Mast Cell Growth Factor Modulates CD36 Antigen Expression on Erythroid Progenitors From Human Bone Marrow and Peripheral Blood Associated With Ongoing Differentiation

By Joost Th.M. de Wolf, Erik W. Muller, Dick H. Hendriks, Ruud M. Halie, and Edo Vellenga

To study the differentiation process of erythroid progenitors from normal human bone marrow and peripheral blood, CD34+/CD36- sorted cells were cultured in the presence of Erythropoietin (Epo) and Epo plus mast cell growth factor (MGF). The CD34+/CD36- cell fraction from bone marrow supported 74 ± 33 erythroid burst forming units (BFU-E)/10^6 cells (mean ± SD, n = 4) in the presence of Epo, which increased 2.1-fold by coculturing with MGF. However, erythroid colony-forming units (CFU-E) were not cultured from the CD34+/CD36- cell fraction. In contrast, the CD34+/CD36- cell fraction supported CFU-Es in the presence of Epo (152 ± 115/10^6) or Epo plus MGF (189 ± 112/10^6), whereas BFU-Es were hardly noticed. However, the transition of the BFU-E to CFU-E was observed by incubating CD34+/CD36- cells (104/100 μL) in suspension with Epo plus MGF for 7 days followed by Epo in the colony assay. This was reflected by the appearance of CD34+/CD36+/Glycophorin A+/CD14- cells. In addition, high numbers of CFU-Es (1,000 ± 150, n = 4) were cultured from this cell fraction. In contrast to bone marrow erythroid progenitors, no peripheral blood BFU-Es were cultured from either the CD36+ or CD36- fraction, whereas BFU-Es were predominantly present in the CD36- fraction. However, the CD34+ progenitor cell from peripheral blood did have the intrinsic capacity to differentiate to CFU-Es because CD34+/CD36+ cells incubated with Epo plus MGF for 7 days and followed by Epo in the colony assay, supported high numbers of CFU-Es (1,200 ± 400, n = 3). To study whether additional growth factors have similar effects on erythroid progenitors, experiments were performed with interleukin 1 (IL-1), IL-3, and IL-6. IL-1 and IL-6 did not modulate the Epo supported proliferation and differentiation. In contrast, IL-3 in the presence of Epo did support CFU-Es, from CD34+/CD36- cells after 7 days in suspension culture. However, flow cytometry analysis showed that Epo plus IL-3 not only supported CD34+/CD36+/Glycophorin A+ cells but also CD34+/CD14+ cells, indicating the differentiation along different cell lineages. In summary, the data show a phenotypic distinction between bone marrow and peripheral blood erythroid progenitors with regard to CD36 expression. In addition, the results suggest that Epo plus MGF or IL-3 and preincubation in suspension culture are prerequisites for the transition of the BFU-E to the CFU-E.

CD36 IS AN INTEGRAL membrane protein present on platelets, endothelial cells, monocytes, mast cells, and some epithelial cells, and can be considered as one of the cell surface receptors for thrombospondin (TSP). The CD36-TSP interaction is involved in mediating platelet-platelet and platelet-monocyte adhesion, as well as cell attachment to the extracellular matrix. In addition, CD36 functions as a receptor for the adhesion of Plasmodium falciparum-infected red blood cells to endothelial cells. Finally, CD36 has been shown on erythroid and megakaryocytic progenitor cells. With regard to the erythroid lineage it has been shown that CD36 is present on the more mature erythroid progenitor cell from normal bone marrow (BM) and human cord blood mononuclear cells, suggesting that CD36 can be considered as a differentiation marker for the erythroid lineage. Recently, the cytokine mast cell growth factor (MGF) has been identified. It affects the proliferation and differentiation of the erythroid lineage. MGF by itself did not stimulate erythroid burst forming unit (BFU-E) colony formation but enhanced the number and size of BFU-Es stimulated by erythropoietin (Epo), Epo plus interleukin-3 (IL-3), and Epo plus granulocyte macrophage colony stimulating factor (G-CSF) (5 μg/kg) in patients with multiple myeloma.

**MATERIALS AND METHODS**

**BM and Blood**

BM and peripheral blood from healthy controls were obtained after informed consent. In addition, peripheral blood cells were isolated during the regeneration phase after high-dose cyclophosphamide (6 g/m²) in the presence of granulocyte colony-stimulating factor (G-CSF) (5 μg/kg) in patients with multiple myeloma.

**Culture Conditions**

Mononuclear cells (MNC) were isolated by density gradient centrifugation using Lymphoprep (Nycomed AS, Oslo, Norway). The MNC were incubated overnight in RPMI 1640 culture medium (GIBCO Ltd, Paisley, UK) and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) in a plastic culture dish at 37°C in 5% CO₂ to allow cell adherence. Nonadherent cells were collected from the dish and contamination with adherent cells was <4% as shown by CD34+/CD36- cells incubated with Epo plus MGF or IL-3 show a strong increase in CD36 antigen expression. These changes are associated with the appearance of CFU-E.

From the Department of Hematology, University Hospital Groningen, The Netherlands.

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Address reprint requests to Edo Vellenga, MD, Department of Hematology, University Hospital, Oostersingel 59, 9713 EZ Groningen, The Netherlands.

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surface marker analysis (CD14; Becton Dickinson, Mountain View, CA). In separate experiments CD34 and CD36 enriched progenitor cell population from bone marrow and peripheral blood were used. Nonadherent cells were incubated with a phycoerythrin-labeled anti-CD34 monoclonal antibody (MoAb) (HPCA-2; Becton Dickinson) and a fluorescein isothiocyanate-labeled anti-CD36 MoAb (OKM-5, Ortho Diagnosticum, Raritan, NJ). After washing, the cells were sorted with a fluorescence-activated cell sorter (FACStar, Becton Dickinson) in CD34+/CD36-, CD34-/CD36+, CD34+/CD36+, and CD36- and CD36+ subpopulations. In addition, nonadherent monoclonal antibodies from peripheral blood were sorted in CD36+/CD36- and CD34+ cell fractions. The purity of the sorted cell fraction was greater than 95% as tested by re-analysis.

CFU-E and BFU-E assays were performed according to Iscove as previously described.3 CD36+/CD34+ sorted cell fractions were added to 1 mL of culture medium consisting of 1% methylcellulose (Dow Chemical Company, Midland, MI), 20% heat-inactivated FBS, 10% deionized bovine serum albumin (BSA) (Cohn Fraction V; Sigma Chemical Co, St Louis, MO), 0.1% α-thioglycerol (Sigma), and Iscove’s medium (IMDM, Flow Laboratories, Rockville, MD). All cultures were performed in 24-well culture plates (Costar Co, Cambridge, MA) and kept at 37°C in a 5% CO₂ air humidified atmosphere. CFU-Es were scored on day 7 according to their characteristic morphology and BFU-Es were scored on day 14 by using an inverted microscope.

Suspension cultures. CD34+/CD36-, 1 × 10⁶, were incubated in duplicates in 100 µL Iscove’s medium (GIBCO) with 10% FBS with Epo, MGF, and Epo plus MGF. After 3, 5, and 7 days total cell numbers were re-analyzed regarding the expression of CD34, CD36, CD14, and Glycophorin A (GP A, Clone 143; CLB, Amsterdam, The Netherlands). In addition, the cells were washed three times in RPMI (160) medium and used for the in vitro CFU-E colony assay by culturing the cells in the presence of Epo. Similar experiments were performed in serum-free culture conditions. This medium consists of IMDM supplemented with deionized BSA, BSA-absorbed cholesterol, iron-saturated transferrin, insulin, nucleosides, sodium pyruvate, L-glutamine, inorganic salts, and trace elements (all from Sigma).17

Growth factors. Optimal proliferation of erythroid progenitors in the presence of Epo and additional CSFs was examined for the following concentrations: recombinant human Epo 1 U/mL (Amgen, Oakland, CA), MGF 1 U/mL (specific activity 1 × 10⁴ U/mg, Immunex, Seattle, WA), and IL-3 10 ng/mL (Genetics Inst, Cambridge, MA). IL-1β (Immunex) and IL-6 (C.L.B., Amsterdam, The Netherlands) were used at a concentration of 100 U/mL.

Statistical analysis. The standard t-test for paired samples was used to determine the significance of the data.

RESULTS

Effects of Epo and MGF on CD34/CD36 Sorted Cells From Normal BM

By using an MoAb against the CD36 antigen an almost definite separation of BFU-E and CFU-E colony formation could be obtained by culturing the cells in the presence of Epo. CD36- sorted BM cells contained 786 ± 370 CFU-Es/10⁶ CD36- cells (mean (x ± SD, n = 3)) versus 4 ± 6 CFU-Es/10⁶ in the CD36+ cell fraction. However, the CD36+ cell fraction did contain erythroid colony-forming cells because 143 ± 49 BFU-Es/10⁶ (x ± SD, n = 3) were shown. Subsequently the nonadherent fraction from BM cells were sorted in CD34+/CD36-, CD34+/CD36+, and CD34-/CD36- cell fractions and cultured in the presence of Epo and Epo plus MGF. The proportion of each subset (+/-, -/+,-/-) in the total population was 1.55% ± 0.7%, 30.9% ± 18%, and 65.4% ± 17%, respectively (x ± SD, n = 7). Cells cultured with MGF alone did not show erythroid colony formation (data not shown). CD34+/CD36- sorted cells contained 74 ± 33 BFU-E/10⁶ (x ± SD, n = 4) in the presence of Epo and 159 ± 17 BFU-E/10⁶ in the presence of Epo plus MGF (P < .01, Table 1). In contrast, almost no CFU-E colony formation was noticed independent of Epo or Epo plus MGF, 1 ± 1, 1 ± 1, respectively.

The CD34+/CD36- cell fraction contained 3 ± 4 BFU-E/10⁶ (x ± SD, n = 4) in the presence of Epo and 16 ± 13 BFU-E in the presence of Epo plus MGF (NS, not significant). In contrast, 152 ± 115 CFU-E/10⁶ (x ± SD, n = 4) were shown and 180 ± 112 CFU-Es in the presence of Epo plus MGF (NS). Finally, it was shown that the CD34+/CD36+ cell fraction did not contain CFU-E colony-forming cells either in the presence of Epo or Epo plus MGF, whereas few BFU-E colony-forming cells were noticed (2 ± 2/10⁶ cells) in the presence of Epo or Epo plus MGF. These data suggest that the expression of CD36 is associated with the development of the CFU-E colony-forming cell. To underline this observation cell suspension cultures were performed with CD34+/CD36- sorted cells (10⁷/100 µL) and cultured in the presence of medium, Epo, MGF, and Epo plus MGF for 7 days. Re-analysis of the antigen expression on the sorted cell population after 3, 5, and 7 days of culture showed that in the presence of Epo 22% ± 6% of the cells (x ± SD, n = 4) were CD34+/CD36-, whereas 12% ± 1% of the cells (x ± SD, n = 4) were CD34+/CD36+. A representative experiment is depicted in Fig 1. Similar results were obtained with MGF (data not shown). However, by incubating the cells in Epo plus MGF a strong increase in the CD36 expression was noticed. After 7 days of suspension culture in Epo plus MGF 6% ± 3% of the cells were CD34+/CD36- and 79% ± 6% of cells were CD34+/CD36- (x ± SD, n = 4). Similar experiments were performed using serum-free culture conditions. The results showed that after 7 days of culturing in the presence of Epo, 20% of the cells were CD34+/CD36- and 21% of the cells CD34+/CD36-, whereas in the presence of Epo plus MGF less than 6% of the cells were CD34+/CD36- and greater than 78% of the cells were CD34-/CD36-. The increased expression of CD36 was associated with a strong expression of GP A, whereas the expression of CD14 antigen was almost negative (Fig 2). In a separate set of experiments 10⁶ CD34+/CD36- were incubated in suspension with Epo plus MGF for 7 days, washed three times with medium, and cultured with Epo in the colony assay. After 7 days 1,000 ± 150 CFU-Es (x ± SD, n = 3) were noticed, whereas the original CD34+/CD36- cell population did not contain CFU-E colony-forming cells when cultured only with Epo plus MGF in the colony assay. In addition, the suspension culture experiments were repeated with Epo or MGF alone. In these cases no CFU-Es were found. To study whether additional growth factors would have comparable effects on the erythroid differentiation, CD34+/CD36- sorted cells were incubated in suspension with Epo in the presence or absence of IL-1, IL-6, or IL-3. After 7 days of culture with Epo plus IL-1 or IL-6 no change in the antigen expression of CD34 and CD36 was observed compared with the effects of Epo alone (data not shown). Costimulation with IL-3 did induce a marked increase in
Fig 1. Flow cytometry analysis of CD34 and CD36 antigen expression on CD34⁺ sorted BM cells (10⁵/100 µL). CD34 and CD36 expression was analyzed after 3, 5, and 7 days in suspension culture in the presence of medium, Epo, and Epo plus MGF. The original sorted cell population contained greater than 98% CD34⁺/CD36⁻ cells.

Fig 2. Flow cytometry analysis of CD34, CD36, CD14, and Glycophorin antigen expression on CD34⁺/CD36⁻ sorted BM cells (10⁵/100 µL) after 7 days of suspension culture in the presence of Epo plus IL-3 and Epo plus MGF. The original sorted cell population contained greater than 98% CD34⁺/CD36⁻ cells.
CD36 expression. However, the degree of CD36 expression was less pronounced compared with the effects of Epo plus MGF. FACS analysis of the total cell population after 7 days of suspension culture showed 2.5% ± 0.7% CD34+/CD36+; 51% ± 9% CD34+/CD36--; 38% ± 9% GpA+/CD14--; and 21% ± 9% CD14+ cells (x ± SD, n = 3) in the presence of Epo plus IL-3 and 1.3% ± 0.9% CD34+/CD36--; 85% ± 8% CD34-/CD36--; 82% ± 7% GpA+/CD14--; and 5% ± 2% CD14+ cells in the presence of Epo plus MGF. A representative experiment is depicted in Fig 2. In addition, IL-3 affected the differentiation of erythroid progenitors. CFU-Es were cultured from CD34+/CD36- cells, when the sorted cell population (10^6/mL) were stimulated in suspension with Epo plus IL-3 for 7 days followed by Epo in the colony assay (400 ± 80 CFU-Es).

**Effects of Epo and MGF on CD34+/CD36- Sorted Cells From Peripheral Blood**

Nonadherent peripheral blood progenitors were sorted in CD36+/-- fractions and cultured in the presence of Epo and Epo plus MGF. No CFU-E colony-forming cell was noticed in the CD36+ and CD36- cell fraction, whereas BFU-Es could be cultured from both fractions (Table 1). By culturing CD36+ cells, 24 ± 17 BFU-Es/10^3 (x ± SD, n = 7) were demonstrated in the presence of Epo and 40 ± 33 (x ± SD, n = 4, NS) in the presence of Epo plus MGF. In the CD36- cell fraction less BFU-E colony-forming cells were shown: 2 ± 2 BFU-Es/10^3 (n = 8) in the presence of Epo and 3 ± 4 (n = 8) in the presence of Epo plus MGF. To show that the CD34+ progenitor cell from peripheral blood has the intrinsic capacity to differentiate to the BFU-E colony-forming cell, cell-suspension culture assays were performed. 10^6 CD34+ sorted cells/100 μL were incubated in suspension in the presence of Epo plus MGF for 7 days, and washed and cultured in the presence of Epo. After 7 days 1,200 ± 400 CFU-Es were noticed (n = 3). In addition, re-analysis of the antigen expression on the CD34+ cells was performed after 7 days of suspension culture with Epo plus MGF. The results show that less than 6% of the cells were CD34+/CD36+; whereas greater than 80% of the cells were CD34-/CD36-. In addition, the cells were CD14-/GpA- (data not shown).

Finally, CD34/CD36 sorted cells were isolated during the regeneration phase after high-dose chemotherapy and G-CSF to study whether a change did occur in the composition of peripheral blood erythroid progenitors. CD34+/CD36+, CD34-/CD36-, or CD34+/CD36- sorted cells (10^6/well) cultured in the methylcellulose culture assay in the presence of Epo plus MGF did not support BFU-E colony formation. However, by incubating the CD34+ cells (10^6/100 μL) with Epo plus MGF for 7 days followed by Epo in the colony assay, CFU-Es were noticed (1,300 ± 300). This was associated with a change in the antigen expression after 7 days in suspension culture with Epo and MGF. Of the total cell population 5% ± 3% of the cells were CD34+/CD36- (x ± SD, n = 3), whereas 77% ± 6% were CD34+ after 7 days of culture (Fig 3). In addition, 77% ± 4% of the cells were GpA-. Similar experiments were performed with Epo plus IL-3 and showed that 51% ± 6% of the cells were CD34+ after 7 days and 38% ± 7% were GpA-, indicating a less pronounced effect of Epo plus IL-3 on the erythroid lineage.

**DISCUSSION**

The present study shows that the BFU-E colony-forming cell from BM can be cultured from the CD34+/CD36- cell population, whereas the BFU-E colony-forming cell originates from the CD34+/CD36+ cell fraction. In contrast, circulating BFU-Es are found predominantly in the CD36+ faction. This phenotypic distinction between BM and peripheral blood erythroid progenitors is in accordance with findings of Emerson et al who showed that the MoAb EP-3 detected 60% to 90% of the BM-derived BFU-E but none of the circulating BFU-E. Our previous findings of differences in responsiveness to growth factors like IL-4 suggested that the difference between peripheral and BM erythroid progenitors is not merely phenotypic but also functional. Like fibronectin-receptors, CD36 is often found during the differentiation process of erythroid progenitors. CD36 is rarely found on primitive BFU-E and fully expressed on CFU-Es. At the end of the CFU-E stage, the CD36 antigen expression is less pronounced, hence reticulocytes and erythrocytes show low expression of CD36. The data suggest that loss of CD36 antigen expression in concert with loss of additional adhesion molecules, like fibronectin, allows the migration of mature cells to the circulation. However, the CD36 antigen cannot be the most relevant adhesion molecule because the BFU-E colony-forming cell in the peripheral blood is especially noticed in the CD36+ cell fraction. However, the expression of the CD36 antigen may play some role because erytrocyes infected with *P. falciparum* show an increased adhesion to endothelial cells caused by the increased expression of the CD36 antigen on the cell sur-

**Table 1.**

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<td>CD34+/CD36+</td>
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<td>Epo</td>
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<td>CFU-E</td>
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<td>BFU-E</td>
<td>74 ± 33</td>
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CD34+/CD36+ (10^6/mL), CD34+/CD36- (10^6/mL), CD34-/CD36- (10^6/mL) sorted BM cells and CD34+/CD36+ (2 × 10^6/mL) sorted peripheral blood cells were cultured in the methylcellulose culture assay in the presence of Epo and Epo plus MGF. After 7 days the number of BFU-Es were scored and after 14 days the BFU-E were scored. The results of BM cultures represent mean ± SD of four experiments and the results of peripheral blood represent mean ± SD of seven experiments.
MGF AND CD36 ANTIGEN EXPRESSION

Fig 3. Two-color analysis of CD34+ sorted peripheral blood cells after 0 and 7 days in suspension culture with Epo plus MGF with FITC anti-CD36 and PE anti-CD34.

This study further underscores the relevance of MGF for the proliferation and differentiation of erythroid progenitors. First, MGF increases the number of BFU-E colony-forming cells from normal BM in the presence of Epo, which was not observed in the CFU-E assay. Second, MGF was a critical factor for the transition of the BFU-E to the CFU-E colony-forming cell. MGF was not the only growth factor that affected the differentiation of the erythroid lineage. IL-3 in the presence of Epo also supported CFU-Es from CD34+/CD36- sorted cells. However, in contrast to MGF, the differentiation of CD34+/CD36- in the presence of Epo plus IL-3 resulted in less CD34-/CD36+/GpA- cells and in cells belonging to different cell lineages.

In addition, the presence of MGF or IL-3 was not the only prerequisite for the transition of BFU-E to the CFU-E colony-forming cell. Cell-cell contact is also of importance because CD34+ cells cultured in the clonal methylcellulose assay in the presence of Epo plus MGF or IL-3 did not support CFU-Es unless the CD34+ cells were pre-incubated with EPO plus MGF or IL-3 in suspension. The data indicate that in addition to cytokines, additional factors determine the differentiation process of erythroid progenitor cells. Similar results were recently obtained by Papayannopoulou et al with cell suspension cultures. It is conceivable that the suspension culture assay mimics the BM microenvironment and allows the differentiation of erythroid progenitor cells, whereas CD34+ cells circulating in the peripheral blood and lacking the microenvironment are unable to undergo the transition of BFU-E to CFU-E colony-forming cell. However, the peripheral blood CD34+ cell retains the intrinsic capacity to differentiate to CFU-Es unless the appropriate conditions are provided. In addition, the differentiation process in vitro is associated with the loss of the CD34 antigen and the appearance of the CD36 antigen, which reflects the normal differentiation process of the erythroid progenitor in the BM.

Finally, it was shown that the circulating CD34+ cell present in high numbers after high-dose chemotherapy and G-CSF had the same properties as the normal circulating erythroid progenitor. The CFU-E colony-forming cell was not mobilized during the regeneration phase with G-CSF suggesting a selective mobilization of early erythroid progenitors from BM to peripheral blood.

In summary, the data show a phenotypic distinction between BM and peripheral blood erythroid progenitors with regard to CD36 expression. In addition, the results suggest that the differentiation of BFU-E to CFU-E colony-forming cell in the BM depends on cytokines as well as the BM microenvironment. The absence of CFU-Es in the peripheral blood may be caused by the lack of one of these factors.

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Mast cell growth factor modulates CD36 antigen expression on erythroid progenitors from human bone marrow and peripheral blood associated with ongoing differentiation

JT de Wolf, EW Muller, DH Hendriks, RM Halie and E Vellenga