Differentiation of Primitive Human Multipotent Hematopoietic Progenitors into Single Lineage Clonogenic Progenitors Is Accompanied by Alterations in Their Interaction with Fibronectin

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Summary

We have previously demonstrated that primitive progenitors from human bone marrow termed long term bone marrow culture initiating cells (LTBMC-IC) adhere avidly to irradiated bone marrow stroma, while more mature clonogenic progenitors fail to do so. In this study we examine the interaction between these progenitors and components of the bone marrow stroma. (*a*) We demonstrate that both primitive LTBMC-IC and more mature clonogenic progenitors adhere to intact fibronectin. (*b*) Primitive LTBMC-IC and multi-lineage CFU-MIX progenitors adhere to the 33/66 kD COOH-terminal heparin-binding cell-adhesion promoting fragment of fibronectin, but adhere significantly less to its 75 kD RGDS-dependent cell-binding fragment. In contrast, more differentiated single-lineage progenitors adhere equally well to the 33/66 kD RGDS independent and the 75 kD RGDS-dependent cell-adhesion fragments of fibronectin. (*c*) Both primitive LTBMC-IC and clonogenic progenitors adhere to the three known cell-attachment sites in the 33/66 kD cell-adhesion promoting fragment, FN-C/H I, FN-C/H II and CS1. However, LTBMC-IC and CFU-MIX progenitors adhere significantly better to FN-C/H II than to the flanking FN-C/H I and CS1 cell-attachment sites. In contrast, single-lineage progenitors adhere equally well to all three cell attachment sites in the 33/66 kD cell-adhesion promoting fragment. (*d*) Finally, adhesion of primitive LTBMC-IC to intact irradiated stroma can be inhibited partially by peptide FN-C/H II and almost completely by a combination of FN-C/H II and peptide FN-C/H I and CS1. This study demonstrates that adhesive interactions between primitive hematopoietic progenitors and the extracellular matrix component fibronectin can occur. Specific changes in adhesion to the 33/66 kD cell-adhesion promoting fragment and the 75 kD RGDS-dependent cell-adhesion fragment of fibronectin are associated with differentiation of primitive multi-lineage progenitors into committed single-lineage progenitors. Such differences in adhesive interaction with fibronectin may allow hematopoietic progenitors at various stages of differentiation to interact with specific supportive loci of the bone marrow microenvironment. Finally, the ability to block adhesion of LTBMC-IC to intact irradiated stroma with peptides FN-C/H II, FN-C/H I and CS1 suggests that receptors responsible for this interaction may be important in the homing of primitive progenitors to the bone marrow.

The role of bone marrow stromal cells and/or extracellular matrix molecules in promoting proliferation and differentiation of primitive hematopoietic cells has been well established through the development of long term stromal based cultures for both myeloid hematopoietic cells (1) and lymphoid cells (2). The stromal elements of such cultures can sustain proliferation of primitive self-renewing hematopoietic cells (3, 4) and support differentiation and maturation of primitive progenitor cells into committed progenitors and mature blood elements (1, 2, 5–8). The influences of stromal elements on the early events which induce primitive, multi-lineage hematopoietic progenitors to proliferate, differentiate or assume single lineage commitment are largely unknown. Bone marrow derived stroma consists of several cell types (1) and a variety of extra-cellular matrix molecules. Extracellular matrix components such as fibronectin (9–15), laminin (16), hemonectin (17, 18), thrombospondin (19), proteoglycans (20, 21) and glycosaminoglycans (22) have been implicated in the adherence of committed clonogenic progenitors to stroma. Adhesive interactions of more primitive hematopoietic progenitors with stromal elements have been less well studied.

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Fibronectin is abundantly present in normal adult bone marrow (23, 24). Its role in the differentiation and maturation of erythroid and B lymphoid progenitors has been extensively studied (9–15). The possible role of fibronectin in homing of more primitive progenitors from human bone marrow and their differentiation into committed hematopoietic elements has, however, not yet been examined. Fibronectin has served as the prototype molecule for studies involving adhesion of cells with the extracellular matrix. Interaction of different cell types with fibronectin is important for their localization into organs (25). Fibronectin may have an instructive and permissive role in allowing immature cells to respond to soluble mediators and other mitogenic signals (26). Since fibronectin is universally present, specificity and selectivity for the adhesion of different cell types or the same cells at different stages of maturation to fibronectin is provided by both tissue specific expression of various forms of fibronectin (25) and differential expression of multiple fibronectin cell surface receptors (27, 28).

Various domains of the fibronectin molecule which interact with different cell surface receptors have been identified. A 75–120 kD proteolytic fragment in the center of the fibronectin molecule contains the sequence arginyl-glycyl-aspartyl-serine (RGDS)[1] (Fig. 1) (29, 30). The $\alpha5\beta1$ cell surface integrin complex (27, 30) is responsible for the interaction of different cell types with this RGDS-containing fragment of fibronectin. Adhesion to fibronectin can also occur in an RGDS-independent fashion via the 33/66 kD "heparin-binding fragment" at the COOH-terminal end of the fibronectin molecule (31–36). Three RGDS-independent cell-adhesion promoting peptide sequences have been identified within the 33/66 kD COOH-terminal fragment of fibronectin, named the 'CS1' peptide (32–34), the 'FN-C/H I' peptide (35, 36) and the 'FN-C/H II' peptide (35, 36) (Fig. 1). The $\alpha4\beta1$ integrin complex acts as the receptor for the CS1 region (33, 34), while cell surface heparin-like molecules are implicated in mediating cell adhesion to the FN-C/H I and FN-C/H II cell adhesion sites (35, 36).

We have recently demonstrated that very primitive bone marrow derived hematopoietic progenitors with long-term in vitro repopulating capacity (long term bone marrow culture initiating cells [LTBMC-IC]) demonstrate significantly greater capacity of adhering to irradiated allogeneic stromal layers than more differentiated clonogenic progenitor cells (5). In the present study, we demonstrate that primitive LTBMC-IC, multilineage CFU-MIX progenitors and single lineage BFU-E and CFU-GM adhere to the 33/66 kD heparin-binding cell-adhesion promoting fragment of fibronectin. In contrast, single lineage BFU-E and CFU-GM but neither primitive LTBMC-IC nor multi-lineage CFU-MIX adhere to the 75 kD RGDS-dependent cell-binding fragment of fibronectin. We demonstrate that the three adjacent cell-attachment sites in the 33/66 kD RGDS-independent cell-adhesion promoting fragment of fibronectin are important in the adhesion of hematopoietic progenitors to this fragment of the fibronectin molecule. Furthermore, differentiation from primitive LTBMC-IC and multi-lineage CFU-MIX progenitors to more differentiated single lineage BFU-E and CFU-GM progenitors is associated with changes in cell adhesion to the three RGDS-independent cell-attachment sites in the COOH-terminal cell-adhesion promoting fragment. These studies suggest that maturation and differentiation of hematopoietic elements is accompanied by acquisition of new functional cell surface receptors which may allow progenitor cells to migrate to distinct loci in the bone marrow microenvironment. Finally, adhesion of LTBMC-IC to intact allogeneic irradiated stroma can in part be blocked by FN-C/H II alone, while adhesion can be blocked to a greater extent by a combination of peptides FN-C/H I, FN-C/H II, and CS1. These findings indicate that receptors present on primitive LTBMC-IC responsible for their adhesion to these cell attachment sites may be important for homing of primitive hematopoietic progenitors to the bone marrow microenvironment and in their close interaction with elements of the bone marrow stroma.

Materials and Methods

Marrow Samples. Bone marrow samples were obtained from normal healthy volunteers after informed consent was obtained using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota. Heparinized bone marrow samples were obtained by aspiration from the posterior iliac crest. Bone marrow mononuclear cells (BMMNC) were isolated by Ficoll-Hypaque (Sigma Diagnostics, St. Louis, MO) density gradient centrifugation (sp. grav. 1.077) for 30' at 37°C and 400 *g*.

Selection of Purified Progenitor Populations

BMMNC were enriched for primitive hematopoietic progenitors by an immunomagnetic depletion of committed myeloid cells, monocytes, and lymphoid precursors. Further separation was accomplished by a positive FACS selection for cells with low/very low horizontal and low vertical light scatter properties and according to CD34 and HLA-DR antigen expression.

Immunomagnetic depletion was performed as published (5): BMMNC were incubated for 30' at 4°C with a mixture of 50 ng/10^6 cells mAbs with overlapping specificity for T and B lymphocytes, NK cells, monocyte and myeloid lineage including anti-CD2 (leu 5), anti-CD3 (leu 4), anti-CD16 (leu 11), anti-CD11 (leu 15), anti-CD15 (MY-18), anti-CD19 (leu 12), anti-CD56 (leu 19), and anti-CD71 (transferrin) (Becton-Dickinson and Co., Mountain View, CA), washed twice and incubated with immunomagnetic beads coated with goat anti-mouse IgG and IgM (Advanced Magnetics Inc., Cambridge, MA) for 30' at 4°C with periodic agitation (30–40 × 10^6 beads were used per 10^6 cells). Rosettes and free particles were removed using a BioMag Separator (Advanced Magnetics Inc.). The rosette-free fraction was called lineage negative BMMNC.

FACS Sort. Lineage negative cells obtained from immunomagnetic separation were first incubated with 100 μg goat F(ab)'2 anti-mouse IgG (Tago Inc.) to block any mouse IgG mAb still present after immunomagnetic depletion. The cells were next incubated with 500 μg mouse IgG (Sigma Diagnostics) to block any unbound active site on the goat F(ab)'2 anti-mouse IgG. Cells were incubated with 0.25 μg of anti-CD34 antibody/10^6 cells (HPCA-1) (Becton Dickinson) for 30' at 4°C and washed twice. We then treated the cells with 0.25 μg/10^6 cells fluorescein conjugated goat

[1] *Abbreviations used in this paper:* LTBMC-IC, long term bone marrow culture initiating cells; RGDS, arginyl-glycyl-aspartyl-serine.

F(ab)'2 anti–mouse IgG (Tago Inc.) for 30' at 4°C and washed twice. Cells were then incubated with 500 μg mouse IgG (Sigma Chemical Co.), followed by a 30' incubation with 0.25 μg of anti–HLA-DR-PE antibody/10^6 cells (L234) (Becton-Dickinson and Co.). After washing, cells were sorted on a FACS-Star laser flow cytometry system (Becton-Dickinson and Co.) equipped with a consort 40 computer. A first selection consisted of gating in for cells with low vertical and very low/low horizontal light scatter properties. The sorting gates were then set to isolate cells expressing high density CD34 antigen and either absence (referred to as DR^- cells) or presence (referred to as DR^+ cells) of HLA-DR antigens.

Short Term Methylcellulose Assay. Cells recovered in the adherent fraction of adhesion assays performed with DR^+ cells were plated in methylcellulose (final concentration 1.12%) (Fisher Scientific, Fair Lawn, NY) with IMDM (Gibco Laboratories, Grand Island, NY) supplemented with 30% FCS (HyClone Laboratories, Logan, UT), antibiotics (penicillin 1,000 U/m and streptomycin 100 U/ml; Gibco Laboratories), 5×10^{-5} M 2-ME, 3 IU recombinant erythropoietin (Epoetin) (Amgen, Thousand Oaks, CA) and 10% conditioned media from the bladder carcinoma cell line 5637. Cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 18–21 d. The cultures were assessed at day 18–21 of culture for the presence of CFU-MIX, CFU-GM and BFU-E as previously described (5). Likewise, cells harvested at week 5 from LTBMC initiated with DR^- cells were plated in short term methylcellulose culture and secondary CFU-MIX, CFU-GM and BFU-E were enumerated at day 14–16 of culture.

Long-Term Bone Marrow Cultures (LTBMC). Bone marrow derived stromal cell cultures were prepared as described (5). DR^- (10^4/well) cells were then plated or panned (5) on allogeneic irradiated stroma in 0.5 ml of LTBMC media (IMDM with 12.5% FCS, 12.5% horse serum [HyClone Laboratories], 2 mM L-glutamine, penicillin 1,000 U/m and streptomycin 100 U/ml and 10^{-6} M hydrocortisone [A-Hydrocort] [Abbott Laboratories, North Chicago, IL]). Cells recovered in adherent fractions from adhesion assays initiated with DR^- cells were also plated in LTBMC for 5 wk. All LTBMC cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. At weekly intervals the cultures were fed by removing half of the supernatant and replacing it with fresh media. At 5 wk of culture, nonadherent cells present in the overlying supernatant and adherent cells recovered from the stromal layers after treatment with 0.1% collagenase were assayed for the presence of clonogenic progenitors in short term methylcellulose assay.

It is believed that committed progenitor cells present in the initial bone marrow population fail to produce clonogenic progenitor growth in LTBMC 4–5 wk after initiation of such cultures (5–8). The number of clonogenic progenitors recovered from both adherent and nonadherent layers of LTBMC at 5 wk of culture may therefore represent progeny of more primitive progenitors which can sustain long term in vitro hematopoiesis and are termed LTBMC initiator or LTBMC-IC (5–8).

Purification of Fibronectin and Fibronectin Fragments. Human plasma fibronectin was purified as a by-product of Factor VIII production by sequential ion exchange and gelatin chromatography (31). A 75 kD tryptic fragment containing the cell binding fragment RGDS was purified from long-term (90') tryptic digestion as previously described (31). The 33 kD and 66 kD COOH-terminal heparin binding fragments were purified from short term (15') tryptic/catheptic digest of intact fibronectin by sequential heparin and antibody column chromatography as described (31). The purity of intact fibronectin, the 33/66 kD fragment and the 75 kD fragment of fibronectin was verified by SDS-PAGE and Coomassie brilliant blue staining.

Peptide Synthesis and Characterization. Peptides from fibronectin were synthesized at the Microchemical Facility of the University of Minnesota by Dr. Robert Wohlheuter using a peptide synthesizer (System 990; Beckman Instruments Co., Fullerton, CA). The procedures used were based on the Merrifield solid-phase system as described previously (37). Lyophilized crude peptides were purified by preparative reverse-phase HPLC on a C-18 column, using an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. The purity and composition of the peptides was verified by HPLC analysis of hydrolysates prepared by treating the peptides under nitrogen in 6 N HCl overnight at 110°C. The peptides include heparin binding peptide I, FN-C/H I, and heparin-binding peptide II, FN-C/H II which have the sequences YEKPGSPPREVVPRPRPGV (35, 36) and KNNQKSEPLIGRTKKT (35, 36) respectively and peptide CS1, which has the sequence DELPQLVTLPHPNLHPGEILDVPST (32–34) (Fig. 1). The sequences use the single amino acid code (K = lysine, R = arginine, H = histidine, E = glutamic acid, D = aspartic acid, Q = glutamine, N = asparagine, P = proline, G = glycine, S = serine, T = threonine, V = valine, I = Isoleucine, L = leucine, Y = tyrosine).

Conjugation of the Peptides to Ovalbumin. The synthetic peptides were chemically conjugated to ovalbumin with 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Sigma Chemical Co.) as described (35, 36). Briefly, equal amounts (by weight) of the peptides and ovalbumin are solubilized in water and mixed with a 10-fold excess (by weight) of EDC dissolved in water. The samples were mixed overnight at 4°C on a circular rotator. The coupled peptides were dialyzed extensively in PBS to remove excess EDC (Spectrapore 6, 10 kD exclusion; Spectrum Medical Industries, Los Angeles, CA). The molar ratio of each of the peptides to BSA was 1:12 for peptide CS1, 1:4 for FN-C/H I and 1:5 for FN-C/H II. The conjugates were stored at −20°C until use.

Generation and Purification of Polyclonal Antibodies Against Synthetic Peptides. Polyclonal antibodies were generated against the different synthetic peptides coupled to keyhole limpet hemocyanin (KLH; Sigma Chemical Co.) using carbodiimide as a coupling reagent (35, 36, 39–40). After the coupling reaction, the coupled peptide was dialyzed in Spectrapore 6 tubing (Spectrum Medical Industries) with an 1,000-D exclusion so that only the excess carbodiimide was removed and any uncoupled peptide will remain in the tubing. This mixture was then concentrated in Acquacide II (Calbiochem-Behring Corp., La Jolla, CA) to a final concentration of 10 mg/ml, and aliquots were stored at −70°C. The coupling of the peptide was verified by the use of radioiodinated peptide in a parallel reaction.

The coupled mixture was then used to immunize New Zealand White Rabbits. Immunization was performed by mixing an equal volume of peptide/KLH conjugate with CFA and injecting this mixture into multiple sites of the shaved back of the rabbits (2 mg of the conjugate/rabbit). Subsequently biweekly boosts in incomplete Freund's adjuvant were injected intra muscular into the hind legs of rabbits. Sera were collected 7–10 d after the sixth immunization, and tested by ELISA for reactivity against the uncoupled peptides and various other ligands.

IgG was purified from pooled immune sera by precipitation with a final concentration of 45% ammonium sulphate overnight at 4°C. This precipitate was pelleted by centrifugation, resolubilized, and dialyzed against 0.035 M NaCl in 0.025 M Tris, pH 8.2. The IgG was purified over a DEAE anion exchange column as described previously (35, 36). Purity of the IgG was determined by SDS-PAGE and Coomassie brilliant blue staining of the gel. Retained immunoreactivity of the purified IgG was verified by ELISA.

Cell Adhesion Assays. Substrata were prepared as follows: fibronectin, proteolytic fragments from fibronectin and synthetic peptides were diluted to the appropriate concentrations in Voller's

carbonate buffer (31). Proteins were adsorbed to wells of 48 well plates (Costar, Cambridge, MA) overnight in a humidified atmosphere at 37°C. Nonspecific sites were blocked the following day with 5 mg/ml BSA (BSA, fatty acid free, Miles, Naperville, IL) in PBS, pH 7.4, for 2–3 h. Control wells were adsorbed with BSA 5 mg/ml or ovalbumin-coupled ovalbumin and then blocked with BSA in PBS. DR^- cells and DR^+ cells were suspended at 4×10^4 cells/ml in IMDM + 1% BSA (Sigma Diagnostics) or LTBMC media and 250 μl cell suspension was plated in ligand coated or control wells for 3 h at 37°C and 5% CO_2. The nonadherent and loosely attached cells were removed after 3 h by 4-5 consecutive washings with warm IMDM after standardized horizontal shaking of the plates (30 sec, 100 excursions per min). Since we were unable to demonstrate significant differences between the adhesion of DR^- cells or DR^+ cells diluted in either IMDM/BSA or LTBMC media, results presented are pooled data of adhesion assays performed either in IMDM/BSA or LTBMC media. Cells present in the adherent fractions of adhesion assays initiated with DR^- cells were evaluated for the presence of LTBMC-IC by culturing in LTBMC for 5 wk and evaluating these cultures for the presence of secondary clonogenic progenitors. Cells recovered in the adherent fraction of adhesion assays initiated with DR^+ cells were evaluated for the presence of single lineage BFU-E and CFU-GM progenitors and multi-lineage CFU-MIX progenitors by culturing in short term methyl cellulose culture. Percent adhesion was calculated as (the number of cells adherent to ligand coated wells − the number of cells adherent to BSA or OVA/OVA control wells, divided by the total input cells) × 100.

Inhibition of Cell Adhesion. Experiments were designed to evaluate the specificity of LTBMC-IC, CFU-MIX, BFU-E and CFU-GM adhesion to the 33/66 kD heparin-binding cell-adhesion promoting fragment of fibronectin and to peptide-ovalbumin conjugates. In the first type of assay, inhibition of cell adhesion to the 33/66 kD fragment of fibronectin or to ovalbumin-coupled peptides (at concentrations inducing half-maximal adhesion of hematopoietic progenitors) was tested by preincubating DR^- and DR^+ cells with increasing concentrations of free synthetic peptides for 30′ before the cell adhesion assays described above. In the second assay, increasing concentrations of purified polyclonal rabbit IgG raised against peptides FN-C/H I, FN-C/H II and CS1 (37, 39) were added to wells which had previously been coated with the 33/66 kD fragment or ovalbumin-coupled peptides (at a concentration inducing half-maximal adhesion of hematopoietic progenitors), and incubated for 30′ at 37°C before the cell adhesion assays. In both above described competition assays, the exogenous peptides and polyclonal IgG remain in the wells for the duration of the cell adhesion assay. Inhibition of adhesion by different synthetic peptides or antibodies was then calculated as 100 − [(number of cells adherent in the treated population/number of cells adherent in the untreated population) × 100].

Statistical Analysis. Results of experimental points obtained from multiple experiments were reported as the $\bar{x} \pm 1$ SEM. Significance levels were determined by two-sided students t-test analysis.

Results

Primitive Multi-lineage Progenitors but not Single-lineage Hematopoietic Progenitors Fail to Adhere to the RGDS-containing 75 kD Cell-binding Fragment of Fibronectin. We first evaluated the adhesive interactions of specific hematopoietic progenitor cells with fibronectin by performing adhesion assays to wells coated with increasing concentrations of fibronectin and its proteolytic fragments. Adherent fractions were then cultured in LTBMC culture (DR^- cells) or in short-term methylcellulose assay (DR^+ cells) and the number of LTBMC-IC, CFU-MIX, BFU-E or CFU-GM present in the adherent cell populations was enumerated. Preliminary studies demonstrated that the 3-h incubation of DR^- and DR^+

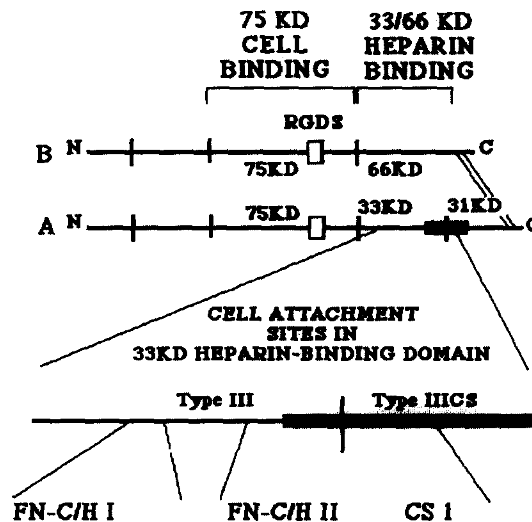

Figure 1. Structure of fibronectin. Location of the 75 kD RGDS-dependent cell-binding fragment, the 33/66 kD major COOH-terminal heparin-binding cell-adhesion promoting fragment and synthetic peptides, FN-C/H I, FN-C/H II and CS1, within the intact fibronectin molecule. Also depicted is the IIIcs region found only in the A-isoform of human plasma fibronectin.

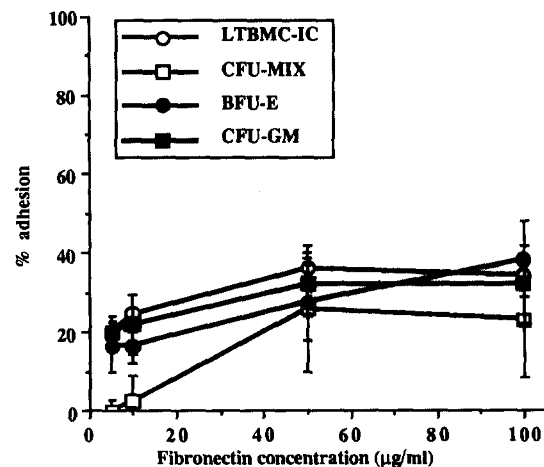

Figure 2. 10^4 DR^- cells (n = 7) and DR^+ cells (n = 7) were plated in fibronectin coated or BSA control wells for 3 h at 37°C and 5% CO_2. Adherent cells were cultured in LTBMC culture (DR^- cells) or in short-term methylcellulose assay (DR^+ cells) and the number of LTBMC-IC, CFU-MIX, BFU-E or CFU-GM present enumerated. Percent adhesion was calculated as (the number of progenitors adherent to ligand coated wells − the number of progenitors adherent to BSA/the total input progenitors) × 100. Mean number of progenitors present in the starting population was: for LTBMC-IC: 374 ± $15/10^4$ cells, for CFU-MIX: 4.66 ± $1.7/10^3$ cells, for BFU-E: 17.4 ± $6.7/10^3$ cells, for CFU-GM: 106 ± $49/10^3$ cells.

cells in ligand coated wells did not significantly decrease their viability and cloning efficiency (data not shown).

Adhesion of primitive LTBMC-IC, immature CFU-MIX and single lineage BFU-E and CFU-GM progenitors to intact fibronectin was not significantly different (Fig. 2). Primitive LTBMC-IC and CFU-MIX adhered to the 33/66 kD COOH-terminal heparin-binding fragment of fibronectin (Fig. 3). However these primitive progenitors adhered significantly less to the 75 kD RGDS-dependent cell-binding fragment (p = 0.007 and p = 0.001) (Fig. 3). In contrast, adhesion of single lineage BFU-E and CFU-GM progenitors occurred equally well to both the 75 kD RGDS-dependent and the 33/66 kD RGDS-independent cell-adhesion fragments (Fig. 3). These findings suggest that upon maturation, primitive progenitor cells acquire functional receptors which allow more differentiated single-lineage progenitors to interact with fibronectin through both an RGDS–dependent and an RGDS-independent mechanism.

Adhesion of Hematopoietic Progenitors to the 33/66 kD Cell-adhesion Promoting Fragment Can Occur through at Least Three RGDS-independent Cell-attachment Sites. Three adjacent cell adhesion promoting sites contained within synthetic peptides FN-C/H I, FN-C/H II, and CS1, have been described in the cell adhesion promoting heparin-binding fragment of fibronectin. In a second set of experiments we established the role of these three sites in the adhesion of hematopoietic progenitors. Although all experiments were performed for LTBMC-IC, CFU-MIX, BFU-E, and CFU-GM, we elected for the sake of simplicity to present only specific data on adhesion of the CFU-GM progenitors. As can be seen in Fig. 4,

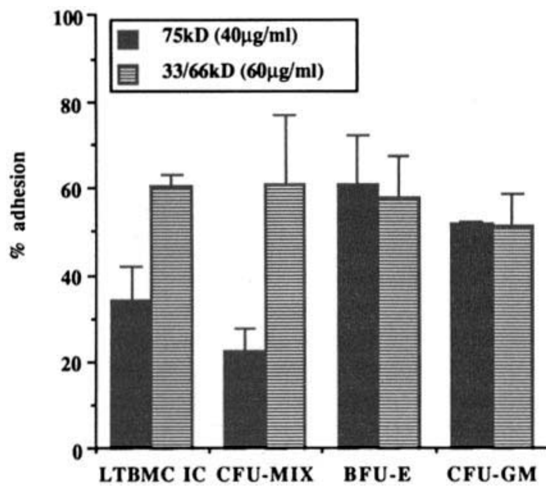

Figure 3. 10^4 DR$^-$ cells (n = 7) and DR$^+$ cells (n = 7) were plated in ligand coated wells or BSA control wells for 3 h at 37°C and 5% CO_2. Adherent cells were cultured in LTBMC culture (DR$^-$ cells) or in short-term methylcellulose assay (DR$^+$ cells) and the number of LTBMC-IC, CFU-MIX, BFU-E or CFU-GM present enumerated. Percent adhesion was calculated as (the number of progenitors adherent to ligand coated wells − the number of progenitors adherent to BSA control wells/the total input progenitors) × 100. Mean number of progenitors present in the starting population was: for LTBMC-IC: 374 ± 15/10^4 cells, for CFU-MIX: 4.66 ± 1.7/10^3 cells, for BFU-E: 17.4 ± 6.7/10^3 cells, for CFU-GM: 106 ± 49/10^3 cells.

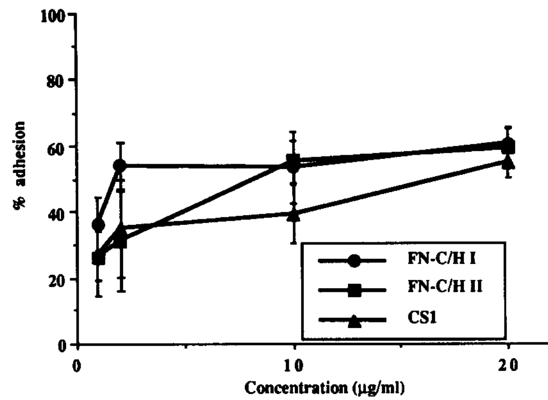

Figure 4. 10^4 DR$^+$ cells (n = 7) were plated in wells coated with increasing concentrations of OVA-coupled FN-C/H I, FN-C/H II or CS1 peptides or OVA-OVA coated control wells for 3 h at 37°C and 5% CO_2. Adherent cells were cultured in short-term methylcellulose assay and the number of CFU-GM present enumerated. Percent adhesion was calculated as (the number of progenitors adherent to ligand coated wells − the number of progenitors adherent to control wells/the total input progenitors) × 100. Mean number of progenitors present in the starting population was 106 ± 49 CFU-GM/10^3 cells.

purified, immobilized synthetic peptide FN-C/H I, peptide FN-C/H II and peptide CS1 were equally effective at promoting adhesion of CFU-GM progenitors.

We then examined the mechanisms through which hematopoietic progenitors adhere to peptides FN-C/H I and FN-C/H II by performing adhesion inhibition experiments with both free synthetic peptides (Fig. 5) and polyclonal IgG antibodies raised against the peptides (Fig. 6). In a first set of experiments FACS sorted hematopoietic progenitor popula-

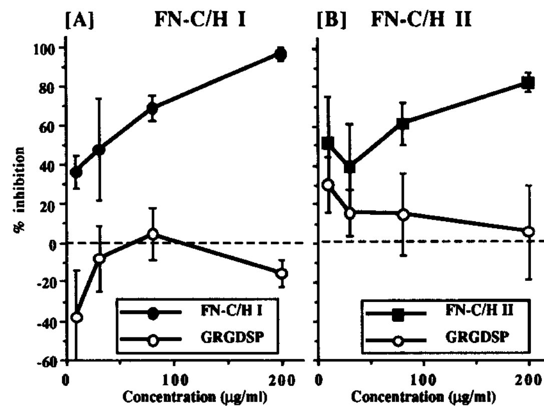

Figure 5. 10^4 DR$^+$ cells were preincubated with increasing concentrations of free synthetic peptides FN-C/H I, FN-C/H II or GRGDSP for 30' before the adhesion experiments. Cells were then allowed to adhere to substrata coated with OVA coupled peptide FN-C/H I (A) or FN-C/H II (B) for an additional 3 h and this in the presence of the free peptides. Adherent cells were plated in methylcellulose assay and the number of CFU-GM present enumerated. Inhibition of adhesion by synthetic peptides was calculated as 100 − ([number of progenitors adherent in the treated population/number of progenitors adherent in the untreated population] × 100). Mean number of progenitors present in the starting population 152 ± 36.8 CFU-GM/10^3 cells (n = 5).

tions were preincubated with increasing concentrations of synthetic peptide GRGDSP, the sequence of minimal recognition in the centrally located 75 kD cell-binding fragment of fibronectin, or with free synthetic peptides FN-C/H I and FN-C/H II for 30′ before the adhesion experiments. Adhesion of CFU-GM progenitors to substrata coated with OVA-coupled peptides FN-C/H I (Fig. 5 *A*) and FN-C/H II (Fig. 5 *B*) could be inhibited significantly and in a dose dependent fashion by preincubating cells with increasing concentrations of free synthetic peptides FN-C/H I or FN-C/H II respectively, but not by free synthetic peptide GRGDSP. We also evaluated the adhesion of hematopoietic progenitors to substrata coated with OVA coupled peptides FN-C/H I and FN-C/H II after preincubating the wells with increasing concentrations of normal rabbit IgG, an antibody raised against an irrelevant synthetic peptide representing a cell adhesion fragment of collagen type IV (anti–IV-H I polyclonal rabbit IgG antibody) (38) or polyclonal rabbit IgG antibodies raised against peptides FN-C/H I and FN-C/H II (Fig. 6). Adhesion of CFU-GM to substrata coated with OVA-coupled peptides FN-C/H I (Fig. 6 *A*) and FN-C/H II (Fig. 6 *B*) was inhibited significantly and in a dose dependent fashion by preincubating the wells with polyclonal IgG antibodies raised against the peptides FN-C/H I and FN-C/H II respectively, but not by rabbit IgG or anti–IV-H I.

We next determined the importance of these three synthetic peptides for the interaction of hematopoietic progenitors with the intact 33/66 kD cell-adhesion promoting fragment of fibronectin by performing adhesion inhibition experiments with free synthetic peptides (Fig. 7 *A*) or polyclonal antibodies raised against these peptides (Fig. 7 *B*). Preincubation of FACS purified hematopoietic progenitors with

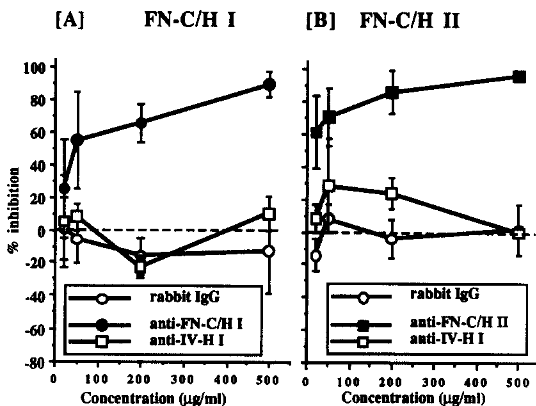

Figure 6. Substrata coated with OVA coupled peptide FN-C/H I (*A*) or peptide FN-C/H II (*B*) were preincubated with increasing concentrations of polyclonal IgG rabbit antibodies raised against synthetic peptide FN-C/H I (*A*) or peptide C/H II (*B*), peptide IV-H I or normal rabbit IgG for 30′ before the adhesion experiments. 10^4 DR$^+$ cells were then allowed to adhere to the preincubated substrata for an additional 3 h and this in the presence of the antibodies. Adherent cells were plated in methylcellulose assay and the number of CFU-GM enumerated. Inhibition of adhesion by antibodies was calculated as 100 − ([number of progenitors adherent to the preincubated substrata/number of progenitors adherent to the untreated substrata] × 100). Mean number of progenitors present in the starting population was 111.7 ± 16.7 CFU-GM/10^3 cells (n = 4).

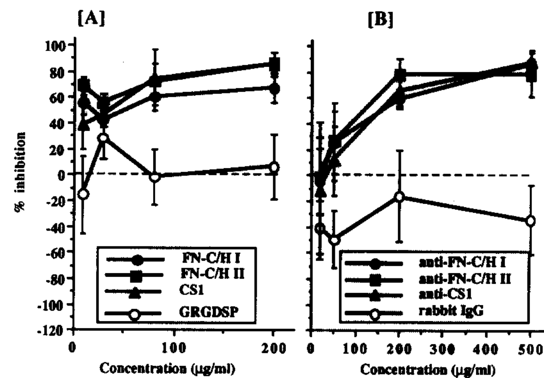

Figure 7. (*A*) 10^4 DR$^+$ cells were preincubated with increasing concentrations of free synthetic peptides FN-C/H I, FN-C/H II, CS1 or GRGDSP for 30′ before the adhesion experiments. Cells were then allowed to adhere to substrata coated with the 33/66 kD heparin-binding cell-adhesion promoting fragment of fibronectin for an additional 3 h and this in the presence of the free peptides. (*B*) Alternatively, wells coated with the 33/66 kD proteolytic fragment of fibronectin were preincubated for 30′ before the adhesion assay with increasing concentrations of polyclonal IgG rabbit antibodies raised against synthetic peptide FN-C/H I, peptide FN-C/H II, peptide CS 1 or rabbit IgG. 10^4 DR$^+$ cells were then allowed to adhere to the preincubated substrata for an additional 3 h and this in the presence of the antibodies. Adherent cells were plated in methylcellulose assay and the number of CFU-GM enumerated. Inhibition of adhesion was calculated as 100 − ([number of progenitors adherent in the treated population/number of progenitors adherent in the untreated population] × 100). Mean number of progenitors present in the starting population was 106 ± 49 CFU-GM/10^3 cells (n = 5) (*A*) and 111.7 ± 16.7 CFU-GM/10^3 cells (n = 5) (*B*).

free synthetic peptides FN-C/H I, FN-C/H II and CS1, but not peptide GRGDSP, prevented the adhesion of CFU-GM progenitors to the 33/66 kD cell-adhesion promoting fragment in a dose dependent fashion. Similarly, preincubation of wells coated with the 33/66 kD cell-adhesion promoting fragment with polyclonal antibodies raised against peptides FN/CH-I, FN-C/H II and CS1 inhibited significantly the adhesion of CFU-GM progenitors to the heparin-binding cell attachment fragment, while such inhibition was not seen with normal rabbit IgG (Fig. 7 *B*).

These studies confirm that, as for other cell types, all three synthetic adhesion-promoting sites in the heparin-binding cell-adhesion promoting fragment of fibronectin are effective at promoting adhesion of human hematopoietic progenitors. Moreover, adhesion of hematopoietic progenitors to the heparin-binding cell-adhesion promoting fragment of FN occurs in an RGDS independent fashion.

Adhesion of Primitive, Multilineage Progenitors to the 33/66 kD Cell-adhesion Promoting Heparin-binding Fragment of Fibronectin May Involve Different Mechanisms Than That of More Committed Single-lineage Progenitors. We next determined the mechanism through which the adhesion of primitive LTBMC-IC, multi-lineage CFU-MIX progenitors and more committed single-lineage BFU-E and CFU-GM progenitors adhere to the 33/66 kD cell-adhesion promoting fragment of fibronectin. We first determined the adhesion of all four cell populations to FN-C/H I, FN-C/H II and CS1 (Fig. 8). Primitive LTBMC-IC and immature multilineage CFU-MIX

progenitors adhered significantly better to peptide FN-C/H II than to peptides FN-C/H I and CS1 (Fig. 8). In contrast, single lineage BFU-E and CFU-GM progenitors adhered equally well to peptide FN-C/H 1, peptide FN-C/H II and peptide CS1 (Fig. 8).

We next verified the mechanism underlying the adhesion of primitive LTBMC-IC, multi-lineage CFU-MIX and single-lineage BFU-E and CFU-GM to the 33/66 kD heparin-binding cell-adhesion promoting fragment of FN by performing adhesion inhibition experiments. We first evaluated the adhesion of all four progenitor populations to the heparin-binding fragment in the presence of free synthetic peptides FN-C/H I, FN-C/H II and CS1. As shown in Fig. 9, adhesion of primitive LTBMC-IC and multi-lineage CFU-MIX progenitors to the 33/66 kD cell-adhesion promoting fragment was inhibited by preincubating FACS sorted hematopoietic progenitors with free synthetic peptide FN-C/H II ($p = 0.02$ and $p < 0.001$, respectively), but less so by peptides FN-C/H I ($p = 0.06$ and $p = 0.08$, respectively) and CS1 ($p = 0.08$ and $p = 0.08$, respectively). In contrast, adhesion of single-lineage BFU-E and CFU-GM to the 33/66 kD cell-adhesion promoting fragment could be inhibited equally well by preincubating the FACS sorted DR$^+$ cells with either free synthetic peptide FN-C/H I ($p = 0.01$ and $p = 0.05$, respectively), peptide FN-C/H II ($p = 0.05$ and $p = 0.01$, respectively) and peptide CS1 ($p = 0.02$ and $p = 0.01$, respectively) before the adhesion assay (Fig. 9). Similar results were obtained in adhesion inhibition experiments in which the adhesion of hematopoietic progenitors to substrata coated with the 33/66 kD cell-adhesion promoting fragment of fibronectin was inhibited by polyclonal rabbit IgG anti-

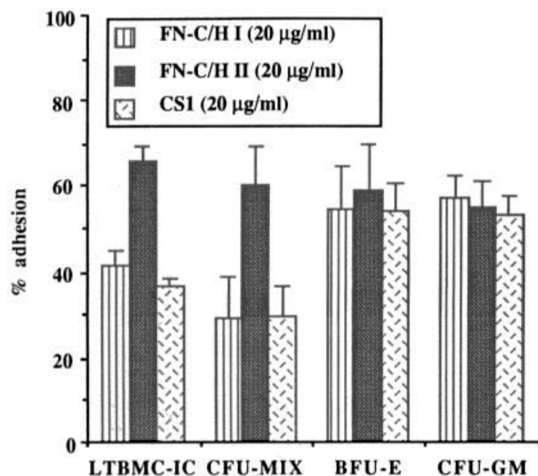

Figure 8. 10^4 DR$^-$ cells (n = 7) and DR$^+$ cells (n = 7) were plated in ligand coated or OVA-OVA control wells for 3 h at 37°C and 5% CO_2. Adherent cells were plated in either LTBMC (DR$^-$ cells) or methylcellulose assay (DR$^+$ cells) and the number of LTBMC-IC, CFU-MIX, BFU-E and CFU-GM enumerated. Percent adhesion was calculated as (the number of cells adherent to ligand coated wells − the number of cells adherent to control wells/the total input progenitors) × 100. Mean numbers of progenitors present in the starting population was: for LTBMC-IC: 374 ± 15/10^4 cells, for CFU-MIX: 4.66 ± 1.7/10^3 cells, for BFU-E: 17.4 ± 6.7/10^3 cells, for CFU-GM: 106 ± 49/10^3 cells.

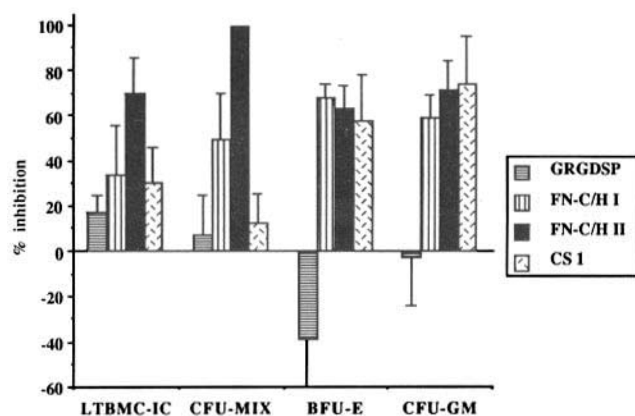

Figure 9. 10^4 DR$^-$ (n = 4) and DR$^+$ (n = 5) cells were preincubated with increasing concentrations of free synthetic peptides FN-C/H I, FN-C/H II for 30′ before the adhesion experiments. Cells were then allowed to adhere to substrata coated with the 33/66 kD heparin-binding cell-adhesion promoting fragment of fibronectin for an additional 3 h and this in the presence of the free peptides. Adherent cells were plated in either LTBMC (DR$^-$ cells) or methylcellulose assay (DR$^+$ cells) and the number of LTBMC-IC, CFU-MIX, BFU-E and CFU-GM enumerated. Inhibition of adhesion by synthetic peptides was calculated as 100 − ([number of progenitors adherent in the treated population/number of progenitors adherent in the untreated population] × 100). Data represent results obtained at 80 µg/ml free synthetic peptide blocking concentration. Mean number of progenitors present in the starting population was: for LTBMC-IC: 424 ± 39.4/10^4 cells, for CFU-MIX: 4.66 ± 1.7/10^3 cells, for BFU-E: 17.4 ± 6.7/10^3 cells and for CFU-GM: 106 ± 49/10^3 cells.

bodies raised against peptide FN-C/H I, peptide FN-C/H II and peptide CS 1 (Fig. 10).

These studies suggest that the FN-C/H II cell attachment site has a more dominant role in the adhesion of primitive LTBMC-IC and CFU-MIX to the 33/66 kD heparin-binding cell-adhesion promoting fragment while the adjacent cell-attachment sites FN-C/H I and CS1 may be less important in this interaction. In contrast, adhesion of more differentiated single-lineage BFU-E and CFU-GM to the 33/66 kD heparin-binding cell-adhesion promoting fragment may involve all three defined cell attachment sites.

Adhesion of Primitive LTBMC-IC to Intact Irradiated Stroma Can Be Inhibited Partially by Peptide FN-C/H II and Almost Completely by a Combination of FN-C/H II with Peptides FN-C/H I and CS1. Finally, we evaluated the ability of peptides FN-C/H I, FN-C/H II and CS1 to inhibit the adhesion of LTBMC-IC to intact allogeneic stroma (Fig. 11). DR$^-$ cells were incubated for 30′ at 37°C in a humidified atmosphere in the absence or presence of either FN-C/H I, FN-C/H II or CS1 before panning on irradiated allogeneic stromal layers (5). After 2 h, non-adherent cells were removed by extensive washing with warm IMDM. Cells adherent to the irradiated stromal layers were then cultured further in LTBMC. At week 5 of culture, stromal and supernatant fractions were evaluated for the presence of LTBMC-IC as described (5).

As we have previously demonstrated, virtually all LTBMC-IC adhere to irradiated stroma when panned for 2 h (94.6 ± 14.6% recovery of LTBMC-IC in LTBMC initiated with

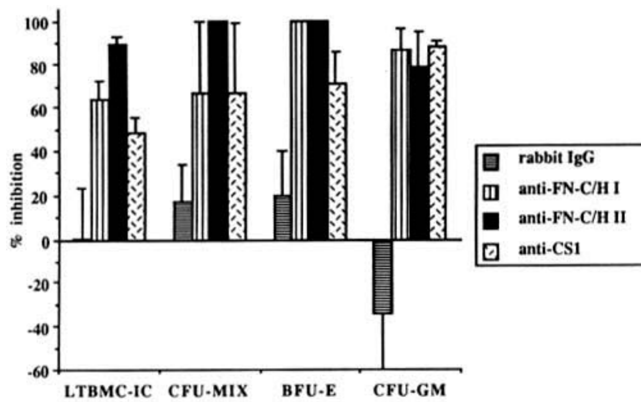

Figure 10. Substrata coated with the 33/66 kD heparin-binding cell-adhesion promoting fragment of fibronectin were preincubated with increasing concentrations of polyclonal IgG rabbit antibodies raised against synthetic peptides FN-C/H I, FN-C/H II, CS1 or rabbit IgG for 30′ before the adhesion experiments. Purified DR^- (n = 4) and DR^+ (n = 5) populations (10^4 cells/well) were then allowed to adhere to the preincubated substrata for an additional 3 h and this in the presence of the antibodies. Adherent cells were plated in either LTBMC (DR^- cells) or methylcellulose assay (DR^+ cells) and the number of LTBMC-IC, CFU-MIX, BFU-E and CFU-GM present enumerated. Inhibition of adhesion by antibodies was calculated as 100 − ([number of progenitors adherent to the preincubated substrata/number of progenitors adherent to the untreated substrata] × 100). Data represent results obtained at antibody concentration of 500 μg IgG/ml. Mean number of progenitors present in the starting population was: for LTBMC-IC: 270 ± 21.8/10^4 cells, for CFU-MIX: 4 ± 1.35/10^3 cells, for BFU-E: 13.4 ± 5/10^3 cells, for CFU-GM; 111.7 ± 16.7/10^3 cells.

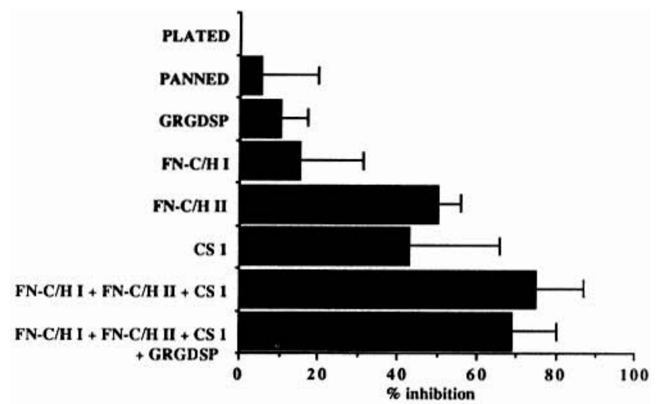

Figure 11. DR^- cells (n = 3) were incubated for 30′ at 37°C in a humidified atmosphere in the absence or presence of either GRGDSP (500 μg/ml), FN-C/H I (200 μg/ml), FN-C/H II (200 μg/ml) or CS1 (200 μg/ml) diluted in 500 μl IMDM + 1% BSA either alone or in combination. Preincubated DR^- cells (10^4 cells/well) were then panned on irradiated allogeneic stromal layers for 2 h. Nonadherent cells were removed by extensive washing with warm IMDM. Cells adherent to the irradiated stromal layers were cultured further in LTBMC. At week 5 of LTBMC, stromal and supernatant fractions were evaluated for the presence of LTBMC-IC. Inhibition of adhesion by different synthetic peptides either alone or in combination was calculated as 100 − ([number of LTBMC-IC adherent in the treated population/number of LTBMC-IC present in LTBMC initiated with plated cells] × 100). The mean number of LTBMC-IC present in LTBMC initiated with plated DR^- cells was: 432 ± 51/10^4 cells.

panned DR^- cells compared to plated DR^- cells). Preincubation of DR^- cells in the presence of peptide GRGDSP did not significantly alter the adhesion of LTBMC-IC to irradiated stroma (p = 0.24). Preincubation of DR^- cells in the presence of either peptide FN-C/H I (p = 0.5) or peptide CS1 (p = 0.2) inhibited the adhesion of LTBMC-IC to irradiated stroma although this inhibition did not attain significance. In contrast, preincubation of DR^- cells with peptide FN-C/H II inhibited significantly the adhesion of primitive LTBMC-IC to irradiated stroma (p = 0.002). This inhibition was even more pronounced when DR^- cells were preincubated in the presence of all three synthetic peptides representing different cell-attachment sites in the 33/66 kD RGDS–independent cell-adhesion promoting fragment of fibronectin. Addition of peptide GRGDSP to the preincubation mixture containing peptides FN-C/H I, FN-C/H II and CS1 did not change the degree of inhibition observed in the presence of the combination of FN-C/H I, FN-C/H II and CS1. These data indicate that cell surface receptors present on LTBMC-IC important for the adhesion of these primitive progenitors to peptide FN-C/H II and less so to FN-C/H I and CS1 may play an important role in their adhesion to the bone marrow microenvironment.

Discussion

In the present paper we extend our earlier studies which demonstrated that primitive LTBMC-IC present in the DR^- cell fraction, but not more differentiated clonogenic progenitors in the DR^+ cell fraction of bone marrow, adhere avidly to irradiated human bone marrow derived stromal layers when panned for 2 h (5). We define here additional differences in adhesive characteristics between primitive multilineage hematopoietic progenitors and their more differentiated single-lineage clonogenic counterparts by examining the interaction of these progenitors with the prototype adhesion molecule fibronectin.

There is evidence that fibronectin serves as an adhesion molecule for primitive $B220^+$ murine pre-B cells with which it interacts through α4β1 and α5β1 integrin receptors (15, 40). Similar observations were made for human primitive B cell progenitors which have the capacity of adhering to fibronectin (14). Similar to what we demonstrate for LTBMC-IC present in the DR^- cell fraction of human bone marrow, adhesion of the most primitive $CD34^+/CD10^+$ B cell precursors to bone marrow derived fibroblast layers was significantly greater than to wells coated with fibronectin, indicating that multiple molecules present on stromal cells or in the extracellular matrix may be important for the adhesion of primitive precursors to bone marrow derived stroma.

The murine IL-3 dependent cell line, FDCP-MIX, murine $CFU\text{-}S_{12}$ and murine repopulating stem cells are capable of adhering to murine stromal cell lines and isolated fibronectin molecules (41). These murine progenitors interact poorly with intact fibronectin, but bind avidly to the CS1-containing 33kD heparin-binding fragment of fibronectin. This adhesion can be inhibited by anti-α4β1 antibodies. Depletion of α4β1 positive hematopoietic progenitors from murine bone marrow

does, moreover, prevent successful engraftment of murine BMMNC in lethally irradiated syngeneic animals. Engraftment can also be prevented by preincubation of murine BMMNC with anti-$\alpha4\beta1$ antibodies, suggesting that murine hematopoietic stem cells may express the $\alpha4\beta1$ integrin receptor and that this receptor may have a role in 'homing' of stem cells to the bone marrow.

We demonstrate that the adhesion of primitive human hematopoietic progenitors to stroma can in part be blocked by preincubating these progenitors with peptides FN-C/H II in combination with peptides FN-C/H I and CS1. This suggests that such progenitors may have functional heparin-like cell surface receptors that are important, together with integrin receptors, in their interaction with the bone marrow environment. Several studies suggest that proteoglycans present in the bone marrow micro-environment play an important role in proliferation and differentiation of human hematopoietic progenitors. Excessive accumulation of proteoglycans within hematopoietic organs in vivo inhibits hematopoiesis (42, 43) possibly by preventing normal interactions of primitive hematopoietic progenitors with the stromal microenvironment. Cell-surface chondroitin-sulphate proteoglycans have been detected on primitive multi-potential FDCP-MIX (44) and more differentiated FDCP-2 (45, 46) murine hematopoietic cell lines. Removal of proteoglycans from stromal layers and/or hematopoietic progenitors in LTBMC blocks adhesion of immature hematopoietic progenitors to stromal layers (47, 48) suggesting that proteoglycans present on immature progenitors may play a role in their attachment to stromal components.

These observations demonstrate the need for further evaluation of the role of cell surface integrin and/or proteoglycan receptors in the adhesion and interaction of human primitive LTBMC-IC with their environment. It will be of interest to examine the possible instructive and permissive role of adhesion of such primitive hematopoietic progenitors to fibronectin for proliferation and differentiation events (10, 26).

The interaction of murine and human erythroid progenitors with fibronectin has been extensively studied. Adhesion of human (10–12) and mouse (9) erythroid progenitors to fibronectin can in part be inhibited by the tetrapeptide RGDS (9–12) and antibodies against fibronectin (9, 10, 49). Adhesion of CFU-E and mature BFU-E to fibronectin can also be inhibited by antibodies against the $\alpha5\beta1$ integrin receptor known to interact with the RGDS sequence in the cell binding fragment of fibronectin (10). The present study confirms these findings, but adds significant new information. We demonstrate that immature human BFU-E can also adhere to the COOH-terminal major heparin-binding fragment of fibronectin suggesting that these progenitors may express additional integrin receptors, such as $\alpha4\beta1$ (33, 34) and/or cell surface heparin-like receptors (35, 36) important in cell-extracellular matrix and possibly cell-cell interactions in the bone marrow microenvironment. Consistent with this hypothesis is the recent observation by Coloumbel et al. (50) who demonstrated that CFU-E express both $\alpha4\beta1$ and $\alpha5\beta1$ integrin receptors.

Several studies have indicated that human bone marrow derived CFU-GM progenitors adhere poorly to intact fibronectin (11, 12). Although adhesion of human CFU-GM progenitors to the intact fibronectin molecule is relatively poor, the present study demonstrates that such progenitors can adhere to the isolated 75 kD cell-binding or 33/66 kD cell-adhesion promoting fragment. These findings indicate that, in addition to committed BFU-E progenitors, CFU-GM progenitors may express integrin receptors and/or cell surface proteoglycan receptors capable of interacting with the cell-binding fragment of fibronectin as well as with the major COOH-terminal heparin-binding fragment.

In conclusion, we demonstrate that primitive human hematopoietic progenitors adhere to the 33/66 kD RGDS-independent heparin-binding cell-adhesion promoting fragment of fibronectin. Adhesion of the most primitive hematopoietic progenitors, the LTBMC-IC, to intact irradiated stromal layers is, however, significantly greater than the maximal level of adhesion observed to the 33/66 kD fragment of fibronectin. Moreover, adhesion of LTBMC-IC to intact irradiated stroma can only in part be blocked by preincubation of purified DR^- cells with free peptide FN-C/H II alone or in combination with the other cell attachment sites from the 33/66 kD cell-adhesion promoting fragment of fibronectin. These observations indicate that, adhesion receptors involved in the interaction between LTBMC-IC and the 33/66 kD cell-adhesion promoting fragment of fibronectin may play an important role in the binding of such primitive progenitors to the bone marrow microenvironment. However, adhesion of these primitive progenitors to intact stromal layers will likely involve additional adhesion mechanisms. In contrast to primitive LTBMC-IC and multi-lineage CFU-MIX progenitors, more differentiated BFU-E and CFU-GM acquire the ability to adhere to an RGDS-dependent 75 kD cell-binding fragment and to adhere avidly to additional cell-attachment sites in the RGDS-independent 33/66 kD cell-adhesion promoting fragment of fibronectin. This indicates that differentiation and maturation of human hematopoietic progenitors seems to be accompanied by alterations in cell surface adhesion receptor expression and/or function. Such alterations may then allow cells at different stages of differentiation to interact specifically with distinct supportive loci within the medullary cavity where additional signals for maturation may become available (51). It will be of interest to determine whether further differentiation and maturation of erythroid or myeloid progenitors is accompanied by additional alterations in the expression and/or activity of adhesion receptors that will ultimately allow mature blood elements to leave the bone marrow microenvironment.

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