Leukocyte function associated antigen 1 (LFA-1) is an adhesion molecule indispensable in immune and inflammatory reactions, but its role in hematopoiesis remains obscure. Since LFA-1 is predominantly expressed by leukocytes, it is considered as a marker of late stage stem cell maturation when expressed on CD34+ bone marrow cells, and represents more mature hematopoietic progenitor cells. We observed that freshly isolated CD34+ bone marrow cells express LFA-1, and that the level of expression is highly variable. Interestingly, the expression of the LFA-1 specific activation epitope L16 on these cells is low, even after culture. This demonstrates that LFA-1 is not activated, as was confirmed by low adhesion to ICAM-1. Culturing sorted CD34+LFA-1+ cells in single cell per well assays in medium supplemented with SCF, Epo, IL-3, IL-6, GM-CSF, and G-CSF revealed that they gave rise to dispersed macrophage-like colonies, supporting the notion that CD34+LFA-1+ cells indeed consist of a mature committed cell population. In contrast, sorted CD34+LFA-1- cells had high proliferative potential and developed into large multilineage colonies within 14 days of culture. Unanticipated, in time course experiments we observed that these CD34+LFA-1- cells expressed LFA-1 within 24 hours upon culture. This induction was neither caused by the monoclonal antibody used to tag CD34 cells, nor dependent on growth factors present in the medium. These findings demonstrate that two populations of CD34+LFA-1+ cells can be discriminated: leukocyte lineage committed CD34+ cells in freshly isolated bone marrow cells, and multipotent CD34+ cells that acquired LFA-1 upon in vitro culture. These in vitro findings support the hypothesis that once contacts with bone marrow stroma are lost, LFA-1 is upregulated by default, due to the lack of negative regulating signals from stromal cells. This might also explain the widely variable expression of LFA-1 as a result of crowding of cells in the bone marrow with subsequent loss of contact with stroma and upregulation of LFA-1, providing those cells with adhesion receptors enabling migration in the periphery.

The role of the β2-integrins in, however, less well understood. Although β2-integrins probably are not likely involved in maintaining the association of hematopoietic cells with the stroma,22 since antibodies directed to β2-integrins were unable to mobilize progenitors into the bloodstream,22,28 recent data showed the interaction of β2-integrins with its ligands are involved in several cell-cell adhesion and signaling processes,29,30 as well as in the production of growth inhibiting cytokines.31 Recent evidence showed that the β2-integrin pathway is activated by signals mediated by CD34, resulting in enhanced homotypic cell adhesion.23,24 In this study we performed a detailed analysis of individual CD34+LFA-1+ and CD34+LFA-1- cells to establish the potential role of LFA-1 on growth and differentiation of CD34+ bone marrow cells. In this analysis we included expression of the LFA-1 activation epitope L16, which is a marker for...
a clustered distribution of LFA-1, a prerequisite for strong adhesion. The results demonstrate that LFA-1 was expressed on part of freshly isolated CD34+ cells in an inactive state and coincided with a mature progenitor cell population. Interestingly, CD34+LFA-1- cells acquire LFA-1 during in vitro culture, but despite this upregulation of LFA-1 in vitro, those cells still comprise a pluripotent cell population suggesting that once microenvironmental factors are lacking LFA-1 is upregulated by default.

MATERIALS AND METHODS

Cells. Bone marrow (BM) cells were obtained from normal allogeneic BM donors after informed consent. BM was diluted with phosphate buffered saline (PBS) and mononuclear cells were isolated by Ficoll-Hypaque density centrifugation (specific gravity, 1.077). After washing, the cells were resuspended with anti-CD34 monoclonal antibody (MoAb) coated magnetic beads (Dynal, Oslo, Norway) for 60 minutes at 4°C with gentle rotation. CD34 positive cells were collected by magnetic force and subsequently released from the CD34 beads with DETACHaBEAD (Dynal). Isolated cells were free from beads and their purity exceeded 90% as determined with flow cytometric analysis. Batches of 200,000 CD34 positive cells were frozen and stored in liquid nitrogen until use.

Staining with MoAb. CD34+ cells were thawed and subsequently washed with PBS containing 10% normal human serum. Appropriate dilutions of the MoAbs (anti-CD34 labeled with phycoerythrin: HPCA-2; Becton Dickinson, San Jose, CA; anti-LFA-1 labeled with FITC: SPV-17, directed to the αL chain of LFA-1; NKK-L16, directed to an activation epitope on the αL chain of LFA-1) were added. For determining L16 expression, cells were suspended in 50 μL PBS containing 10% normal mouse serum treated with Chelex-100 (BioRad, Richmond, CA) to remove divalent cations. Incubations with control antibodies labeled with either FITC or phycoerythrin were run in parallel. Cells were incubated with the MoAbs for 45 minutes at 0°C. After washing in PBS the cells were suspended in 1 mL PBS and used for cell sorting.

Cell sorting. Dual color membrane immunofluorescence was performed by simultaneous labeling with FITC and PE labeled MoAbs. Fluorescence was analyzed using a Coulter Epics Elite (Coulter, Hialeah, FL) flow cytometer with an argon laser tuned at 488 nm. Light-scatter was set such to exclude subcellular particles and dead cells. Single cells positive for CD34 and either positive or negative for LFA-1 were sorted in a 96-well plate at a density of 10,000 cells per well. The cells were cultured over 48 hours in IMDM medium containing 10% FCS. The cells were collected and stained with CD34-PE and LFA-1-FITC. Single LFA-1 positive and negative cells were sorted in a 96-well plate containing IMDM medium and growth factors and growth was recorded over 14 days.

Phenotypic analysis of growing colonies. Nine days after initiating the single cell per well culture the growing cells were collected and stained with Glycoporphin PE (Immunotech, Marseille, France) CD15-PE and CD14-FITC (Becton Dickinson, San Jose, CA) to establish putative multilineage potential.

Adhesion assays. Cells were isolated from freshly collected normal bone marrow and subsequently sorted into a CD34+LFA-1- and a CD34+/LFA-1+ population. Part of those cells was used directly in adhesion assays while the remaining cells were cultured for 2 days before the adhesion assays were performed. ICAM-1 fusion protein consisting of the five extracellular domains of ICAM-1 fused to a human IgG1 Fc fragment was used in the adhesion assays. Twenty thousand sorted cells were added to immobilized ICAM-1 Fc chimeric protein and incubated for 30 minutes at 37°C. After washing, the number of adhered cells was determined. Adhesion properties of activated LFA-1 were deduced from assays in which 50 nmoL PMA was added during incubation.

RESULTS

Expression of LFA-1 by CD34 positive cells. The results in Fig 1 demonstrate that the expression of LFA-1 on CD34 positive cells was highly variable as found for 18 different donors. The percentage LFA-1 positive cells ranged from 5% to 70% with a mean of 33%. No correlation with the age of the donor was found. The LFA-1 and CD34 double staining fluorescence pattern is shown in Fig 2. The upper row shows the forward and side scatter (frame 1), double fluorescence obtained with control antibodies (frame 2), and...
double fluorescence obtained with LFA-1-FITC and CD34-PE antibodies (frame 3). Staining for LFA-1 expression was generally dim as exemplified in the upper panel of Fig 2. The lower row shows the results obtained with a different donor. Furthermore, CD34⁺ cells lacked expression of the L16 activation epitope demonstrating the expression of an inactive form of LFA-1 (data not shown).

**Growth characteristics of LFA-1 positive and negative cells.** LFA-1 expression was found to correlate to more mature progenitor cells when cultured on allogeneic stromal cells. To gain insight into possible effects of the stromal layer on the growth characteristics, single CD34⁺LFA-1⁻ as well as CD34⁺LFA-1⁺ cells were cultured for 14 days in liquid medium containing several cytokines. From the growth curve (Fig 3), it is concluded that the majority of the CD34⁺LFA-1⁻ cells grew rapidly with a doubling time of approximately 24 hours. The same growth characteristics were found for five individual donors. The growth potential of the CD34⁺LFA-1⁻ cells was much less. After reaching 50 to 100 cells the cells stopped growing any further. Further analysis demonstrated that the majority of the CD34⁺LFA-1⁻ cells belong to the category representing the largest colony size (Fig 4). On the contrary, the majority of the CD34⁺LFA-1⁺ cells belong to the category that grows to less than 100 cells (Fig 4).

**Morphology of the growing cells.** Because LFA-1 has been implicated in lineage commitment, the morphology of both CD34⁺LFA-1⁻ and CD34⁺LFA-1⁺ colonies was noted using the video image. CD34⁺ cells that do not express LFA-1 grew to be very large colonies (>1,000 cells). However, CD34⁺LFA-1⁺ cells grew rapidly with a doubling time of approximately 24 hours. The same growth characteristics were found for five individual donors. The growth potential of the CD34⁺LFA-1⁻ cells was much less. After reaching 50 to 100 cells the cells stopped growing any further. Further analysis demonstrated that the majority of the CD34⁺LFA-1⁻ cells belong to the category representing the largest colony size (Fig 4). On the contrary, the majority of the CD34⁺LFA-1⁺ cells belong to the category that grows to less than 100 cells (Fig 4).
Fig 4. Growth of CD34 positive bone marrow cells. Cells were sorted based on their LFA-1 expression. Growth was scored as the highest number of cells obtained during 14 days of culture in medium containing cytokines. Three categories were defined: 1, colonies containing more than 1,000 cells (I); 2, colonies containing more than 100 but less than 1,000 cells (II); and 3, colonies containing less than 100 cells (III). The results for the different categories were expressed as a percentage of the total number of wells containing growing cells.

Donor 1

<table>
<thead>
<tr>
<th>LFA+</th>
<th>LFA-</th>
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<tbody>
<tr>
<td>&gt;1000</td>
<td>&lt;100</td>
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number of cells/colony

Donor 2

<table>
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<th>LFA+</th>
<th>LFA-</th>
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<tr>
<td>&gt;1000</td>
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<td>0</td>
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number of cells/colony

Donor 3

<table>
<thead>
<tr>
<th>LFA+</th>
<th>LFA-</th>
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<tr>
<td>&gt;1000</td>
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number of cells/colony

Donor 4

<table>
<thead>
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<th>LFA+</th>
<th>LFA-</th>
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number of cells/colony

different sizes were noted as exemplified by the upper row of Fig 5. Erythroid, granulocytic, monocytic, as well as mixed colonies were found as deduced from staining pooled colonies with MoAbs directed to glycophorin A, CD15, and CD14. A completely different picture was seen when analyzing the LFA-1+ cells. The size of these colonies was very small. The majority of the cells grew to be less than 100 cells. The cells were dispersed throughout the well and had an irregular appearance, resembling macrophages (Fig 5, lower row). Essentially, the same growth pattern was observed for all tested donors.

Induction of LFA-1 during culture. Next we investigated whether in vitro culture affected LFA-1 expression. CD34+ cells were stained with an antibody directed toward LFA-1, and subsequently the LFA-1 negative cells sorted in a 24-well plate. Surprisingly, the majority of the cells acquired LFA-1 expression during culture for 48 hours. The level of induced expression of LFA-1 was variable (data not shown). Up to 90% of the CD34+LFA-1- cells acquired LFA-1 during culture. To investigate whether induction of LFA-1 is caused by the growth factors present, we cultured CD34+LFA-1- cells in the presence and absence of growth factors. Essentially the same results were observed (Fig 6). These findings suggest that induction of LFA-1 is likely due to the lack of factors provided by the stroma (soluble factors, adhesion molecules). Furthermore, to exclude that MoAbs against CD34 used to isolate the cells induced LFA-1 expression, we labeled freshly isolated bone marrow cells with anti-LFA-1 antibodies and sorted the LFA-1 negative fraction. After culturing for 48 hours, the LFA-1 expression on the CD34+ subpopulation was determined after staining with anti-CD34 antibodies. LFA-1 expression on these cells was comparable with cells from the same donor obtained after 48 hours culture initiated with CD34+LFA-1- cells. These findings demonstrate that induction of LFA-1 is not due to prior labeling with CD34 antibodies.

Expression of the L16 epitope and adhesion of LFA-1 expressing CD34+ cells. The activation status of LFA-1 expressed on CD34+ cells was deduced from staining with MoAb NKI-L16. The results are shown in Table 1. CD34+ cells cultured for 48 hours in medium were regarded as L16- suggesting they express an inactive form of LFA-1. Likewise, hardly any adhesion was found for CD34+LFA-1+ cells to ICAM-1. The CD34+LFA-1- cells that became partially LFA-1 positive during culture for 48 hours showed hardly any adhesion to ICAM-1. In accordance those cells are L16- (Table 1). The functional capability of LFA-1 was demonstrated by adhesion assays in the presence of PMA (Fig 7). For both cell populations adhesion was clearly demonstrated. Adhesion of resting PBL to ICAM-1 is also di-
Fig 5. Morphology of the growing cells. Video image of representative wells in which either CD34'LFA-1- or CD34'LFA-1' bone marrow cells were seeded.

Comparison of growth properties of cells that do or do not acquire LFA-1 expression in culture. Although LFA-1 cells acquired LFA-1 expression in culture, large differences in growth characteristics between LFA-1+ and LFA-1- cells were observed, indicating that induction of LFA-1 does not coincide with lineage commitment. Therefore, CD34'LFA-1-' cells were sorted and cultured for 48 hours. Subsequently, the cells were harvested and after staining with an anti-LFA-1 MoAb sorted to follow the progeny of single cells. We noticed that the growth capacity of cells that acquire LFA-1 expression in vitro is much larger than cells that remain LFA-1 negative (Fig 8). In both cases, the obtained colonies comprised granulocytic, monocytic, and erythroid cells as deduced from light microscopy, indicating their multilineage properties. To support this visual observation, cells were collected after 9 days of culture and stained with Glycophorin-PE, CD14-PE, and CD15-FITC. The results are depicted in Fig 9. Scatter analysis indicated that

Table 1. Expression of LFA-1 and Its Activation Epitope NKI-L 16 by Cultured CD34' Cells

<table>
<thead>
<tr>
<th></th>
<th>CD34'LFA-1-</th>
<th>CD34'LFA-1+</th>
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<tbody>
<tr>
<td>Control</td>
<td>2 (190)</td>
<td>1 (134)</td>
</tr>
<tr>
<td>L7</td>
<td>88 (462)</td>
<td>34 (228)</td>
</tr>
<tr>
<td>L16</td>
<td>23 (289)</td>
<td>21 (205)</td>
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Mononuclear bone marrow cells were sorted into a CD34'LFA-1' and CD34'LFA-1- fraction and cultured for 2 days. Collected cells were stained for the indicated MoAbs. The percentage positive cells are presented and the mean fluorescence channel is indicated in parentheses.
the LFA-1- cells that remain negative showed less side scatter than the cells that became LFA-1+, indicating more mature myeloid cells in the subpopulation that acquired LFA-1 expression. From the staining with MoAbs it is evident that both cell populations contained erythroid, myeloid, and granulocytic cells, demonstrating that this in vitro induced LFA-1 expression is not linked to lineage commitment.

**DISCUSSION**

The expression of LFA-1 coincided with more mature CD34+ bone marrow cells as deduced from colony forming capacity of sorted cells on allogeneic irradiated stromal layers. The data obtained in this report with sorted CD34+LFA-1+ in single cell per well assays support and extend this finding. Here, we also demonstrate that CD34+LFA-1- bone marrow cells become LFA-1+ after in vitro culture. Notwithstanding, this upregulation of LFA-1 cultures initiated with CD34+LFA-1- bone marrow cells contained colonies of several lineages. In both populations CD15+, CD14+, CD14+CD15+, and glycophorin+ cells were detectable, indicating the multilineage potential of both sub-populations.

It is remarkable that LFA-1 is not activated since expression of the LFA-1 activation epitope L16 was low. This indicates that LFA-1 is not likely to be involved in strong binding of the hematopoietic cells to the stroma. This is in accordance with mobilization data in which MoAbs toward LFA-1 were unable to mobilize hematopoietic cells in contrast to anti-VLA-4 antibodies. Recently, it was reported that the β1-integrins VLA-4 and VLA-5 are expressed on steady state bone marrow in a low affinity configuration that upon treatment with cytokines transiently increase their adhesive function. Similarly, the β2-integrin LFA-1 is also expressed on CD34+ bone marrow cells in a nonactivated form, but in addition, expression of LFA-1 is rapidly upregulated by in vitro culture independent from cytokine treatment. It is tempting to speculate about the physiologic function in vivo of upregulation of LFA-1. We hypothesize that in steady state bone marrow LFA-1 is downregulated due to interactions with the stromal cells. During episodes in which the immune system is activated (ie, infection), a transient lowering of the ratio between growth inhibiting and growth stimulating cytokines will initiate cell proliferation, resulting in crowding of proliferating progenitor cells in the bone marrow. Therefore, significant numbers of cells will not be able to make firm contacts with the stroma since all space is already occupied. As a result, these cells will rapidly upregulate LFA-1, thus equipping these cells with surface molecules that allow migration into the periphery. Such a mechanism predicts that the percentage CD34+ cells that express LFA-1 is highly variable. The wide variation in LFA-1 expression as was found here supports this hypothesis. These data emphasize that not only activation, but also upregulation of adhesion molecules, plays an important role in maintaining normal steady state bone marrow. This notion
is supported by Mullersieburg and Deryugina, who suggested a similar mechanism.\(^39\)

It was reported earlier that LFA-1 expressing CD34 positive cells comprised a more mature progenitor subset.\(^37\) Indeed, we show here that this subset is largely committed to the macrophage lineage based on the growth potential of single CD34 positive cells. Recently it was described that the LFA-1 positive precursors isolated from fetal liver contained all progenitor types, whereas LFA-1 negative cells contained only erythroid progenitors.\(^40\)

However, at that stage erythropoiesis is predominant and granulomonocytopenesis is minimal. Moreover, their CD18 nonadherent fraction did not express CD34 explaining the low progenitor content. We sorted CD34\(^+\)LFA-1\(^+\) and CD34\(^+\)LFA-1\(^-\) cells and hence our CD18\(^-\) fraction contained CD34\(^+\) progenitors. Others found that upon culturing of CD34 positive cells, the expression of LFA-1 diminishes after 15 days of culture.\(^41\)

This indicates again that the expression of LFA-1 is not linked to the differentiation state of the bone marrow cells, since upon culture cells become more mature. Lack of linkage between the expression of surface molecules and differentiation was recently also observed for DR expression on primitive cells.\(^42\)

The extremely rapid upregulation of LFA-1 in vitro highlights the dynamic behavior of CD34 positive cells when placed outside the microenvironment of the bone marrow. Cells that acquire LFA-1 in vitro showed a higher plating efficiency than the cells that remained LFA-1 negative. No commitment to a certain lineage was observed for both sub-populations. This is different from the results obtained with the LFA-1 positive cells.

Activation of LFA-1 and subsequent adhesion is a multistep process. The expression of the L16 epitope is an important step in this process.\(^35\) Lack of L16 expression in CD34\(^+\) bone marrow cells indicates that LFA-1 is not actively involved in adhesion. Expression of LFA-1 is necessary to enable LFA-1 mediated migration. At the migration site LFA-1 will be activated. Hence, L16 expression will be low and upregulated at the place of migration. Inactive LFA-1 was also found on KG1 cells. On those cells, LFA-1 mediated adhesion was induced by signals mediated by CD34.\(^22,33\)

The expression of LFA-1 on CD34\(^+\) bone marrow cells as reported here is significantly lower compared with data by others.\(^27,43,44\) In the context of the present findings, this discrepancy in expression is likely due to differences in isolation procedure of the CD34 positive cells. We isolated the CD34 positive cells by means of magnetic bead isolation, and the collected cells were frozen immediately after isolation. Such an isolation procedure is completed within 5 hours after collecting the bone marrow and is largely performed at 0°C. Labeling experiments were performed directly after thawing of the cells. This is different from other reports in which the isolated CD34\(^+\) cells were incubated for at least 24 hours in medium containing serum at 37°C before analyzing LFA-1 expression.\(^27,45\) Culturing of these cells in the presence of IL-3 further increased the expression of LFA-1.\(^45\)

This indicates that the higher level of LFA-1 expression, as
was reported earlier, is at least partially due to this rapid induction in vitro.

In conclusion, we found that CD34 positive cells showed variable expression of LFA-1, and that the expression of LFA-1 coincides to a mature lineage committed cell population. Moreover, LFA-1 is present in an inactive state. Furthermore, upon initial in vitro culturing, LFA-1 negative cells rapidly obtain LFA-1, but no induction of lineage commitment was observed. These observations indicate that LFA-1 does not play a pivotal role in adhesion of hematopoietic cells to the bone marrow matrix but rather prepares cells for their life in the periphery. It will be extremely interesting to identify the elements (soluble factors, adhesion molecules) that suppress LFA-1 expression in the bone marrow.

ACKNOWLEDGMENT

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REFERENCES

4. Verfaillie CM: Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. Blood 82:2045, 1993
31. Lukacs NW, Strieter RM, Elner VM, Evanoff HI, Burdick


Induction of LFA-1 on pluripotent CD34+ bone marrow cells does not affect lineage commitment

R Torensma, RA Raymakers, Y van Kooyk and CG Figdor