The Association of Erythroblasts With Macrophages Promotes Erythroid Proliferation and Maturation: A 30-kD Heparin-Binding Protein Is Involved in This Contact

By Manjit Hanspal and Jatinder S. Hanspal

Although the association of erythroblasts with macrophages has been well documented in the human bone marrow, where erythropoiesis occurs in distinct anatomic units called erythroidic islands. In sections of normal marrow, islands of maturing erythroblasts surround a central macrophage. Studies of hematopoietic differentiation in long-term bone marrow cultures have shown that areas of erythropoiesis contain central macrophages. Although these studies have unequivocally established an intimate association between erythroblasts and macrophages, the functional significance of these interactions and the role of the central macrophage in erythropoiesis, if any, is not known.

Macrophages are known to produce a host of factors that modulate cellular growth and differentiation. These include interleukin-1, interferon γ, tumor necrosis factor, a variety of mitogens, and a number of myeloid colony-stimulating factors, including the multipotent granulocyte-macrophage colony-stimulating factor, the unipotent macrophage colony-stimulating factor, and granulocyte colony-stimulating factor. In addition, macrophages release a number of cytokines that act on erythroid progenitor cells, eg, burst-promoting activity, an insulin-like growth factor, and erythropoietin. Cytokines produced by macrophages act both as positive and negative regulators of erythropoiesis.

It has become apparent that the adhesion of hematopoietic progenitor cells to bone marrow stromal cells (eg, macrophages, monocytes, and T lymphocytes) allows their localization to the bone marrow and supports their proliferation and differentiation. A direct contact of the stromal cells with hematopoietic progenitor cells plays an important role in erythropoiesis. This cell:cell contact involves multiple adhesive molecules, eg, integrins, whose expression is developmentally regulated. Integrins represent dimeric glycoproteins consisting of a common β subunit noncovalently bound to a different α subunit. They are subdivided according to their common β subunit. Integrins of the β1 family are the primary mediators of all extracellular matrix adhesions, whereas integrins of the β2 family, present only in hematopoietic cells, are of importance in cell-cell and cell-endothelial interactions. In the erythroid cell lineage, β2 integrins are present only in early progenitors (burst-forming units-erythroid [BFUe]), whereas β1 integrins of the VLA-4 (very late antigen) type are highly expressed at the progenitor and precursor levels, but are lost during terminal maturation. This is consistent with progressively decreased binding of maturing erythroid cells to fibronectin, a ligand for VLA-4 receptor.

In the present study, we have examined the role of erythroblast-macrophage contact in the terminal maturation of erythroid cells and have begun to identify the molecule(s) involved in this contact. Using a two-phase liquid culture of
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human peripheral blood-derived erythroid progenitors, we have shown that the association of erythroblasts with macrophages promotes erythroid proliferation and terminal maturation leading to their enucleation. In the absence of macrophages, erythroid cells mature to the late erythroblast stage but fail to enucleate. Furthermore, using a cell attachment assay involving the incubation of solubilized surface-labeled erythroblasts with macrophage membrane proteins transferred to a nitrocellulose membrane, we have shown that erythroblast surface proteins specifically attach to a 30-kD heparin-binding protein from macrophage membranes. An apparently similar protein is present in erythroblast membranes as well and is capable of binding homotypic and heterotypic cells.

MATERIALS AND METHODS

Two-phase liquid cultures of human peripheral blood erythroid progenitors. Peripheral blood was obtained from healthy donors and mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. The mononuclear cells were collected and cultured using some modifications of the two-phase liquid culture system described originally by Fibach et al,24 Wada et al,25 and, recently, us.26 The mononuclear cells were cultured in the first phase at a density of 10^6/mL in Iscove’s modified Dulbecco’s medium (IMDM; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and 10% conditioned medium collected from cultures of the 5637 bladder-carcinoma cell line.27 After 1 week of incubation at 37°C in an atmosphere of 5% CO2, the nonadherent cells were harvested and treated with the carbonyl iron method28 to remove phagocytic cells. The floating, nonphagocytic cells were recultured in IMDM containing 30% FCS, 1% deionized bovine serum albumin, 10 mM L-2-mercaptoethanol, 1.5 mM glutamine, 300 μg/mL iron-saturated transferrin, and 2 μM human recombinant erythropoietin (generously provided by Ortho Biotech, Raritan, NJ). These secondary cultures were incubated at 37°C in a humidified atmosphere containing 5% O2 and 5% CO2. On day 4 or 5 of the second phase, when clusters of proerythroblasts start to appear, the cells were harvested (the medium was saved), suspended in 2 mL of the culture medium, layered on a 2 mL solution of 45% Percoll (Pharmacia, Uppsala, Sweden) in phosphate-buffered saline (PBS; final density, 1.0585 g/mL), and centrifuged at 1200g for 5 minutes at room temperature. The upper layer containing predominantly proerythroblasts and macrophages were collected, washed, and resuspended in the original culture medium and incubation continued for another 10 to 11 days at 37°C in an atmosphere of 5% O2 and 5% CO2. The pellet containing greater than 90% of the lymphocytes was discarded. After 1 week in the second phase, viable cells were counted by trypan blue exclusion. Hemoglobin-containing cells were scored with use of the acetic acid-benzidine peroxidase reaction29 and cellular morphology was assessed by preparing cytopsin slides stained with Wright-Giemsa.

Cell lines. HeLa cells, a human epithelial-like cell line, were obtained from the American Type Culture Collection (Rockville, MD). HeLa cells were cultured to confluence in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% FCS and antibiotics at 37°C in a 5% CO2/95% air incubator. Cells reached confluence within 4 to 5 days, at which time they were detached from the flask by a 15-minute incubation in cell dissociation solution in PBS (cat. no. C 5914; Sigma, St Louis, MO) at 37°C. The cells were washed and either subcultured or used for experiment. K562 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FCS and antibiotics at 37°C in a 5% CO2/95% air incubator.

Removal of macrophages and preparation of macrophage-conditioned medium. For studies involving macrophage-depleted cultures, macrophages were removed using monoclonal antibodies as described earlier.30 The mixture of proerythroblasts and macrophages collected from top of Percoll after 4 days of second phase were suspended at a concentration of about 10^7/mL in IMDM containing 1:20 rat anti-human Mo2 monoclonal antibodies (Coulter Cytometry, Hialeah, FL) and incubated at 4°C for 45 minutes. After three washings in cold IMDM, the cells were resuspended in IMDM containing 1:40 fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG and incubated for a further 45 minutes. The cells were then washed, resuspended in IMDM, and incubated with sheep anti-FITC antibody attached to magnetic particles for 30 minutes at 4°C. MO2-positive cells were removed by magnetic separation using a Bio-Mag separator (Advanced Magnetic Inc, Cambridge, MA). The control cultures, ie, macrophage-containing cultures, were treated identically but were not exposed to Mo2 monoclonal antibodies. Cell viability was tested in both the control and macrophage-depleted cultures before and after the magnetic separation procedure.

To prepare macrophage-conditioned medium, macrophages were obtained from human peripheral blood mononuclear cells essentially as described previously.13 Macrophage-conditioned medium was prepared by culturing 2 x 10^6 macrophages in serum-free IMDM for 24 hours at 37°C. The medium was harvested and stored at -20°C until use.

Radiolabeling of cells. For surface labeling, erythroblasts and macrophages were washed three times with PBS and then labeled using the glucose oxidase-lactoperoxidase method.3 Briefly, washed cell pellet was incubated with 500 μL PBS containing 20 μg/mL lactoperoxidase, 0.25 U/mL glucose oxidase, 200 μCi/mL 125I-Iodine (New England Nuclear, Boston, MA) and 2.5 mM glucose for 5 minutes on ice. Glucose (2.5 mM) was added again and the reaction continued for 5 more minutes. The reaction was stopped with 3 mL of PBS containing 0.02% sodium azide. The cells were washed three more times with PBS containing azide and then either exposed to radiolabeled antibodies or used as intact labeled cells or they were solubilized in a buffer containing NP-40 (0.1 mol/L Tris-HCl, pH 8.5, 150 mM NaCl, 5 mM/L EDTA, 0.5% NP-40; NP-40 buffer) and centrifuged at 500g for 5 minutes to remove nuclei. The supernatant was stored at -20°C in aliquots. The cells were then labeled with [35S]methionine (30 μCi/mL, 1000 Ci/mmol; ICN Biomedicals, Irvine, CA) for 4 hours or overnight. At the end of the labeling period, 10 vol of 155 mM/L choline chloride and 5 mM/L HEPES, pH 7.1, was added and the cells were harvested by centrifugation. Labeled cells were solubilized in the NP-40 buffer and centrifuged to remove nuclei. The supernatant was stored at -20°C in aliquots.

Preparation of cell membranes. Macrophage membranes were prepared by hypotonic lysis.32 Cultured macrophages were incubated with 5 mM/L Tris-HCl, pH 7.4, and allowed to swell for 5 minutes on ice. An aliquot of 60% sucrose in 5 mM/L Tris-HCl was added to restore isotonicity (final concentration of sucrose, 10%). The macrophages were collected and disrupted by 10 strokes of a tight-fitting Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at 500g for 5 minutes. The cell membranes were pelleted by centrifugation at 48,000g for 20 minutes and washed twice with 5 mM/L Tris-HCl, pH 7.4. Erythroblast membranes were prepared essentially as described previously.34 HeLa and K562 cell membranes were prepared by hypotonic lysis in 10 mM/L...
Tris-HCl, pH 7.5, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L leupeptin, and 10 μg/mL aprotinin, followed by cell disruption with 10 strokes of a tight-fitting Dounce homogenizer. Isotonicity was restored by adding sucrose to a final concentration of 10%. Nuclei were removed and cell membranes were pelleted as described above for macrophage membranes.

Macrophage and erythroblast membranes were treated with 1 mol/L NaCl in 5 mmol/L phosphate buffer, pH 7.4, containing 1 mmol/L CaCl2 and 1 mmol/L PMSF, for 30 minutes at room temperature. The eluate was collected by centrifugation at 48,000g for 20 minutes. Similarly, macrophage and erythroblast membranes were extracted with 1% Triton X-100 in PBS containing 1 mmol/L CaCl2 and 1 mmol/L PMSF by incubating on ice for 30 minutes followed by centrifugation. The Triton-insoluble pellet was washed once with the Triton buffer and the resulting pellet was suspended in sodium dodecyl sulfate (SDS) sample buffer.

The cell attachment assay. The attachment of erythroblasts to macrophages or erythroblasts to erythroblasts was studied using some modifications of the previously described method.31 For the erythroblast-macrophage attachment, macrophage membranes were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane using the standard transfer procedure.32 After completion of electrophoretic transfer, the nitrocellulose membrane was rinsed in PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at 22°C. Erythroblasts were surface labeled with 125I (1 μCi/106 cells), as described above. Either intact or NP-40 solubilized radiolabeled cells were diluted 10-fold with PBS and then incubated over the nitrocellulose membrane for 2 hours at 4°C. Thereafter, the membrane was washed four or five times with PBS, air-dried, and exposed to x-ray film for autoradiography.

Inhibition of cell:cell adhesion by heparin. The cell attachment assay described above was performed in the presence of 50 U/mL of heparin sodium (sterile, preservative-free, Porcine Intestine mucosa, 1,000 USP units/mL; Elkins-Sinn Inc, Cherry Hill, NJ). For some experiments, macrophage membrane proteins isolated from metabolically labeled macrophages were solubilized in 10 mL of the NP-40 buffer (0.1 mol/L Tris-HCl, pH 8.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP-40). A 3-mL suspension of heparin-bound agarose (Sigma) was added and incubated at 4°C for 2 hours. The agarose beads were removed by centrifugation and the supernatant apparently free of heparin-binding proteins was used in the cell attachment assay by incubating over nitrocellulose membrane containing electrophoretically transferred erythroblast membrane proteins.

RESULTS

The formation of erythroblastic islands in two-phase liquid cultures of human peripheral blood erythroid progenitors. On day 7 or 8 and beyond, in the second phase of the liquid cultures, we observed the formation of distinct colonies. Wright-Giemsa staining of the cytopsin preparations showed that these colonies were consisted of a central macrophage surrounded by a ring of erythroblasts (Figs 1 and 2). The presence of such colonies called erythroblastic islands has been shown previously in the human bone marrow. In addition to the morphologic identification, we confirmed the presence of the central macrophages in these islands by immunofluorescence studies using monoclonal antibodies against macrophage surface antigens, CD36 and CD14 (data not shown). Between day 7 and day 10 of the second phase, the cells surrounding the central macrophage appear to be at the proerythroblast and basophilic normoblast stage with large nucleus and very little hemoglobin (Fig 1). On day 12 or 13 of the second phase, approximately 90% of the cells are benzidine-positive and appear to be at the late erythroblast stage as seen by Wright-Giemsa-stained cytopsin preparations. At around day 14 or 15 in the second phase of the culture, more mature cells in the erythroblastic islands start to enucleate and get released from the macrophage (Fig 2B, D, and E). In addition to erythroblastic islands, several macrophage-free colonies of erythroblasts were seen in our cultures (Fig 2C).

A close association of erythroblasts with macrophages promotes erythroid proliferation and maturation leading to their enucleation. To study the role of macrophages in erythroid development, macrophages were removed from cultures using a monoclonal antibody (Mo2) against macrophage surface antigen. In the absence of macrophages, the number of erythroid cells, as determined by benzidine staining, was markedly decreased as compared to the macrophage-containing cultures (Table I). Cell viability, as tested by trypan blue exclusion staining, was found to be greater than 95% before and after the removal of macrophages. In addition, in macrophage-depleted cultures, the erythroid cells matured to the late erythroblast stage but they failed to enucleate, suggesting a role of macrophages in erythroid proliferation and maturation. To determine whether the effect of macrophages was through the release of soluble growth factors, we reconstituted the cultures with macrophage-conditioned medium. Erythroid cell proliferation and maturation was considerably less compared with when intact macrophages were present (Table I). These results suggest that a close association of erythroblasts with macrophages promotes erythroid proliferation and maturation. An association of late-stage erythroblasts with macrophages, as illustrated in Fig 2A and B, is more likely to function in enucleation because the proliferative capacity of these cells is minimal. It should be cautioned that macrophages may exert at least some of their effect through a soluble factor(s) because we cannot rule out the possibility of proteolytic degradation in the macrophage-conditioned medium.

A 30-kD membrane protein is involved in erythroblastic-macrophage contact. Next, we examined the molecule(s) responsible for the interaction between erythroblasts and macrophages using a modification of the cell attachment assay previously used for the detection of hemonectin in the extracellular matrix of bone marrow.33 The macrophage membrane proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking the non-specific sites with BSA, the nitrocellulose membrane was incubated with surface-labeled erythroblasts solubilized in NP-40. Erythroblast surface proteins specifically attached to a 30-kD protein from macrophage membranes, whereas no adhesion was seen to the protein standards (Fig 3). Identical results were obtained when surface-labeled intact erythroblasts (without solubilization) were used (data not shown). A similar approach was used to determine the putative attachment site for macrophages on erythroblasts. Erythroblast membrane proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The latter was then
incubated with surface-labeled macrophages solubilized in NP-40. Surprisingly, macrophage surface proteins also attached to a 30-kD protein on erythroblast membranes (Fig 3, lane 6). Furthermore, because, in addition to erythroblast-macrophage colonies, we have observed a significant number of macrophage-free erythroblast colonies in our cultures (Fig 2C), we asked what are the molecules involved in erythroblast-erythroblast interaction. The cell attachment assay described above showed that a 30-kD protein is also involved in erythroblast-erythroblast contact (Fig 3, lane 4). An additional adhesive peptide of about 28 kD was observed in erythroblast membranes (Fig 3, lanes 4 and 6). The amount of this peptide remained the same even when protease inhibitors were omitted during the preparation of membranes (data not shown), suggesting that it is not the result of proteolytic degradation. Nevertheless, tryptic digestions of the two peptides (Hanspal et al, unpublished data) suggest that they are related. The attachment with the same 30-kD/28-kD protein bands was seen when metabolically labeled erythroblasts and macrophages, solubilized in NP-40, were used (data not shown).
Table 1. Effect of Macrophages and Macrophage-Conditioned Medium on Erythroid Cell Proliferation and Maturation

<table>
<thead>
<tr>
<th>No. of benzidine-positive cells (×10^4)</th>
<th>− Macrophages</th>
<th>+ Macrophages</th>
<th>Macrophage CM</th>
</tr>
</thead>
<tbody>
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<td>4 ± 0.5</td>
<td>23 ± 2</td>
<td>7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>No. of enucleated cells (×10^4)</td>
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<td>9 ± 0.5</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>% Enucleation</td>
<td>0</td>
<td>35 ± 5</td>
<td>4 ± 1</td>
</tr>
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The number of benzidine-positive cells and enucleated cells were determined on day 15 of the second phase in macrophage-depleted cultures (− Macrophages), macrophage-containing cultures (+ Macrophages), and in cultures devoid of intact macrophages but containing 10% macrophage-conditioned medium. (Macrophage CM). Percent enucleation represents values relative to total cell number (the number of benzidine-positive cells is approximately 90% of total cell population in all the cultures). The data represent mean ± SD.

shown), indicating that these adhesive proteins are actually synthesized by the cells and are not adsorbed from the serum during in vitro cultures.

To determine whether the 30-kD protein mediates the attachment of macrophages to macrophages, macrophage membranes were incubated with radiolabeled macrophages in the cell attachment assay described above. In contrast to erythroblast-erythroblast and erythroblast-macrophage interactions, our cell attachment assay did not detect interaction of macrophages to macrophages (data not shown). This result is not necessarily surprising, because macrophages do not seem to grow in clusters or form colonies in vitro. This observation suggests that either the ~30-kD protein from macrophage and from erythroblast membranes are two different proteins or that the concentration of this protein on cell surface is important for cell:cell contact.

The results presented above show that a 30-kD protein is exposed on the membrane of both erythroblasts and macrophages. Treatment of purified cell membranes with 1 mol/L NaCl resulted in the release of this protein in the eluates (Fig 4A, lane 1), suggesting that the 30-kD protein is a peripheral membrane component. In addition to its mobility on SDS polyacrylamide gels, the presence of the 30-kD protein was confirmed by the cell attachment assay (Fig 4A, lane 2). Furthermore, to determine whether this protein is linked to an integral membrane protein, we extracted macrophage cell membranes with 1% Triton X-100. As shown in Fig 4B, the 30-kD protein remained exclusively bound with the Triton-insoluble fraction, suggesting that it is linked to an integral membrane protein, which, in turn, is bound to the membrane skeleton. The 30-kD protein from erythroblast membranes behaved identically in response to 1 mol/L NaCl and 1% Triton X-100 extractions (data not shown).

The 30-kD protein-mediated cell:cell adhesion is specific for erythroid and macrophage cell types. To determine the specificity of the 30-kD protein-mediated erythroblast-macrophage interactions that regulate erythroid maturation, we examined the attachment of solubilized surface-labeled erythroblasts with HeLa cells, a human epithelial cell line. Plasma membranes from HeLa cells were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The latter was then incubated with solubilized surface-labeled erythroblasts. As shown in Fig 5, solubilized surface-labeled erythroblasts did not attach to HeLa cell membrane proteins, whereas they specifically attached to the 30-kD protein from macrophage membranes. In addition, because the 30-kD protein also mediates erythroblast-erythroblast interaction, plasma membranes from K562 cells, a human erythroleukemia cell line, were used in the cell attachment assay. Again, solubilized surface-labeled erythroblasts specifically attached to a 30-kD protein present in K562 cell membranes (Fig 5). These results show that the 30-kD protein-mediated cell:cell attachment is specific for erythroid and macrophage cell lineages.

Fig 3. Cellular attachment to the 30-kD protein from macrophages and erythroblasts. The cell attachment assay is shown on 10% SDS-PAGE. Lane 1, molecular weight (MW) standards, values are indicated to the left. (A) Radiolabeled erythroblasts solubilized in NP-40 were incubated with MW standards (lane 2), macrophage membranes (lane 3), and erythroblast membranes (lane 4) transferred to a nitrocellulose membrane. Autoradiographs are shown. (B) Radiolabeled macrophages solubilized in NP-40 were incubated with MW standards (lane 5) and erythroblast membranes (lane 6) transferred to a nitrocellulose membrane followed by autoradiography. No attachment is seen to protein standards in lanes 2 and 5, but a distinct band of 30 kD is seen in lanes 3, 4, and 6. An additional band of 28 kD is seen in lanes 4 and 6 containing erythroblast membranes.
Next, we examined the possibility that the 30-kD adhesive protein is related to thrombospondin (TSP), an extracellular matrix protein, or is a proteolytic fragment of TSP that remained associated with cell membranes during membrane preparation. This possibility was examined because monoclonal antibodies (MA-11) against the amino terminus of TSP showed a strong cross-reactivity with a protein of about 30 kD present in both macrophage and erythroblast membranes (data not shown). To determine if the 30-kD adhesive protein represents the amino terminal domain of TSP, we used purified amino terminal heparin-binding domain of TSP in our cell attachment assays. Neither macrophages nor erythroblasts showed any attachment to the purified fragment of TSP (data not shown; anti-TSP antibodies, MA-II, and the purified amino terminal heparin-binding domain of TSP were generously provided by Dr. Jack Lawler, Harvard Medical School, Boston, MA). This result is in agreement with a previous report that showed that TSP binds primary hematopoietic progenitor cells and that the cell-binding domain resides toward the carboxyl terminal of the molecule. Hence, the 30-kD adhesive protein we have detected in macrophages and erythroblasts is not TSP but it could be a TSP-like protein.

In addition, a possibility was examined that the 30-kD protein is related to some other adhesive molecules such as fibronectin, vitronectin, and types I, III, and IV collagen. Purified proteins (purchased from Sigma) were used in the cell attachment assay. Solubilized surface-labeled erythroblasts attached to fibronectin (Fig 6, lane 2), but not to the other proteins tested (data not shown). To determine whether the 30-kD protein was involved in the attachment of erythroblasts to fibronectin, radio-labeled erythroblasts were preincubated with 50 μg/mL fibronectin, solubilized in NP-40 buffer, and then used in the cell attachment assay with macrophage membranes and pure fibronectin. As shown in Fig 6, the 30-kD protein-mediated attachment of erythroblasts to macrophages was unaffected (Fig 6, lane 3), whereas the attachment of erythroblasts to fibronectin was completely

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HANSPAL AND HANSPAL

Probed with Radiolabeled Erythroblasts

Probed with Radiolabeled Eryth. Preincubated with Fn

HANSPAL AND HANSPAL

Probed with Radiolabeled Erythroblasts

Probed with Radiolabeled Eryth. Preincubated with Fn

abolished (Fig 6, lane 4) by preincubation of erythroblasts with fibronectin. We conclude that the 30-kD protein is not involved in the attachment of erythroblasts to fibronectin.

The 30-kD protein-mediated cell:cell adhesion is Ca\(^{2+}\)-independent. Cell:cell adhesion is functionally operated either by a Ca\(^{2+}\)-dependent mode, as exhibited by the cadherin family of adhesion proteins,\(^{39}\) or by a Ca\(^{2+}\)-independent mode, as observed with the neural-cell adhesion molecules (N-CAMS) and related molecules.\(^{40}\) We examined the dependence of 30-kD protein-mediated adhesion on extracellular Ca\(^{2+}\) by measuring the attachment of radiolabeled erythroblasts with macrophage or erythroblast membranes either in the presence or absence of EGTA. As shown in Fig 7, removal of extracellular Ca\(^{2+}\) did not affect 39-kD protein-mediated cell adhesion, in common with the mode of adhesion mediated by N-CAMS.

The 30-kD protein-mediated cell:cell adhesion is specifically inhibited by heparin. To determine the possible inhibitors of adhesion between erythroblasts and macrophages or erythroblasts and erythroblasts, we examined the effect of heparin, which is known to bind to several other adhesive proteins such as fibronectin\(^{41,42}\) and thrombospondin.\(^{43,44}\) The cell attachment assay involving the incubation of radiolabeled erythroblasts with macrophage membranes was performed in the presence and absence of heparin. As shown in Fig 8A, the 30-kD protein-mediated attachment was completely abolished in the presence of heparin. Furthermore, in an attempt to remove the 30-kD protein from macrophages, radiolabeled macrophage membrane proteins solubilized in an NP-40 buffer were incubated with heparin-bounded agarose. The agarose beads were removed and the resulting supernatant, containing macrophage membrane proteins but depleted of heparin-binding proteins, was collected. The latter was then incubated over a nitrocellulose membrane containing electrophoretically transferred erythroblast membrane proteins. As shown in Fig 8B, the attachment of macrophage membrane proteins with erythroblast membranes, mediated by the 30-kD protein, was almost completely abolished. These results show that the 30-kD adhesive protein is heparin-binding and that the removal of this protein from macrophages abolishes their attachment with erythroblasts. Similarly, the removal of the 30-kD protein from solubilized radiolabeled erythroblast membrane proteins abolished their
attachment with macrophage membrane proteins on a nitrocellulose membrane (data not shown).

To study the effect of heparin on the function of erythroblast-macrophage contact, increasing amounts of heparin were added to the culture medium. In the absence of heparin, big clusters containing several hundred cells in close contact with each other were seen (Fig 9A). But, with increasing concentrations of heparin present in the culture medium, the clusters were disrupted resulting in very small colonies (Fig 9B and C) and several single cells (Fig 9D). Furthermore, in the presence of increasing concentrations of heparin, there was a progressive decline in the number of erythroid cells, as judged by benzidine staining, as well as in the number of enucleated cells (Table 2). One other batch of purified heparin (Porcine intestine mucosa, 170 USP units/mg; tissue culture tested; Sigma) was also used in these experiments to rule out the possibility of the presence of a toxic contaminant. Identical results were obtained with both batches of heparin. These results clearly indicate that the association of erythroblasts with macrophages promotes erythroid cell proliferation and maturation and that this association is mediated by a 30-kD heparin-binding protein. To rule out the possibility that the inhibition of cell proliferation in presence of heparin is nonspecific, HeLa cell cultures were subjected to 20 and 50 U/mL of heparin. After 4 days of culture, cells were detached from the surface and washed two times with PBS and viability was tested by exclusion of trypan blue. No change in cell number or viability was seen in 4 days of culture (data not shown).

**DISCUSSION**

Although the presence of erythroblastic islands, the anatomical unit of erythropoiesis, consisting of a central macrophage surrounded by a ring of developing erythroblasts, has been well documented both in vivo and in vitro, the function and identification of the intimate contacts between these two cell types is not known. To study such interactions and their role in erythropoiesis, we have used a two-phase liquid culture system that supports the proliferation and maturation of human erythroid progenitors and macrophages. In the second phase of this culture system, morphologically identified erythroid cells and macrophages form colonies, with each colony consisting of a central macrophage surrounded by a ring of developing erythroblasts. These colonies are identical to the previously reported erythroblastic islands. Between day 7 and day 10 of the second phase, the cells surrounding the central macrophage appear to be at the proerythroblast and basophilic normoblast stage. On day 12 or 13 in the second phase, the majority of erythroid cells appear to be at the polychromatophilic and orthochromatophilic stage that eventually undergo enucleation. The extruded nuclei are occasionally seen in the macrophages by Wright-Giemsa staining of cytacentrifuged preparations, suggesting that they are probably ingested by the central macrophages.

The removal of macrophages from cultures using specific monoclonal antibodies resulted in a marked decrease in the number of erythroid cells without affecting cell viability. In addition, in the absence of macrophages, erythroid cells matured to the late erythroblast stage but they failed to enucleate, suggesting that the presence of macrophages in the cultures promotes erythroid proliferation and maturation leading to enucleation. Macrophages are known to produce a number of growth factors that act both as positive and negative regulators of erythropoiesis.13 However, when we reconstituted macrophage-depleted cultures with either macrophage-conditioned medium or a mixture of growth factors produced by macrophages (data not shown), erythroid cell proliferation and maturation (as evidenced by the number of enucleated cells) remained considerably less compared with the level found when intact macrophages were present. These results suggest that a close association of erythroblasts (of varying maturity) with macrophages promotes proliferation and enucleation of erythroid cells. An association of macrophages with immature erythroblasts (proerythroblasts and basophilic normoblasts; Fig 1) is likely to increase their proliferative capacity, whereas an association of macrophages with late-stage erythroblasts, as shown in Fig 2A and B, is likely to affect their enucleation because the proliferative capacity of these cells is minimal.
To examine the molecule(s) involved in erythroblast-macrophage contacts, we have used a cell attachment assay involving the incubation of either intact or solubilized surface-labeled erythroblasts with macrophage membranes. The results presented here show that erythroblast surface proteins specifically attach to a 30-kD protein in macrophage membranes. Furthermore, an apparently similar protein of 30 kD is also present in erythroblasts and is involved in erythroblast-erythroblast interaction in addition to erythroblast-macrophage interaction. Hence, the 30-kD protein is involved in homophilic interactions and is capable of binding homotypic and heterotypic cells. Our results show that the 30-kD protein is exposed on the membrane of macrophages and erythroblasts and could be released from membranes by high salt, but remains bound with the Triton-insoluble skeletal fraction, suggesting that the 30-kD adhesive protein is attached to the membrane skeleton via a transmembrane component. We further show that cell-cell adhesion mediated by the 30-kD protein is functionally operated by a Ca²⁺-independent mode and is specifically inhibited by heparin. Hence, the ~30-kD protein from macrophage and from erythroblast membranes seems to have similar properties but further characterization is necessary to determine if indeed they are the same adhesion protein. This question is presently under investigation.

A number of other adhesive molecules with very different molecular weights, cellular localization, and binding specificities are present in hematopoietic tissues, eg, hemoecin (60 kD), sialoadhesin (185 kD), vascular adhesion molecule-1 (VCAM) (100 kD), and TSP (450 kD). Hemoecin, an extracellular matrix protein is specific for the adhesion of immature granulocytes; Sialoadhesin, a membrane glycoprotein of stromal macrophages interacts selectively with myelomonocytic precursors; a homologue of VCAM-1 is expressed by bone marrow stromal cells and is a ligand for VLA-4; and TSP, an extracellular matrix protein is synthesized by both macrophages and erythroblasts and is capable of binding primary hematopoietic progenitor cells. Recently, a protein of molecular weight 37 kD has been reported in murine bone marrow stromal cells and murine hematopoietic progenitor cells. This protein is not only

Table 2. Effect of Heparin on Erythroid Cell Proliferation and Maturation

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<th>Amount of Heparin Added (U/mL)</th>
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</tbody>
</table>

Increasing amounts of heparin were added to cultures on day 4 of the second phase. On day 15 of the second phase, number of erythroid cells as judged by benzidine staining and the number of enucleated cells (from Wright-Giemsa cytocronged preparations) was counted. Percent enucleation represents values relative to total cell number (the number of benzidine-positive cells is approximately 90% of total cell population in all the cultures). This experiment was performed three times with similar results.
different from our adhesive protein in size but also in its solubility in Triton X-100, its Ca\(^{2+}\)-dependent mode of adhesion, and its nonheparin binding property.

Taken together, we have shown the formation of erythroblast islands in cultures of peripheral blood-derived progenitors. The removal of macrophages from the cultures or reconstitution with macrophage-conditioned medium resulted in a marked decrease in the number of erythroid cells as well as their stage of maturation, suggesting that the association of macrophages with erythroblasts promotes proliferation and maturation of erythroid cells. In this context, it has been shown that the proliferation and development of hematopoietic stem cells takes place in close association with marrow stromal cells\(^\text{14,15}\) and has been well established that the growth factors synthesized by stromal cells do not need to be free in the surrounding medium to act on their target cells. In fact, growth factors anchored to the membrane of the stromal cells are biologically active and act as ligands for hematopoietic precursors through specific receptors.\(^\text{20}\)

It will be interesting to examine if this mechanism is involved in erythroblast-macrophage association. Furthermore, using a cell attachment assay involving incubation of either intact or solubilized surface-labeled cells with membrane proteins, we have shown the presence of (a) novel 30-kD heparin-binding protein(s) on the surface of macrophages and erythroblasts. This (these) protein(s) mediate(s) erythroblast-macrophage and erythroblast-erythroblast interactions in a homophilic manner.

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REFERENCES


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The association of erythroblasts with macrophages promotes erythroid proliferation and maturation: a 30-kD heparin-binding protein is involved in this contact

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