Myeloid and Erythroid Progenitor Cells From Normal Bone Marrow Adhere To Collagen Type I

By Michael Koenigsman, James D. Griffin, Jennifer DiCarlo, and Stephen A. Cannistra

One of the mechanisms by which normal hematopoietic progenitor cells remain localized within the bone marrow microenvironment is likely to involve adhesion of these cells to extracellular matrix (ECM) proteins. For example, there is evidence that uncommitted, HLA-DR-negative progenitor cells and committed erythroid precursors (BFU-E) bind to fibronectin. However, fibronectin is not known to mediate binding of committed myeloid (granulocyte-macrophage) progenitors, raising the possibility that other ECM proteins may be involved in this process. We investigated the binding of the M07 myeloid cell line to a variety of ECM proteins and observed significant specific binding to collagen type I (56% ± 5%), minimal binding to fibronectin (18% ± 4%) or to laminin (19% ± 5%), and no binding to collagen type III, IV, or V. Similarly, normal bone marrow myeloid progenitor cells (CFU-GM) demonstrated significant specific binding to collagen type I (46% ± 8% and 47% ± 12% for day 7 CFU-GM and day 14 CFU-GM, respectively). The ability of collagen to mediate binding of progenitor cells was not restricted to the myeloid lineage, as BFU-E also showed significant binding to this ECM protein (40% ± 10%). The binding of M07 cells and CFU-GM was collagen-mediated, as demonstrated by complete inhibition of adherence after treatment with collagenase type VII, which was shown to specifically degrade collagen. Binding was not affected by anti-CD29 neutralizing antibody (anti-β1 integrin), the RGD-containing peptide GRGDTP, or divalent cation chelation, suggesting that collagen binding is not mediated by the β1 integrin class of adhesion proteins. Finally, mature peripheral blood neutrophils and monocytes were also found to bind to collagen type I (25% ± 8% and 29% ± 6%, respectively). These data suggest that collagen type I may play a role in the localization of committed myeloid and erythroid progenitors within the bone marrow microenvironment.

EMATOPOIESIS OCCURS through a series of orderly steps involving proliferation, commitment, and differentiation of early bone marrow progenitor cells, ultimately leading to the release of mature cells into the peripheral blood. To ensure that hematopoietic progenitors are provided with appropriate conditions for development, including the availability of humoral growth factors, it is likely that specific mechanisms exist for the adhesion of these cells to bone marrow stromal cells or to extracellular matrix (ECM) proteins during maturation. For example, early murine progenitors with high proliferative capacity are preferentially located in the adherent cell layer of long-term marrow cultures. In human studies, uncommitted, HLA-DR-negative progenitors capable of repopulating long-term marrow cultures have been shown to avidly bind to the stromal cell layer, whereas more committed progenitors exhibit significantly reduced binding. Although the mechanism for the adhesion of early progenitors to the stromal cell layer is not fully understood, there is increasing evidence that the ECM protein fibronectin plays an important role in this process. For example, uncommitted, HLA-DR-negative progenitors selectively bind in vitro to the heparin binding domain II of fibronectin, an epitope that is recognized by the VLA-4 molecule of the β1 integrin family. Interestingly, as early progenitors become more committed, as indicated by expression of HLA-DR, their binding to fibronectin is altered in two major ways. First, binding of committed progenitors appears to be mediated through an additional region of the fibronectin molecule termed the cell binding domain, an area recognized by the VLA-5 integrin. Second, only the erythroid subset of committed progenitors appears to maintain significant binding avidity for fibronectin, with other committed progenitors such as granulocyte-macrophage colony-forming units (CFU-GM) demonstrating significantly reduced binding to this ECM protein. The binding of erythroid progenitors to fibronectin is differentiation-dependent, with progressive loss of binding as the cells mature into erythrocytes, thereby providing a potential mechanism for the release of mature red blood cells (RBCs) from the bone marrow into the peripheral blood.5,7 Thus, both the pattern of progenitor cell binding to fibronectin as well as the recognition sites for binding to this molecule change as the progenitor cell acquires a more committed phenotype. Although fibronectin may partly mediate the binding of uncommitted, HLA-DR-negative progenitors and committed erythroid precursors, it is likely that this protein does not play a major role in the localization of committed myeloid progenitors such as CFU-GM within the bone marrow. However, other ECM proteins in the bone marrow that could be involved in the binding of committed myeloid progenitors include hemonectin, collagen types I, III, and IV, and laminin. Hemonectin is a 60-Kd protein purified from rabbit ECM that has been observed to support the binding of murine CFU-GM and, to a lesser extent, erythroid burst forming units (BFU-E) in vitro.8 Myeloid binding to hemonectin appears to be differentiation-associated, with more mature cells such as bands and polymorphonuclear leukocytes exhibiting decreased adhe-
sion to this molecule when compared with undifferentiated blasts. Although the molecular structure of hemonectin and its relationship to other adhesion proteins are unknown, the ability of this protein to support the binding of CFU-GM in a differentiation-associated fashion deserves further investigation.

The role of collagen in the localization and development of progenitor cells within the bone marrow is of interest for several reasons. Collagen types I, III, and IV are major constituents of the bone marrow ECM, and hematopoietic precursor cells express molecules such as CD44 that are known to recognize collagen. Furthermore, inhibition of collagen synthesis by cis-4-hydroxyproline interferes in a differentiation-associated fashion deserves further investigation.

Reagents. Purified ECM proteins were obtained as follows: collagen type I (bovine), collagen type IV (murine), and laminin (murine) from Collaborative Research (Bedford, MA); collagens type III (human) and V (human) from Sigma (St Louis, MO) (Sigma designations X and IX); fibronectin (human) from Telios (San Diego, CA), Rehounan human granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and erythropoietin were kindly provided by Dr Steven Clark (Genetics Institute, Cambridge, MA). The following murine monoclonal antibodies that recognize adhesion proteins were used as part of this study: 4B4 (anti-CD29, β1 integrin), 8F2 (anti-VLA-4), 2H6 (anti-VLA-5), and IF7 (anti-CD26) were gifts of Dr Chikao Morimoto (Dana Farber Cancer Institute [DFCI], Boston, MA); TS2/7 (anti-VLA-1) was a gift of Dr Martin Hemler (DFCI); 12F1 (anti-VLA-2) was a gift of Dr Virgil Woods (University of San Diego, CA); J143 (anti-VLA-3) was a gift of Dr Anthony Albino (Memorial Sloan Kettering Institute, New York, NY); GoH3 (anti-VLA-6) was a gift of Dr Arnon Sonnenberg (University of Amsterdam, Amsterdam, The Netherlands); and 514 (anti-CD44) was a gift of Dr Goeffrey Kanss (DFCI). Fetal calf serum (FCS) for colony cultures was purchased from HyClone (Logan, UT). Materials used for serum-free cell culture were obtained as follows: crystalline bovine serum albumin (BSA) (globulin-free), bovine insulin, and cholesterol from Sigma; human iron-saturated transferrin from Boehringer-Mannheim (Indianapolis, IN); Dulbecco's minimal essential medium (DMEM) from Whittacker (Walkersville, MD). Serum-free medium (SFM) consisted of DMEM containing BSA 15 mg/mL, insulin 1 µg/mL, transferrin 7.7 × 10^{-4} mol/L, cholesterol 7.8 µg/mL. Highly purified collagenase type VII from Clostridium histolyticum was obtained from Sigma. The synthetic peptides GRGESP and GRGDTP were from Telios. Chromium (1 mCi/mL, 200 Ci/g) and tritiated thymidine (1 mCi/mL, 2 Ci/mmol) were purchased from New England Nuclear (Boston, MA).

 MATERIALS AND METHODS

Source of cells. The GM-CSF- and IL-3-dependent cell line, M07, was obtained from Dr Steven Clark, and was originally derived by Avanzi et al from the peripheral blood of an infant with acute megakaryocytic leukemia. This line was maintained in DMEM supplemented with 20% FCS (20% DMEM) and 10 ng/mL of both GM-CSF and IL-3. Bone marrow progenitor cells were obtained from healthy donors after informed consent by posterior iliac crest aspiration into heparinized syringes. Mononuclear cells were recovered by Ficoll density centrifugation, and macrophages were removed by plastic adherence for 1 hour at 37°C as previously described. Neutrophils and monocytes were simultaneously prepared from heparinized peripheral blood donated by healthy volunteers. After Ficoll density centrifugation, neutrophils were isolated from the RBC pellets by dextran sedimentation. Monocytes were obtained from the mononuclear cell fraction by plastic adherence for 1 hour at 37°C, as previously described. Both neutrophil and monocyte preparations were greater than 90% pure as assessed by Wright-Giemsa staining.

Immunophenotyping. M07 cells were screened for the expression of known collagen adhesion receptors and for the myeloid marker CD33 by indirect immunofluorescent staining. Briefly, 1 × 10^6 cells were stained for 30 minutes, 4°C with 100 µL of either an irrelevant control antibody (3C11C8, anti-γ-interferon antibody), or with the previously described antibodies that recognize CD29, VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, CD44, or IF7. After washing two times in phosphate-buffered saline (PBS), the cells were labeled with fluorocein-isotiocyanate-labeled goat antimouse Ig (FITC) (Tago, Burlingame, CA) for 30 minutes, 4°C. After two additional washes, the cells were analyzed on a Coulter Epics C flow cytometer (Coulter, Hialeah, FL).

Chromium assay for cell adhesion. The binding of M07 cells, neutrophils, and monocytes to ECM proteins was quantitated by assessing "chromium release of adherent cells. Cells, 10^5, were labeled with 100 µCi of "chromium (200 Ci/g) for 1 hour at 37°C, and then washed two times in PBS and resuspended at 0.5 to 1 × 10^6/mL of SFM. Flat-bottom 96-well microtiter wells (Falcon, Oxnard, CA) were coated with 10 to 20 µL of individual ECM proteins (20 to 70 µg/well, 0.28 cm^2/well) and allowed to air dry for 1 to 3 hours at room temperature. Control coating was performed with either the respective solvents (0.1N acetic acid for collagen preparations, Tris/NaCl for laminin, H_2O for fibronectin) or with 1.5% BSA. BSA was used as a control to ensure that cells were not adhering to protein in a nonspecific fashion in these experiments.

None of the cell types used in this study was observed to bind to BSA. "Chromium-labeled cells (50 to 100 × 10^3) were added to each well in a total of 100 µL of SFM, and binding was allowed to occur for 2 to 4 hours at 37°C. The nonadherent cells were then removed by three washes with PBS, followed by lysis of bound cells with 0.1% NP40. The radioactivity of each lysate was measured in a gamma counter. The percentage of cells adhering to an individual ECM protein (percent specific binding) was calculated as follows: percent specific binding = 100 × (mean cpm [ECM-coated surface] - mean cpm [control surface])/CPM (total).

For some experiments, "chromium-labeled cells were pretreated with anti-CD29 antibody (1:200 dilution) or control antibody (anti-CD33, which reacts with M07 cells) for 30 minutes at 4°C before performing the bind, with continued presence of antibody during the duration of the bind. Likewise, the effects of RGD-containing peptides was determined by preincubation of cells in 200 µg/mL of either GRGESP or GRGDTP for 30 minutes at room temperature, followed by binding in the continued presence of peptide. The effects of divalent cation chelation were assessed by performing the bind in EDTA (10 mmol/L final concentration per well) in some studies. Finally, collagen-coated wells were pretreated with collagenase in some experiments to determine the

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specificity of binding to collagen. For these studies, collagen-coated or control wells were pretreated with 20 μL of either collagenase buffer (50 mmol/L Tris, 2.5 mmol/L CaCl₂, pH 7.5) or collagenase (0.5 U/mL) at room temperature and allowed to air dry before adding ²⁵chromium-labeled cells.

**Assay for progenitor cell adhesion.** Nonadherent bone marrow mononuclear cells from healthy donors were added to 96-well plates coated with either ECM protein, control solvent, or with 1.5% BSA as described. To ensure that a reasonable number of colonies would be obtained per well after binding, cell numbers were added in a range of 10 to 100 x 10⁵ per well. Progenitor cells were allowed to bind for 4 hours at 37°C, followed by removal of nonadherent cells by washing three times in PBS. For determination of CFU-GM binding, each well was overlaid with 0.3% agar in Iscove’s modified Dulbecco’s MEM (IMDMEM) containing 10% (vol/vol) 5637 conditioned medium as a source of CSFs. Myeloid progenitor cells were scored using an inverted microscope. Clusters (8 to 40 cells) were counted on day 7 and colonies (>40 cells) were counted on day 14. For determination of CFU-mix and BFU-E binding, each well was overlaid with 0.9% methylcellulose in IMDMEM containing 4 U/mL erythropoietin, and 5 ng/mL of both GM-CSF and IL-3. CFU-mix and BFU-E were enumerated on day 14. The total number of CFUs added per well was determined under identical growth conditions in collagen- or control-coated plastic wells. It should be noted that there was no difference in the growth of CFU-GM in the presence or absence of collagen in any of the experiments performed in this study. The percentage of CFUs adherent to ECM (percent specific binding) was calculated as follows: percent specific binding = 100 x (mean CFU [ECM-coated surface] – mean CFU [control surface])/total CFU. To establish consistency between experiments, all data were corrected to reflect cluster or colony number per 10⁵ cells added. For some experiments, the effects of control antibody (anti-CD33), anti-CD29, EDTA, and collagenase on progenitor cell binding to collagen type I were assessed under the same incubation conditions as described for ²⁵chromium-labeled cells. Specifically, bone marrow mononuclear cells were first pretreated with either control or anti-CD29 antibody, with continuous exposure to antibody during the binding period.

**Cell proliferation assay.** The effect of ECM proteins on the proliferation of MO7 cells was examined in 96-well plates coated with either ECM protein or control solvent. MO7 cells, 20 x 10⁵ per well, were incubated for 60 hours at 37°C in the presence or absence of 5 to 25 ng/mL of GM-CSF in either 20% FCS or in SFM. Tritiated thymidine (1.6 μCi, 2 Ci/mmol) was added to each well for the last 12 hours of incubation, followed by cell harvesting and determination of radioactivity with a beta counter (Packard Liquid Scintillation Analyzer 2000 CA).

**Assessment of collagenase activity.** To determine the specificity of collagenase VII for collagen type I, 7 μL of either laminin, fibronectin, or collagen (approximately 20 μg of each protein) were treated with 43 μL of either control buffer (50 mmol/L Tris, 2.5 mmol/L CaCl₂, pH 7.5) or with collagenase type VII (approximately 430 mU) in a total volume of 50 μL at room temperature for 1 hour. In some experiments, 7 μL of collagen was treated with 43 μL of neutrophil supernatant (86% vol/vol) under the same conditions to test for the possibility of endogenous collagenase production by neutrophils. After addition of 50 μL of 2X sample buffer with mercaptoethanol, the samples were boiled at 100°C for 5 minutes and loaded onto a 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel. After electrophoresis, the protein bands were visualized by Coomassie blue staining.

**Statistical analysis.** Significance levels for comparison between treatment groups were determined using the two-sided Student’s t-test for paired samples.

**RESULTS**

Adhesion receptor expression and pattern of ECM binding of MO7 cells. Initial binding experiments were performed using the factor-dependent myeloid leukemic cell line, MO7. Immunophenotyping analysis was initially performed to determine whether these cells expressed receptors for collagen (VLA-1, -2, and -3, CD44, and CD26), fibronectin (VLA-3, and -4), and laminin (VLA-1, -2, and -6).³⁹ The percentage of MO7 cells specifically positive for each adhesion protein was: VLA-1 = 0%, VLA-2 = 41%, VLA-3 = 0%, VLA-4 = 48%, VLA-5 = 94%, VLA-6 = 64%, CD29 (VLA-β1-chain) = 95%, CD44 = 89%, and CD26 = 0%. The cells also expressed the CD33 molecule (90%). These data suggest that MO7 cells express adhesion proteins for collagen, fibronectin, and laminin. Therefore, ²⁵chromium-labeled MO7 myeloid cells were allowed to bind to microtiter wells previously coated with 20 to 70 μg of either collagen types I, III, IV, and V, fibronectin, or laminin for 4 hours at 37°C, with subsequent washing and measurement of specifically bound cells as previously described. As shown in Table 1, MO7 cells demonstrated significant binding to bovine collagen type I (56% ± 5%), with a significantly lesser amount of binding to human fibronectin (18% ± 4%) and to murine laminin (19% ± 5%). There was no appreciable binding of MO7 cells to collagen type III, IV, or V. Increasing the absolute amounts of any coated ECM protein did not increase binding further. The kinetics of binding of MO7 cells to collagen type I showed peak binding at 2 hours of incubation.

Adhesion of normal bone marrow progenitor cells to collagen type I. In view of the significant degree of MO7 binding to collagen type I, the remainder of our studies were designed to assess the relevance of this observation for normal bone marrow progenitor cells. Adherence-depleted bone marrow mononuclear cells were allowed to bind to collagen type I-coated microtiter wells for 4 hours at 37°C, followed by washing and overlying the bound cells with either 0.3% agar or 0.9% methylcellulose under conditions suitable for CFU-GM colony formation. The percentage of M07 cells specifically positive for each adhesion protein was: VLA-1 = 0%, VLA-2 = 41%, VLA-3 = 0%, VLA-4 = 48%, VLA-5 = 94%, VLA-6 = 64%, CD29 (VLA-β1-chain) = 95%, CD44 = 89%, and CD26 = 0%. The cells also expressed the CD33 molecule (90%). These data suggest that MO7 cells express adhesion proteins for collagen, fibronectin, and laminin. Therefore, ²⁵chromium-labeled MO7 myeloid cells were allowed to bind to microtiter wells previously coated with 20 to 70 μg of either collagen types I, III, IV, and V, fibronectin, or laminin for 4 hours at 37°C, with subsequent washing and measurement of specifically bound cells as previously described. As shown in Table 1, MO7 cells demonstrated significant binding to bovine collagen type I (56% ± 5%), with a significantly lesser amount of binding to human fibronectin (18% ± 4%) and to murine laminin (19% ± 5%). There was no appreciable binding of MO7 cells to collagen type III, IV, or V. Increasing the absolute amounts of any coated ECM protein did not increase binding further. The kinetics of binding of MO7 cells to collagen type I showed peak binding at 2 hours of incubation.

**Table 1. Adhesion of the Myeloid Leukemic Cell Line MO7 to ECM Proteins**

<table>
<thead>
<tr>
<th>ECM Protein*</th>
<th>Specific Binding (%)</th>
<th>Range (%)</th>
<th>No. of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I</td>
<td>56 ± 5</td>
<td>48-63</td>
<td>5</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>2 ± 2</td>
<td>0-2</td>
<td>3</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>0</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>Collagen type V</td>
<td>0</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>Laminin</td>
<td>10 ± 5</td>
<td>14-28</td>
<td>5</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>18 ± 4</td>
<td>14-21</td>
<td>5</td>
</tr>
</tbody>
</table>

*Microtiter wells were coated with either control solvent or extracellular matrix proteins as described in text before performing bind with 60 x 10⁵²⁵chromium-labeled MO7 cells for 4 hours at 37°C.

†Percent specific binding of cells adhering to each ECM protein was calculated for each experiment using mean values from quadruplicate wells as described in text. Background binding was 3% ± 2% for all experiments. The value shown is mean ± SD percent specific binding from the indicated number of experiments.

‡Indicates number of separate experiments performed for each ECM protein.
that would support maximum colony growth of CFU-GM, CFU-GEMM, and BFU-E as described. Bone marrow progenitors were depleted of accessory cells to reduce the potential for simultaneous accessory cell binding that might alter the pattern of colony formation due to the secretion of endogenous growth factors. As shown in Table 2, significant binding of day 7 CFU-GM (46% ± 8%, n = 6), day 14 CFU-GM (47% ± 12%, n = 4), and BFU-E (40% ± 10%, n = 5) to collagen type I was observed (P < .001). Interestingly, there were no quantitative differences between the binding of early (ie, day 14 CFU-GM or CFU-GEMM) versus later (day 7 CFU-GM) progenitor cells to collagen type I. Although CFU-mix tended to bind to collagen type I, this effect was not statistically significant, possibly due to the small numbers of colonies observed.

**Specificity of myeloid cell binding to collagen I.** To confirm that the observed binding of M07 cells or normal bone marrow progenitors was mediated by collagen type I, as opposed to a contaminating protein present in the collagen preparation, we studied the effects of pretreatment of collagen-coated microtiter wells with highly purified collagenase type VII. As shown in Fig 1, collagenase pretreatment significantly reduced the binding of both M07 cells and CFU-GM to collagen type I, whereas similar pretreatment with collagenase buffer had no effect. To determine whether the collagenase effect was specific for collagen, we treated fibronectin, laminin, and collagen type I with collagenase for 1 hour at room temperature, with subsequent visualization of the protein products by polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining. As shown in Fig 2, collagenase had no effect on the integrity of laminin or fibronectin, whereas it completely degraded the three major bands associated with collagen type I (115, 125, and 212 Kd).

**Effects of anti-CD29 antibody, RGD-containing peptide, or divalent cation chelation on the binding of myeloid cells to collagen type I.** The 6-1 integrin family of adhesion proteins is comprised of heterodimers containing a common

| Table 2. Adhesion of Normal Myeloid Progenitor Cells to Collagen Type I |
|-------------------------|-----------------|-----------------|-----------------|-----------------|
| Donor | Treatment | CFU-GM D7 | CFU-GM D14 | BFU-E | CFU-mix |
| 1 | Control | 7 ± 7 | 0 ± 0 | 0 ± 0 | 32 ± 2 |
| | Collagen | 57 ± 6 | ND | 5 ± 2 | ND |
| | Total CFU added | 158 ± 32 | ND | ND | ND |
| | % Specific binding | 32 | ND | 31 | 31 |
| 2 | Control | 4 ± 1 | 1 ± 1 | 0 ± 0 | 0 ± 0 |
| | Collagen | 22 ± 6 | 8 ± 2 | 4 ± 2 | 4 ± 2 |
| | Total CFU added | 31 | 12 | 9 ± 4 | 2 ± 1 |
| | % Specific binding | 58 | 67 | 44 | 44 |
| 3 | Control | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| | Collagen | 49 ± 10 | 9 ± 3 | 7 ± 3 | 2 ± 1 |
| | Total CFU added | 109 | 22 ± 1 | 25 ± 4 | 20 |
| | % Specific binding | 45 | 41 | 28 | 10 |
| 4 | Control | 4 ± 2 | 1 ± 0 | 0 ± 0 | 0 ± 0 |
| | Collagen | 25 ± 4 | 7 ± 2 | 9 ± 4 | 2 ± 1 |
| | Total CFU added | 44 ± 6 | 14 ± 6 | 22 ± 8 | 4 ± 2 |
| | % Specific binding | 48 | 43 | 41 | 50 |
| 5 | Control | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| | Collagen | 22 ± 5 | ND | ND | ND |
| | Total CFU added | 44 | 44 | 44 | 44 |
| | % Specific binding | 50 | 50 | 50 | 50 |
| 6 | Control | 2 ± 2 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| | Collagen | ND | ND | 4 ± 2 | 1 ± 0 |
| | Total CFU added | 7 ± 4 | 4 ± 2 | 3 ± 1 |
| | % Specific binding | 57 | 33 | 33 | 33 |
| 7 | Control | 74 ± 6 | 17 ± 4 | ND | ND |
| | Total CFU added | 167 ± 19 | 46 ± 8 | ND | ND |
| | % Specific binding | 43 | 37 | 37 | 37 |

*Bone marrow progenitor cells from seven different normal donors were depleted of monocytes by plastic adherence before performing binding assay.

1Cells (10-100 x 10³) were added to flat bottom 96-well plates precoated with either 0.1% acetic acid (control) or collagen type I (60 μg). After 4 hours at 37°C, the wells were washed to remove nonadherent cells, followed by addition of either agar or methylcellulose as described in text. Appropriate controls were performed in order to assess the total number of CFU-GM and BFU-E added to each well.

2Data expressed as mean ± SD of colony number from quadruplicate wells. To establish consistency between experiments, all data are expressed as cluster (day 7) or colony (day 14) number per 10⁶ total cells added.

3ND, not determined.

4Cumulative mean ± SD of percent specific binding from individual experiments. For each experiment shown, highly significant binding (P < .001, Student's t-test), of CFU-GM and BFU-E to collagen type I was observed. Although CFU-mix tended to bind to collagen-coated wells, this binding was not statistically significant, possibly due to the small number of colonies observed.
Fig 1. Effects of collagenase on binding of M07 cells or normal bone marrow CFU-GM to collagen type I. Control or collagen-coated wells were incubated for 1 hour at room temperature with 20 µL of either buffer (50 mmol/L Tris, 2.5 mmol/L CaCl₂, pH 7.5) or with collagenase type VI (0.5 U/mL) before adding either chromium-labeled M07 cells or adherence-depleted normal bone marrow progenitor cells for determination of specific binding as described in text. Data are expressed as mean ± SD of percent specific binding from two separate experiments for M07 cells and for day 7 CFU-GM.

8-1 chain (CD29) and at least six different α subunits. These six distinct heterodimers are referred to as VLA-1 to VLA-6, and they are known to bind to a variety of ECM proteins. VLA proteins -1, -2, and -3 have been determined to bind to collagen in a calcium-dependent fashion that may be partially blocked by peptides containing the RGD sequence. Because immunophenotypic analysis of the M07 cell line showed expression of VLA-2, we were interested in determining whether collagen binding of this cell line was mediated through this adhesion protein. Therefore, we studied the effects of a neutralizing anti-CD29 antibody (4B4), RGD-containing peptide, or divalent cation chelation with EDTA, on the ability of M07 cells or normal bone marrow CFU-GM to bind to collagen type I. The 4B4 antibody has been previously shown to block VLA-4/VCAM-1 interactions, to neutralize collagen-induced proliferation of T cells, and to neutralize binding of the ovarian cancer cell line OVCAR-3 to both laminin and fibronectin (S.A.C., unpublished observations, June 1991). As shown in Table 3, neither antibody 4B4 nor RGD-containing peptide altered adhesion to collagen type I. Also, divalent cation chelation caused no significant change in collagen binding of M07 cells or of CFU-GM. These results suggest that binding of M07 cells or of CFU-GM to collagen type I occurs through a VLA-independent mechanism.

Because CD44 is also expressed by M07 cells and is known to mediate collagen binding, we next tested the effects of anti-CD44 antibody (515) on the adherence of M07 cells to collagen type I. However, no inhibitory effect of anti-CD44 on M07 binding was observed (data not shown). Because the 515 antibody used in these studies is not known to be neutralizing, M07 cells were continuously exposed to 515 antibody for 72 hours at 37°C in an attempt to study the effects of CD44 downregulation on subsequent binding. However, CD44 expression was not downregulated despite a 72-hour exposure to the 515 antibody (data not shown).

Comparative binding of normal neutrophils, monocytes, and M07 cells to collagen type I. These data suggest the possibility that binding to collagen I may represent one of the mechanisms by which committed myeloid cells remain within the bone marrow microenvironment during development. In an attempt to determine whether mature myeloid cells also bind to collagen, we investigated the ability of chromium-labeled neutrophils, monocytes, and M07 cells to bind to collagen type I, with cumulative results shown in Fig 3. These experiments were performed over a 2-hour incubation (as opposed to 4 hours) at 37°C to maintain neutrophil viability at greater than 90%. In the absence of
Table 3. Effects of Anti-CD29 Antibody, RGD Peptide, or EDTA on Binding of MO7 Cells of CFU-GM to Collagen Type I

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Antibody or RGD peptide: *</th>
<th>Control†</th>
<th>Anti-CD29</th>
<th>RGD</th>
<th>EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO7</td>
<td>100% (n = 3)</td>
<td>100% (n = 1)</td>
<td>114% (n = 3)</td>
<td>ND</td>
<td>79% (n = 9)</td>
</tr>
<tr>
<td>CFU-GM (day 7)</td>
<td>100% (n = 3)</td>
<td>96% (n = 1)</td>
<td>NO</td>
<td></td>
<td>97% (n = 6)</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

*Binding was performed in quadruplicate wells in the continuous presence of either anti-CD29, which is a neutralizing antibody recognizing the β-1 integrin molecule, or RGD peptide (GRGDTP), with details as described in Materials and Methods. Cells were pretreated with either antibody or peptide prior to binding as previously described.

†For experiments using the anti-CD29 antibody, the control antibody was anti-CD33, which recognizes a surface protein with no known adhesion properties. For experiments using RGD peptide, the control peptide was GRGESP. Neither anti-CD33 nor GRGESP peptide altered binding when compared to treatment with media alone.

‡In a separate set of experiments, binding was performed in quadruplicate wells under serum-free conditions with either 20% vol/vol PBS (control) or 20% vol/vol EDTA in PBS (for a final concentration of 10 mmol/L EDTA in SFM).

§Data for each treatment group are expressed as percent of control specific binding (SB) as determined from the indicated number of experiments as follows: % of control SB = 100 x (mean SB [treatment])/ (mean SB [control]).

∥Not significantly different when compared with control (P = .06).

EDTA, background binding of monocytes to control wells was significant (20% to 50% adhesion), precluding an accurate assessment of specific binding to collagen type I under these conditions. Because pilot experiments showed that the binding of monocytes to plastic was significantly blocked by 10 mmol/L EDTA without affecting corresponding binding to collagen I, we performed binding experiments using neutrophils, monocytes, and MO7 cells in the presence of 10 mmol/L EDTA to achieve acceptable levels of background binding (5% to 10%). As shown in Fig 3, the binding of both neutrophils and monocytes to collagen type I was significantly less than that of MO7 cells (25% ± 8% and 29% ± 6% vs 40% ± 7%, P = .001). In addition, comparison of percent specific binding to collagen type I obtained with neutrophils, monocytes, and CFU-GM (Table 2) showed significantly lower levels for either neutrophils or monocytes when compared with either day 17 (46% ± 8%) or day 14 CFU-GM (47% ± 12%) (P < .001).

Although CFU-GM binding was typically performed over a 4-hour period, as opposed to 2 hours for neutrophils and monocytes, we have performed additional experiments which show that peak CFU-GM binding is achieved after a minimum of 1 hour of incubation at 37°C (data not shown). Thus, it is unlikely that the lower binding of neutrophils or monocytes compared with that of CFU-GM is due to differences in binding duration.

During terminal differentiation, myeloid cells acquire the ability to produce collagenase. Therefore, we considered the possibility that the observed decrease in neutrophil and monocyte adhesion to collagen might result from endogenous collagenase secreted by these cells during the incubation period. To evaluate this possibility, media were conditioned by either neutrophils, monocytes, or MO7 cells for 2 hours at 37°C, (5 × 10⁶ cells/mL SFM). Cell-free supernatant was collected and used to treat 20 μg of collagen type I in a total volume of 50 μL (86% vol/vol supernatant) for 1 hour at room temperature, followed by analysis by SDS-PAGE as described. No collagen degradation was observed in any treatment group (data not shown).

Effects of collagen type I on progenitor cell proliferation. To examine whether progenitor cells derive a proliferative advantage during binding to collagen type I, we evaluated thymidine incorporation of MO7 cells (n = 10), as well as colony growth of bone marrow progenitors (n = 9), in the presence or absence of collagen. Experiments were conducted in the presence or absence of GM-CSF or IL-3 over a dose range of 5 to 25 ng/mL. No collagen-induced enhancement of proliferation was observed (data not shown).

DISCUSSION

This study was initially undertaken in an attempt to investigate the ability of several ECM proteins to support the adherence of the MO7 myeloid leukemia cell line. This line was chosen for these experiments in view of its hematopoietic origin and its expression of several potential adhesion proteins, including VLA-2, VLA-4, VLA-6, CD29, and CD44. We observed that collagen type I mediated significant binding of MO7 cells, whereas these cells bound to a considerably lesser degree to fibronectin and laminin. In view of the high level of MO7 binding to collagen type I, we further investigated the role that this ECM protein might play in the binding of normal bone marrow cells.
Both normal CFU-GM and BFU-E were found to significantly bind to collagen type I, and this interaction was completely abolished by pretreatment of collagen-coated plates with collagenase. In contrast to the results of other investigators, who report proliferative effects of collagen type I on T lymphocytes,22 we found no evidence for a mitogenic effect of collagen type I on myeloid cells in the presence or absence of exogenous GM-CSF or IL-3. This observation makes it unlikely that our results are due to a selective growth advantage of cells exposed to collagen-coated wells.

We also observed that mature neutrophils and monocytes bind to collagen type I (Fig 3). Interestingly, this binding was significantly less when compared with that of MO7 cells (Fig 3), suggesting the possibility that adhesion to collagen may decrease with myeloid maturation. It is important to note that although MO7 cells have a blast morphology and represent a less differentiated phenotype when compared with neutrophils or monocytes, the MO7 cell line is leukemic, as well as megakaryocytic, in origin and may not accurately reflect the binding of normal bone marrow progenitors. However, the binding of neutrophils and monocytes to collagen type I was also observed to be significantly lower when compared with that of normal CFU-GM (Table 2). It must be pointed out that these data are not strictly comparable due to technical differences between the measurement of mature cell binding (“chromium-labeling”) and the measurement of CFU-GM binding (colony assay). Unfortunately, in view of the low number of CFU-GM present in normal bone marrow, it is difficult to obtain highly purified progenitor cell populations for use in chromium-labeling studies.

Collagen binds to a heterogeneous group of cell surface receptors. These include the β-1 integrin family of heterodimers (VLA-1, -2, and -3) (24), the CD44 molecule, also known as the class III collagen receptor,14,15 and a 110-Kd protein known as dipetidyl peptidase IV (CD26).25 Although VLA-1, VLA-3, and CD26 were not expressed by the MO7 cell line, both VLA-2 and CD44 were strongly expressed. The interaction of collagen with β-1 integrins is known to be inhibited by RGD-containing peptides,22,26 by divalent cation chelation,27 and by neutralizing antibody against the common β-1 chain.22 However, as shown in Table 3, these interventions did not alter the binding of MO7 cells or of CFU-GM to collagen type I. The concentration of RGD peptide used in this study was based on previous work which demonstrated that this dose is capable of blocking collagen-induced T-cell proliferation as well as HeLa cell attachment to a collagen-based gelatin matrix.23,28 However, higher concentrations of RGD peptide have been required for maximal inhibition of binding in other cell systems.22 Nevertheless, our data suggest that the binding of myeloid cells to collagen type I is not mediated by the β-1 integrins, despite the expression of VLA-2 by MO7 cells. Although VLA expression is sometimes necessary for the binding of cells to ECM proteins such as collagen, it is clear that such expression is not sufficient for binding to occur. For instance, it has been recently shown that activated B cells bind to germinal center follicles through an interaction between B-cell-expressed VLA-4 and the VCAM-1 molecule expressed within the germinal center.21 However, B-cell lines which express VLA-4 do not necessarily bind to germinal centers, suggesting that VLA-4 must be activated (through a conformational change or through association with another surface protein) to recognize the VCAM-1 molecule. A similar requirement for activation has been recently shown for the binding of LFA-1 to ICAM-1,29 and for the binding of LAM-1 to mannose residues.30 Therefore, VLA expression may be necessary, but not sufficient, for binding in some situations. The fact that the MO7 cell line expresses VLA-2 but does not bind to collagen through this molecule is consistent with this hypothesis.

Our studies do not exclude the possibility that CD44 is responsible for mediating the binding of MO7 cells or committed progenitor cells to collagen type I. CD44 expression was noted on greater than 90% of MO7 cells in this report, and it has also been observed in highly purified populations of normal human bone marrow progenitor cells.13 In addition, CD44 expression has been shown to diminish as myeloid cells differentiate toward mature neutrophils.22 Because it is not known whether the anti-CD44 antibody used in this study is able to neutralize collagen binding, and because the CD44 molecule could not be downregulated from the surface of MO7 cells, the role of CD44 in mediating the binding of progenitor cells to collagen type I remains unclear.

It is likely that the binding of hematopoietic progenitor cells to ECM proteins involves the coordinate interaction of several types of adhesion proteins. Thus, uncommitted, HLA-DR-negative progenitors bind to fibronectin,31 whereas committed erythroid progenitors bind to both fibronectin32 and collagen type I, and committed myeloid progenitors bind to both hemonectin33 and collagen type I. The presence of alternative mechanisms of erythroid progenitor cell binding that do not involve fibronectin was also suggested by Coulombel et al.32 who demonstrated that BFU-E adherence to stromal cell-derived ECM could only be partially blocked by antifibronectin antibody. Based on our studies, it is possible that collagen type I may have partly contributed to the fibronectin-independent binding of BFU-E observed by these investigators. Although not specifically addressed in this study, the role of other collagens such as the type III and type IV species in the binding of normal progenitor cells deserves further investigation.

Our data suggest the possibility that collagen type I may partly mediate the adhesion of committed myeloid and erythroid progenitor cells within the bone marrow microenvironment. In addition, mature myeloid cells such as neutrophils and monocytes retain the capacity to bind to collagen type I, with a suggestion that this binding may be decreased compared with that of CFU-GM. Under certain conditions, higher levels of bone marrow progenitor cells circulate in the peripheral blood, such as during rebound myelopoesis after chemotherapy,33 during hematopoietic growth factor administration,34 or during pathologic condi-
tions such as the stem cell expansion characteristic of chronic myeloid leukemia. The ability of hematopoietic growth factors such as GM-CSF or G-CSF to mobilize bone marrow progenitors into the circulating white blood cell pool is particularly noteworthy, because these agents are also known to alter the expression of several adhesion proteins on the surface of myeloid cells. A comparison of the binding of circulating versus bone marrow progenitor cells to collagen type I may permit a better understanding of the physiologic role of this ECM protein in progenitor cell adhesion.

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REFERENCES


Myeloid and erythroid progenitor cells from normal bone marrow adhere to collagen type I

M Koenigsmann, JD Griffin, J DiCarlo and SA Cannistra

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