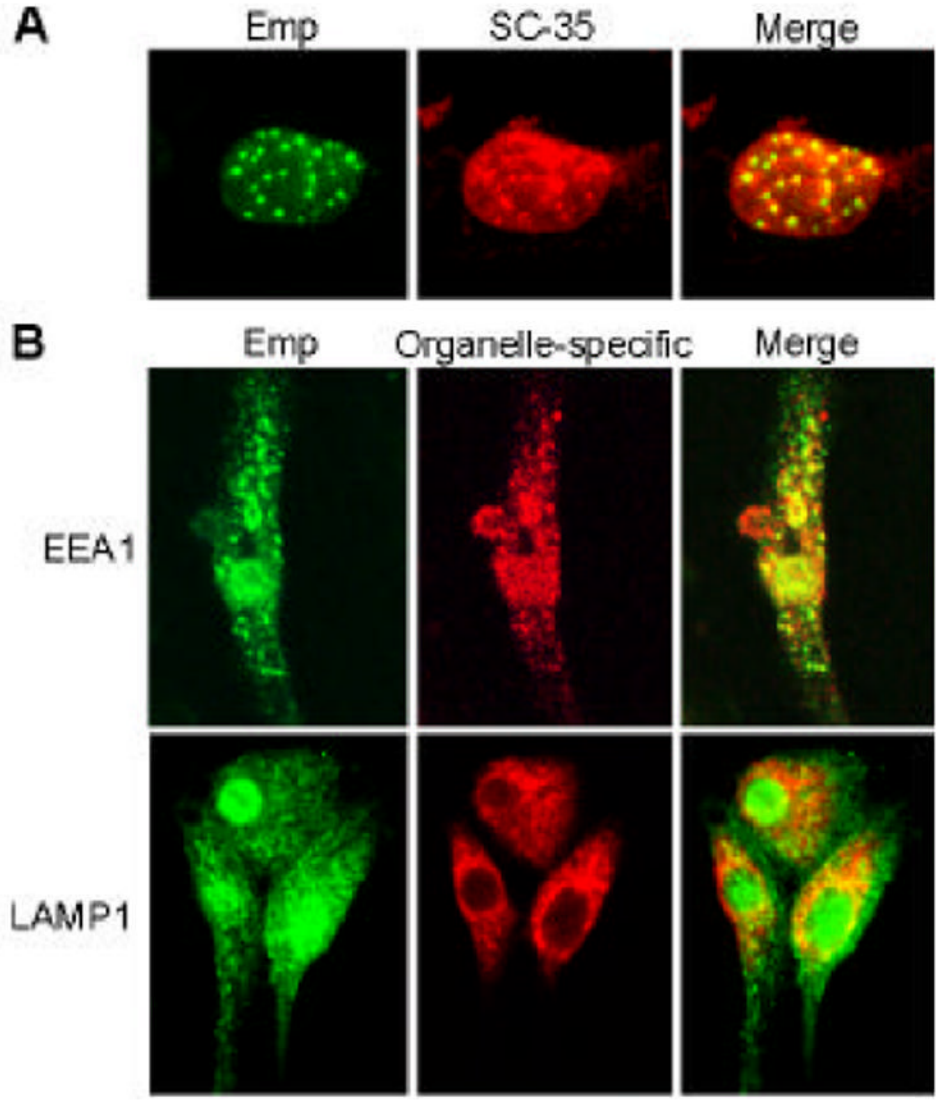

Figure 4.
Intracellular distribution of Emp in macrophages. (A) Nuclear matrix, and (B) Intact macrophages (cultured for 4–6 days) were fixed, permeabilized, and stained with anti-Emp, anti- SC-35, and an organelle-specific antibody.

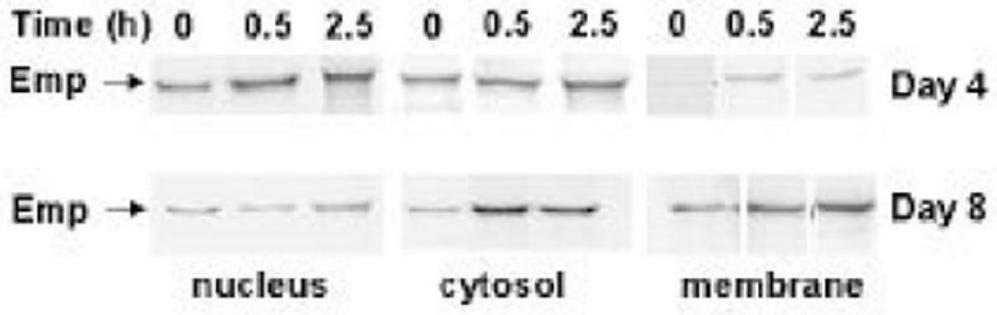

Figure 5.

[^{35}S] metabolic labeling for the newly synthesized Emp in macrophages. Fetal liver macrophages cultured for 4 or 8 days (1 X 10^8 each) were grown in methionine-free medium containing [^{35}S]methionine for 30 min and then chased with cold methionine for 0, 0.5, and 2.5 hours. Newly synthesized Emp was immunoprecipitated from nuclear, cytoplasmic, and plasma membrane fractions and analyzed on SDS-PAGE, and detected by autoradiography.