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Research Article

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VLA-5 Is Expressed by Mouse and Human Long-term Repopulating Hematopoietic Cells and Mediates Adhesion to Extracellular Matrix Protein Fibronectin

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Abstract

Fibronectin (FN), an extracellular matrix protein, is involved in the adhesion and migration of hematopoietic cells and has been shown to enhance retroviral gene transfer into primitive hematopoietic cells by co-localization of target cells and retrovirus when used as a substrate in vitro. We have previously found that mouse hematopoietic stem cells could be transduced on a FN fragment that included the recognition sequence Arg-Gly-Asp (RGD), suggesting that stem cells may express the integrin very late antigen (VLA)-5. To address this, we investigated the binding of mouse and human hematopoietic cells to recombinant peptides that contained one or a combination of the three principle cell-binding domains of FN. These domains included the VLA-5–binding sequence RGD, the VLA-4–binding site CS1, and the high affinity heparin-binding domain. Here we show that mouse long-term in vivo repopulating stem cells, as well as primitive human NOD/SCID mouse repopulating cells, can bind extracellular matrix protein FN by using integrin VLA-5 in vitro. This binding is specific and can be inhibited by antibodies to VLA-5. In addition, preincubation of BM cells with peptide CH-296, which contains all three primary FN-binding domains, decreased the engraftment of cells in the bone marrow in vivo, while intravenous injection of the same peptide induced an increase of progenitor cells in the spleen. In summary, our data demonstrate that VLA-5 is expressed on primitive mouse and human hematopoietic cells and suggest that there may be significant cooperation between integrin receptors and proteoglycan molecules in the engraftment of bone marrow cells and hematopoietic cell adhesion in vivo. (*J. Clin. Invest.* 1998. 102:1051–1061.)
Key words: hematopoietic stem cells • fibronectin • VLA-5

Introduction

Hematopoiesis occurs in a complex environment in vivo, which includes not only cell–cell but also cell–matrix interactions. For several decades, it has been appreciated that the survival of reconstituting hematopoietic stem cells in vitro depends to a large extent on the adhesion of these cells to stromal cells of the hematopoietic microenvironment (1). One of the molecules involved in the adhesion of hematopoietic cells is extracellular matrix protein fibronectin (FN;[1] 2, 3). Recently, there has been a renewed interest in the adhesive properties of FN from the rapidly expanding field of gene therapy, as studies have shown that retroviral gene transfer into primitive hematopoietic cells could be increased by co-localization of retrovirus and target cells on specific FN domains (4–6). Interestingly, it has also become clear that the interaction of hematopoietic cells with FN may trigger intracellular signaling pathways that affect cell survival and proliferation (7–10). These findings may have important implications for the maintenance and manipulation of hematopoietic stem cells ex vivo.

The binding of hematopoietic cells to FN is mediated by a number of integrin receptors. The classical receptor is integrin α5β1 (CD49e/CD29: very late antigen [VLA]-5), which recognizes the minimal binding sequence Arg-Gly-Asp (RGD), as well as two other synergistic VLA-5 binding sites, all of which are located within the cell-binding domain of the FN molecule (11–14). A second well studied receptor is integrin α4β1 (CD49d/CD29: VLA-4), which binds sites within the alternatively spliced IIICS region of the molecule defined by the synthetic peptides CS1 and CS5 (15–17). In addition to these and other integrin receptors (18), the interaction between hematopoietic cells and FN also involves the cell surface complex of chondroitin-sulfate proteoglycan and CD44, which is recognized by the high-affinity COOH-terminal heparin-binding domain of FN (as reviewed in 2, 3).

VLA-4 has been demonstrated to play an important role in the interaction of primitive hematopoietic cells and the bone marrow (BM) extracellular matrix. For instance, mouse long-term repopulating hematopoietic stem cells (HSCs) have been shown to adhere to a chymotryptic fragment of FN (FN 30/35, which contains the heparin-binding site as well as the IIICS region) using VLA-4 (19). Human long-term culture-initiating cells have also been shown to adhere through VLA-4, as was demonstrated using a COOH-terminal chymotryptic/catheptic fragment of FN (20). In addition, intravenous injection of a VLA-4–specific antibody in vivo has been shown to mobilize progenitors from the BM into the blood in primates as well as

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1. *Abbreviations used in this paper:* α-MEM, α-modification of Dulbecco's Modified Eagle's Medium; BM, bone marrow; FN, fibronectin; HEL, human erythroleukemia; HPP-CFC, high proliferative potential colony-forming cell; HSC, hematopoietic stem cell; IMDM, Iscove's Modified Dulbecco's Medium; SCF, stem cell factor; TBI, total body irradiation; VLA, very late antigen.

in mice, while incubation of transplanted BM cells with anti–VLA-4 antibody before transplantation significantly reduced their engraftment (21, 22).

In contrast, much less is known about the role of VLA-5 in the interaction of primitive hematopoietic cells with the extracellular matrix. In long-term BM cultures, binding of human hematopoietic cells to a peptide containing the VLA-5–binding site RGD has been shown to increase with differentiation (20). VLA-5 has also been shown to be differentially expressed during T cell differentiation (23, 24), in erythropoiesis (25, 26), and also in myelopoiesis (27). In vitro, it has been shown that some but not all primitive mouse hematopoietic cells could adhere to FN using VLA-5 (28). However, VLA-5 has not yet been demonstrated on primitive in vivo repopulating cells.

While studying FN-mediated retroviral gene transfer into mouse hematopoietic cells, we noted that the use of a peptide containing the VLA-5–binding sequence RGD appeared to be correlated with the transduction of long-term repopulating cells (6), suggesting that VLA-5 may be expressed by primitive hematopoietic cells. To investigate the potential role of VLA-5 in the binding of primitive hematopoietic cells to FN, we have used various well-defined and highly specific recombinant peptides, containing single domains or combinations of the three principal cell-binding domains of human plasma FN (29). Using cell adhesion assays and antibody blocking studies, we here demonstrate that VLA-5 is expressed on mouse HSC, as defined by long-term engraftment of adherent cells in WBB6F1/J-Kit^{W}/Kit$^{W\text{-}V}$ animals, as well as on primitive human hematopoietic cells as shown by multilineage engraftment in immunodeficient NOD/SCID mice. In addition, we show that preincubation of cells with a recombinant peptide that contains both VLA-4– and VLA-5–binding sites as well as the heparin-binding domain reduced the engraftment of these cells to the marrow, while injection of this peptide induced a change in the distribution of hematopoietic progenitor cells in vivo.

Methods

Animals. Male and female C57BL/6J and WBB6F1/J-Kit^{W}/Kit$^{W\text{-}V}$ animals, 8–16 wk old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained under conventional conditions at the Laboratory Animal Research Center at the Indiana University School of Medicine. B6.*Hbb*^{d}/*Hbb*^{d}, *Gpi-1*^{a}/*Gpi-1*^{a} animals were kindly provided by Dr. M.C. Yoder (Indiana University). A breeding colony of NOD/LtSz-*scid*/*scid* (NOD/SCID) mice (30) was established using breeding pairs kindly provided by Dr. L.D. Shultz (Jackson Laboratory). NOD/SCID animals were housed in a positive airflow ventilated rack (Lab Products, Maywood, NJ) and bred and maintained in microisolators under specific pathogen-free conditions. All mice received autoclaved food and acidified water ad libitum. Before transplantation, NOD/SCID mice received 300 cGy total body irradiation (TBI) at 86 cGy/min using a GammaCell 40 (Nordion International Inc., Ontario, Canada) equipped with two opposing ^{137}Cesium sources. In specific experiments, C57BL/6J mice received a single dose of 920 cGy (TBI). All animal experiments were performed in accordance with institutional guidelines approved by the Animal Care Committee of the Indiana University School of Medicine.

Mouse bone marrow cells. Mouse BM cells were harvested by flushing the femurs with HBBS (GIBCO BRL, Grand Island, NY) containing 25 mMol/L HEPES (GIBCO BRL), 0.1% (wt/vol) BSA (Boehringer Mannheim, Indianapolis, IN), 100 U/mL penicillin, and 100 μg/mL streptomycin (2% Pen/Strep; GIBCO BRL). Mononuclear cells were isolated by buoyant density centrifugation (700 × *g* for 30 min at 25°C) using Histopaque-1083 (Sigma, St. Louis, MO). Cells were then washed and resuspended in α-MEM (α-modification of Dulbecco's modified Eagle's medium; GIBCO), containing 10% FCS (Introgen, Purchase, NY) and 2% Pen/Strep.

Human cells. Human BM and peripheral blood cells were collected from healthy consenting adult human volunteers according to protocols approved by the Institutional Review Board of the Indiana University School of Medicine. BM cells were washed and diluted in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS, 2% Pen/Strep, and 20 U/mL heparin and low density mononuclear cells (< 1.077 g/mL) were isolated by centrifugation (700 *g*, for 30 min at 25°C) on Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). BM CD34^{+} cells were isolated using the Magnetic Activated Cell Sorter (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Human peripheral blood cells were collected by apheresis after 5 d of administration of human granulocyte colony-stimulating factor (G-CSF; Filgrastim Neupogen®, Amgen [Thousand Oaks, CA]; at 10 μg/kg/day) and CD34^{+} cells were isolated by immunomagnetic methods using the Isolex® 300i cell selection device (Baxter Immunotherapy, Irvine, CA). For these studies, cell selection kits and disposables were kindly provided by Baxter Immunotherapy. In specific experiments, magnetically selected CD34^{+} cells were labeled with CD34-FITC and CD38-PE (both from Becton Dickinson, Mountain View, CA) and CD34^{+}38^{-} cells (representing 10% of all CD34^{+} cells) were sorted on a FACStar Plus flow cytometer (Becton Dickinson).

Coating of FN peptides. Recombinant human FN fragments were expressed in *Escherichia coli* and purified as previously described (29). Nontissue culture 96-well plates were coated with FN fragments diluted in PBS at 0.4–200 nmol/cm^2 (4 wells/concentration) for 2–4 h at room temperature. To block nonspecific binding sites, plates were subsequently incubated for 30 min with a 2% (wt/vol) solution of BSA in PBS. Wells were then washed three times with 200 μL PBS, 0.1% (vol/vol) Tween-20 (Sigma) and incubated for 30 min at 37°C with mouse antibodies specific for the heparin-binding domain (FNH3-8; Takara Shuzo, Otsu, Japan) and cell-binding domain (FN12-8; Takara Shuzo) of FN (31). Antibodies were used at a previously determined optimal dilution in PBS, 0.1% Tween-20, and 0.1% (wt/vol) BSA. After incubation, plates were washed with washing buffer and incubated for 30 min at 37°C with horseradish peroxidase-conjugated goat anti–mouse IgG (GIBCO BRL). After washing again, wells were incubated with 100 μL peroxidase substrate solution (1-Step Turbo TMB-ELISA; Pierce, Rockford, IL) for 15 min at room temperature. The reaction was stopped with 100 μL of 1 mol/L H_2SO_4 and the optical density was measured at 450 nm using an ELISA reader (Molecular Devices, Menlo Park, CA).

Cell adhesion assays. Nontissue culture plates were coated with FN fragments at 100 nmol/cm^2 for 2–4 h and blocked with BSA as described above. Before FN adherence, mouse and human hematopoietic cells were depleted of plastic-adherent cells by a short 30-min incubation in tissue culture plates at 37°C. Nonadherent cells were then allowed to adhere to the FN-coated plates for 1 h at 37°C at 2 × 10^6 cells/well in 35-mm petri dishes or 15–20 × 10^6 cells in 10-cm petri dishes in α-MEM or IMDM tissue culture medium containing 10% FCS and 2% Pen/Strep. In specific experiments, cells were preincubated for 30 min at 4°C with 10 μg/mL human or mouse integrin-specific blocking antibody, isotype control antibody, or 500 μg/mL heparin (high molecular weight heparin sodium salt; Sigma), and allowed to adhere to FN-coated plates in the presence of antibody or heparin. Antibodies against mouse VLA-4 (anti-CD49d, clone R1-2, rat IgG2b and 9C10 [MFR4.B] rat IgG2a), mouse VLA-5 (anti-CD49e, clone 5H10-27 [MFR5], rat IgG2a), human VLA-5 (anti-CD49e, clone IIA1, mouse IgG1), as well as isotype control rat IgG2a (clone R35-95), rat IgG2b (R35-38), and mouse IgG1 (anti-TNP, clone 107.3) were purchased from PharMingen (San Diego, CA). All antibodies were unconjugated and contained no sodium azide. After incubation, nonadherent cells were collected by carefully rinsing the plates multiple times with medium. Adherent cells were harvested by vigorously rinsing the plates with PBS, followed by a 1–2-min

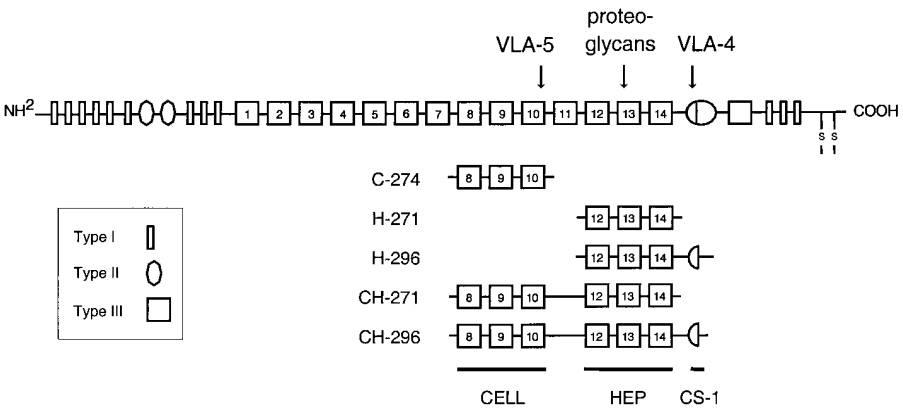

Figure 1. FN and FN peptides. Schematic representation of the A chain of a FN dimer and the corresponding domains present in *E. coli*–produced recombinant FN fragments (29). The designations of the recombinant FN fragments are shown next to schematic drawings of each fragment. Indicated are the types I, II, and III homologous sequence repeats as well as the cell binding site (*CELL*), which contains the Arg-Gly-Asp (RGD) cell adhesion recognition sequence that is recognized by VLA-5, the high-affinity heparin binding site (*HEP*), and the alternatively spliced non–type III connecting segment (III CS) that contains the VLA-4–specific cell-binding site as represented by peptide CS-1.

trypsinization at 37°C. Remaining cells were finally removed from the plates using a cell scraper (Fisher Scientific, Pittsburgh, PA).

Cell lines. Murine lymphocytic leukemia L1210 cells (32), murine myeloid leukemia M1 cells (33), and human erythroleukemia (HEL) cells (34) were obtained from the American Type Culture Collection (Rockville, MD). L1210 cells were cultured in RPMI 1630 (GIBCO BRL), 15% calf serum, 2% Pen/Strep, 2 mM L-glutamine, while M1 and HEL cells were cultured RPMI 1640, 10% FCS, 2% Pen/Strep.

Mouse HPP-CFC assay. High proliferative potential colony-forming cells (HPP-CFC) were enumerated in a standard double-layer agar culture system as previously described (35). In brief, a 1% and 0.6% (wt/vol) solution of warm agar (Difco, Detroit, MI) in distilled water was mixed with an equal volume of double-strength α-MEM tissue culture medium. The agar medium also contained 2 mMol/L L-glutamine, 20% FCS, and 2% Pen/Strep (at final concentrations). The 0.5% bottom agar was plated at 1 mL/dish in 35-mm gridded tissue culture dishes (Sarstedt, Newton, NC) containing 100 ng/mL recombinant rat stem cell factor (SCF; Amgen), 200 U/mL recombinant murine IL-3 (Peprotech, Rocky Hill, NJ), 1,000 U/mL recombinant murine IL-1α (Genzyme, Boston, MA), and 1,600 U/mL recombinant human macrophage colony-stimulating factor (Genetics Institute, Boston, MA). Using triplicate plates, mouse BM cells were cultured in the 0.3% top agar (at 1 mL/dish in 35-mm gridded tissue culture dish) at 37°C in a humidified environment at 5% O_2, 10% CO_2. After 14 d, HPP-CFC colonies were morphologically identified as compact and dense colonies with a diameter of > 0.5 mm.

Progenitor assays. Human progenitor cells were cultured in 1 mL of semisolid tissue culture medium consisting of IMDM (GIBCO BRL), 25% FCS, 10% human plasma, 2% Pen/Strep, 5×10^{-5} mol/L β-mercapto-ethanol, 11 ng/mL human IL-3 (Peprotech), 100 ng/mL human SCF (Amgen), 4 U/mL human erythropoietin (Amgen) and 0.8% methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada). Mouse progenitor cells were cultures in triplicate in α-MEM, 30% FCS, 2% Pen/Strep, 2 mMol/L L-glutamine (GIBCO BRL), 1×10^{-4} mol/L β-mercapto-ethanol, 100 U/mL recombinant mouse IL-3 (Peprotech), 100 ng/mL recombinant rat SCF (Amgen), 4 U/mL recombinant human erythropoietin and 0.8% methylcellulose (Stem Cell Technologies). Colonies were counted after 14 d of culture in a humidified environment at 37°C and 5% CO_2.

Transplantation of adherent mouse BM cells. B6.*Hbb*^{d}/*Hbb*^{d}, *Gpi-1*^{a}/*Gpi-1*^{a} mouse low density BM cells were allowed to adhere to BSA-coated or FN-coated plates, in the presence or absence of anti–mouse VLA-5 blocking antibody (anti-CD49e, clone 5H10-27 (MFR5); PharMingen) or isotype control (rat IgG2a) antibody. After removing the nonadherent cells, various proportions of the adherent cells were transplanted into groups of nonirradiated WBB6F1/J-Kit^{W}/Kit$^{W\text{-}V}$ (*Hb*^{d}/*Hb*^{s}) mice (five mice/group) by lateral tail vein injection. At 3–6 mo after transplantation, peripheral blood hematopoietic chimerism was determined by hemoglobin electrophoresis as has previously been described (36).

Transplantation of adherent human CD34^{+} *cells in NOD/SCID mice.* Human peripheral blood CD34^{+} cells were allowed to adhere to BSA-coated or FN-coated 10-cm petri dishes at 15–20 $\times$ 10^{6} cells/

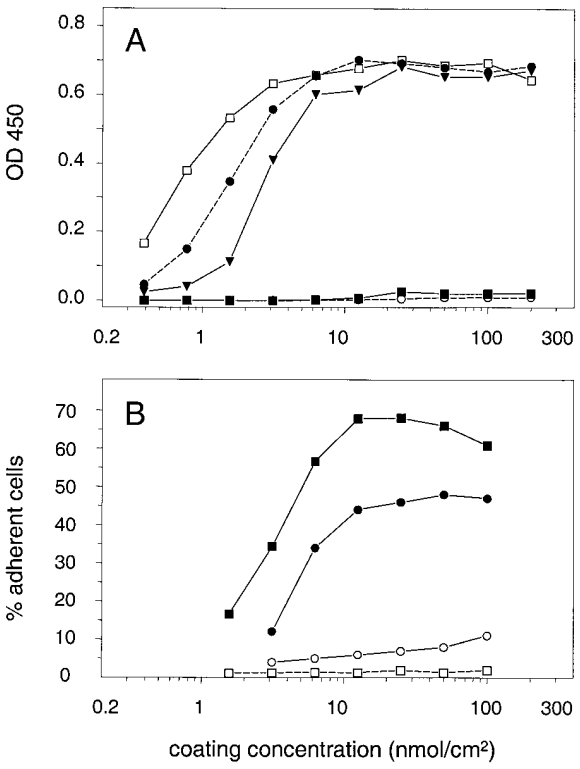

Figure 2. Plastic adherence of FN peptides. Effect of the coating concentration on the adsorption of recombinant FN fragments to nontissue culture–treated plastic (*A*) and on the subsequent binding of mouse BM cells or human HEL cells (*B*). Bound FN was detected by ELISA (average of 4 wells/concentration) using antibody FN12-8 (*A*), which is specific for the CELL-binding domain of FN. (*A*) Fragment H-271 (*filled squares*), C-274 (*open squares*), CH-271 (*filled circles*), H-296 (*open circles*), and CH-296 (*filled triangles*). For clarity, error bars have been omitted. (*B*) Binding of human HEL cells (*squares*) or low-density mouse BM cells (*circles*) to plates coated with H-271 (*open symbols*) or CH-296 (*closed symbols*).

dish. Adhesion to plates coated with peptide C-274, which contains the recognition sequence RGD, was performed in the presence of either anti–human VLA-5 antibody (anti-CD49e, clone IIA1; PharMingen) or isotype control (mouse IgG1) antibody. In these specific experiments the tissue culture medium was supplemented with 100 ng/mL human SCF and 100 U/mL human IL-6, conforming to the protocol we have used previously for gene transfer (6). After 1 h, nonadherent cells were removed and adherent cells were collected and transplanted into NOD/SCID mice preirradiated with 300 cGy TBI. After 8 wk, human–mouse hematopoietic chimerism was determined by FACScan analysis (Becton Dickinson) using anti-CD45 human leukocyte-specific and anti–mouse CD18 monoclonal antibodies. Human B cells and myeloid cells were detected using antibodies specific for CD19, CD33, and HLA-DR (all from Becton Dickinson).

Effect of FN peptides on engraftment. To measure the ability of FN peptides or anti-integrin antibodies to interfere with the marrow repopulating ability (MRA [37]) of mouse low-density mouse BM cells, cells were preincubated for 30 min at 4°C with recombinant FN fragments, or with anti–VLA-4/CD49d (clones R1-2 and 9C10 [MFR4.B]; PharMingen), anti-VLA-5/CD49e (clone 5H10-27 [MFR5]; PharMingen), a combination of both or isotype control antibodies (all at a saturating concentration of 100 μg/mL). Cells were then washed and intravenously injected into irradiated (920 cGy TBI) splenectomized C57BL/6J mice (4 mice/group). Animals had been splenectomized to exclude a possible effect of changes in spleen seeding on the BM engraftment. 10 d after transplantation mice were sacrificed, femoral BM was collected, pooled per group and tested for in vitro colony-forming cells in methylcellulose cultures.

Intravenous injection of FN fragments. To study the effect of FN peptides on the distribution of hematopoietic progenitor cells in vivo, C57BL/6J mice were intravenously injected for 1–4 consecutive days with various amounts of low endotoxin (0.1–0.8 USP EU/mg) recombinant FN fragments, ranging from 0.5 to 7.5 mg/kg/day, in HBBS, 25 mMol/L HEPES, 0.1% BSA. Controls included vehicle alone or human recombinant G-CSF (Filgrastim Neupogen®, at 250 μg/kg/day; Amgen). All injections were given once daily and animals were sacrificed ~ 4 h after the last injection. A single-cell suspension of spleen cells was prepared by gently rubbing the spleen over a nylon 100-μm mesh filter. Cells were washed in PBS and erythrocytes were lysed during a 2-min incubation at 4°C in 155 mMol/L NH_4Cl, 10 mMol/L $KHCO_3$, 0.1 mMol/L EDTA (all from Sigma) at pH 7.4. The number of HPP-CFC in BM and spleen were enumerated in agar cultures as described above.

Statistical analyses. Differences between groups were compared by Student's *t* test, ANOVA, or by using the nonparametric Wilcoxon test or Kruskal-Wallis test. Differences were considered significant at $P < 0.05$.

Results

Plastic adherence of recombinant peptides. To quantify the binding of recombinant FN fragments (Fig. 1) to nontissue culture-treated plastic, plates were coated with equimolar amounts of peptide and tested by ELISA. The antibodies used were specific for the cell-binding domain (FN12-8; Fig. 2 *A*) and high-affinity HEP-binding domain (FNH3-8; not shown) of FN. For all peptides, the plastic adsorption reached a plateau at a concentration of 10–20 nmol/cm^2. As expected, fragments lacking the cell-binding domain (i.e., H-271 and H-296) were not detected by antibody FN12-8 (Fig. 2 *A*), while the fragment that lacked the type III repeats 12–14 (i.e., C-274) was not detected by antibody FNH3-8 (not shown). The adhesion of human HEL cells or mouse low-density BM cells to fragment CH-296 reached a maximum at a similar coating con-

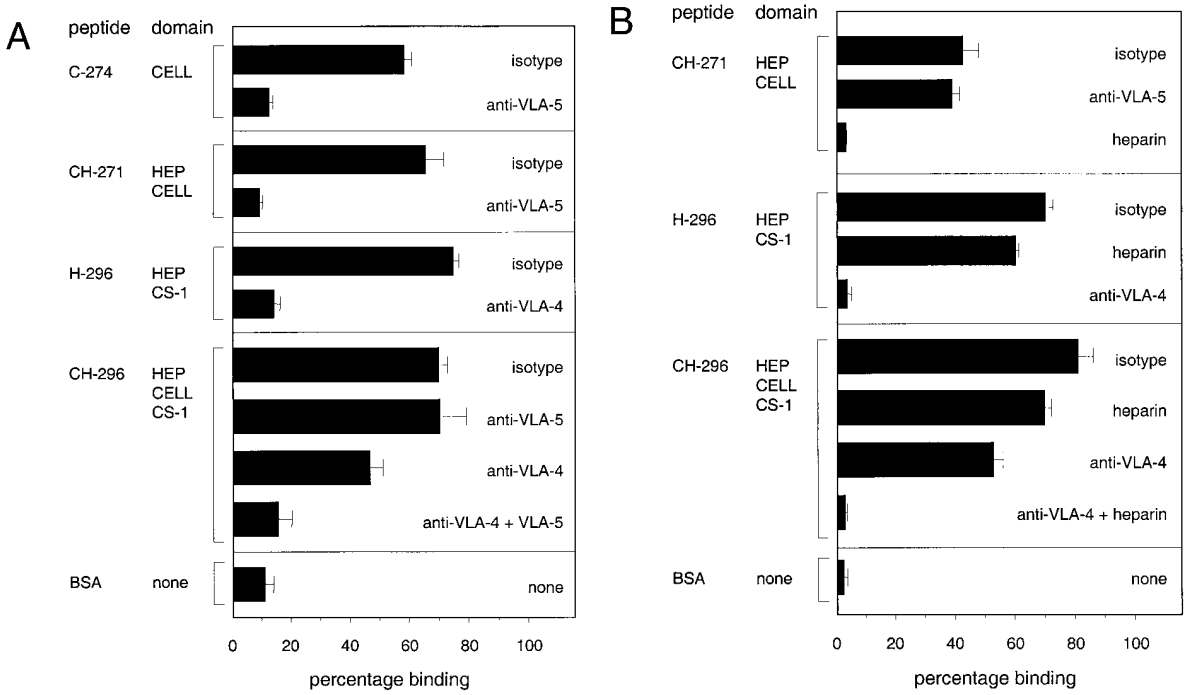

Figure 3. Characterization of FN fragments. Binding of murine M1 (*A*) or L1210 (*B*) leukemia cells to nontissue culture–treated 24-well plates coated with recombinant FN fragments (at 100 nmol/cm^2) in the presence of anti-VLA4 (CD49d, clones R1-2 and 9C10 [MFR4.B]) or anti–VLA-5 (CD49e, clone 5H10-27 [MFR5]) blocking antibodies, isotype control antibodies (all at 10 μg/mL) or heparin (at 500 μg/mL). M1 express both VLA-4 and VLA-5 while L1210 cells only express VLA-4 (not shown). Cells were preincubated for 30 min at 4°C and then allowed to bind to the FN-coated plates in the presence of antibody or heparin. After 1 h incubation at 37°C, nonadherent cells were washed away and the number of adherent cells was determined by Coulter counter. Bars indicate average ±SD of four wells.

centration (Fig. 2 *B*). In contrast, fragment H-271, which lacks the CS-1- and CELL-binding domains but contains the heparin-binding domain, showed only a very low cell-binding activity. Based on these results, a coating concentration of 100 nmol/cm^2 was chosen for all subsequent binding studies. A time-course experiment at this concentration showed that 85% of the peptides that can be bound over a period of 4 h are already adsorbed to the plastic within the first 10 min (data not shown).

Functionality of FN peptide cell-binding domains. Next, we tested whether the various recombinant FN peptides were able to bind hematopoietic cells through their specific cell-binding domains. Plates were coated with recombinant FN peptides at 100 nmol/cm^2, and M1 mouse leukemia cells were allowed to adhere to these peptides in the presence or absence of blocking anti–VLA-4 and/or anti–VLA-5 antibody, isotype control antibody, or heparin. The binding of M1 cells to various FN peptides could specifically be blocked by anti–VLA-4 and/or anti–VLA-5 antibodies (Fig. 3 *A*), indicating that all recombinant peptides used in our study contained functional VLA-4– (CS-1) and VLA-5– (CELL) binding domains. Although, M1 cells did not adhere to the HEP-binding domain of any peptide, binding to this domain could be demonstrated using mouse leukemia cell line L1210 (Fig. 3 *B*). As these cells do not express VLA-5 (not shown), binding to peptide CH-271 (HEP + CELL) could be completely blocked by heparin. Similarly, binding to CH-296 (HEP + CELL + CS-1) could be inhibited by heparin and anti–VLA-4. Surprisingly, binding of L1210 to peptide H-296 (HEP + CS-1) could be blocked by anti–VLA-4, while the addition of heparin hardly affected the binding (Fig. 3 *B*). This suggests that the HEP-binding domain in peptide H-296 did not substantially contribute to cell binding. Similarly, the HEP-binding domain in peptide H-271 was also less active, binding M1 and L1210 cells at only baseline levels (5–12%; not shown). These differences in the activity of the HEP-binding domain between peptides H-271 and CH-271 and H-296 and CH-296, may reflect differences in the tertiary structure imposed on this domain by the inclusion of the CELL-binding domain in CH-271 and CH-296.

Binding of mouse and human low density bone marrow cells and progenitors. Mouse and human progenitor cells (Fig. 4, *A* and *B*, respectively) and mouse and human low-density cells (not shown) bound similarly to recombinant FN peptides. The results show a low level of adhesion to peptide H-271 (HEP), an intermediate level of adhesion to peptides C-274 (CELL) and CH-271 (HEP + CELL), and maximum adhesion to peptides H-296 (HEP + CS-1) and CH-296 (HEP + CELL + CS-1). For mouse as well as human cells and progenitors (Fig. 4, *A* and *B*), no significant difference could be found between the binding to peptide CH-296 (HEP + CELL + CS-1) and H-296 (HEP + CS-1). In addition, a measurable population of mouse as well as human progenitor cells did bind to C-274, a peptide containing the RGD sequence but lacking the CS-1– or HEP-binding sites. These results suggest that the interaction of mouse and human hematopoietic progenitor cells with extracellular matrix protein FN in vitro are dominated by integrin receptor VLA-4, while some cells clearly adhere to RGD-containing peptide C-274.

Adherence of mouse hematopoietic stem cells. To test the adherent properties of more primitive mouse hematopoietic cells, we injected a fixed proportion of FN peptide-adherent low density B6.*Hbb*^{d}/*Hbb*^{d}, *Gpi-1*^{a}/*Gpi-1*^{a} BM cells into groups of nonirradiated WBB6F1/J-Kit^W/Kit$^{W\text{-}V}$, *Hbb*^{s}/*Hbb*^{d} mice. Hematopoietic chimerism was determined by hemoglobin electrophoresis at 6 mo after transplantation (Fig. 5). In Fig. 5, the lanes marked *D* and *R* represent hemoglobin bands from donor and untransplanted recipient animals, respectively. The electrophoretic pattern demonstrated that a low number of stem cells was contained in the BSA-adherent fraction, with donor-type hemoglobin detected in only one out of nine animals (lane *8*). In contrast, all animals transplanted with C274-adherent BM cells showed a complete conversion to donor-type hemoglobin (on average 99.5%, as detected by densitometry; Fig. 5 *B*), indicating that engrafting HSCs were present among the population of cells adherent to RGD-containing peptide C-274. The engraftment was comparable with the engraftment in animals transplanted with CH-296 adherent BM

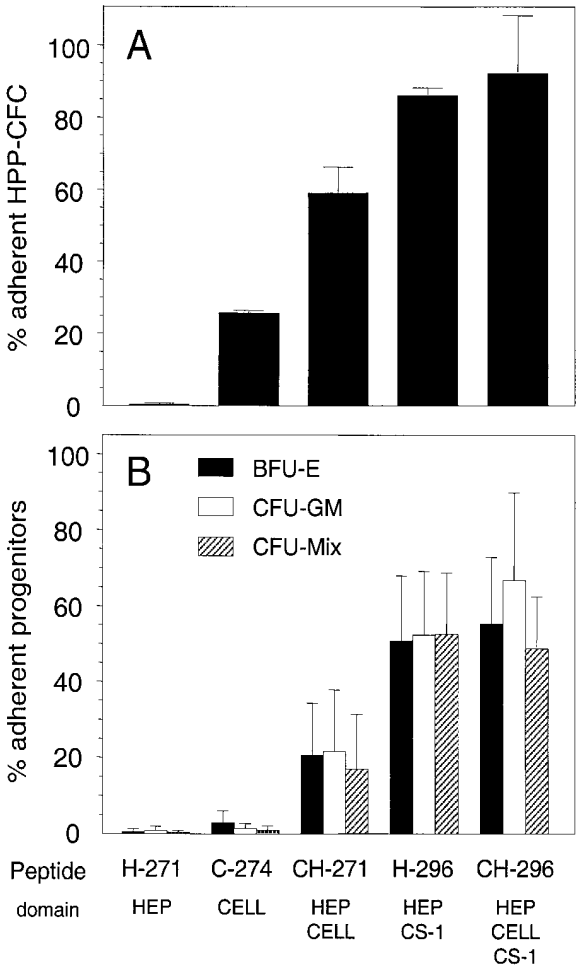

Figure 4. Adhesion of mouse and human clonogenic cells. Binding of (*A*) mouse and (*B*) human clonogenic cells from low-density BM cells to nontissue culture 35-mm plates coated with 100 nmol/cm^2 recombinant FN fragments. Cells (2×10^6 cells per plate) were allowed to adhere for 1 h at 37°C as described in Methods. After removing all nonadherent cells, adherent cells were tested for HPP-CFC (mouse) and BFU-E, CFU-GM, and CFU-Mix (human) using standard clonogenic assays. The different FN peptides, as well as their respective binding domains, are indicated in the graph. Bars denote the average percentage of adherent cells (±SD) of four experiments (after subtraction of the number of progenitors adherent to BSA-coated control plates). All differences are significant ($P < 0.001$) except between H-271 and C-274 (*B*) and H-296 and CH-296 (*A* and *B*).

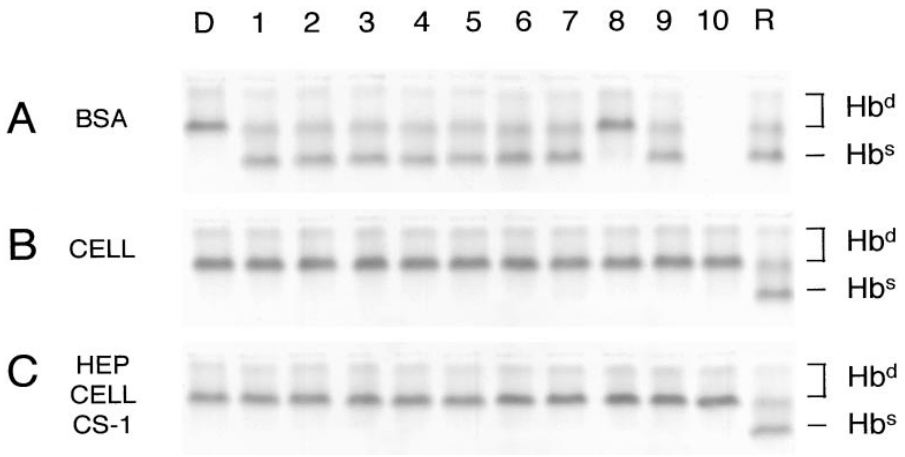

Figure 5. Long-term engraftment of adherent cells. Hemoglobin electrophoresis of RBC lysate from WBB6F1/J-Kit^{W}/Kit$^{W\text{-}V}$ animals transplanted with BSA-adherent or FN-adherent BM cells from B6.*Hbb*^{d}/*Hbb*^{d}, *Gpi-1*^{a}/*Gpi-1*^{a} animals, at 6 mo post-transplantation. Animals in each group received a fixed proportion of the total number of cells adherent to each of the peptides. In this experiment, each animal received the adherent fraction from a total of 7×10^5 to 1×10^6 low-density cells. Shown are animals transplanted with cells adherent to (*A*) *BSA*, (*B*) fragment C-274 (*CELL*), and (*C*) fragment CH-296 (*HEP + CELL + CS-1*). Each gel includes a control lane with donor hemoglobin (homozygous *Hb*^{d}/*Hb*^{d}, indicated with *D*) and untransplanted recipient (heterozygous *Hb*^{d}/*Hb*^{s}, indicated with *R*). Chimerism was quantified by densitometry.

cells (Fig. 5 *C*). These results demonstrate that long-term repopulating stem cells adhere to an FN fragment that contains RGD but not CS1, suggesting that these cells express integrin VLA-5.

Specificity of VLA-5 in adherence of mouse progenitors and long-term repopulating cells. To prove that the interaction of mouse hematopoietic cells and FN peptide C-274 was mediated by VLA-5, we repeated our binding studies in the presence of either an isotype control antibody or anti–mouse VLA-5 blocking antibody (Fig. 6). The addition of anti–VLA-5 significantly reduced the number of low-density cells (Fig. 6 *A*, $P < 0.05$) and HPP-CFC (Fig. 6 *B*, $P < 0.005$) that bound to peptide C-274. Similarly, after transplantation of a fixed proportion of the C-274–adherent cells from plates incubated with anti–VLA-5 antibody (Fig. 6, *C* and *D*, representing a high and a threefold lower fraction of adherent cells injected, respectively), there was a significant decrease in the average level of donor-type hemoglobin when compared with control ($P = 0.0017$). Based on the mean engraftment level in control groups in Fig. 6, *C* and *D* (89.4 and 47.5%, respectively, of which the cell doses are threefold apart), and lower level of engraftment with anti–VLA-5 (66.4% in Fig. 6 *C*), it can be estimated by extrapolation that ~ 50% of the binding to fragment C-274 is mediated by VLA-5. Furthermore, injection of 20% of the nonadherent fraction from the VLA-5–treated plates (from Fig. 6 *C*) induced a chimerism of 75.9±10% (mean± SEM; $n = 5$; data not shown). When compared with the abovementioned engraftment levels and specificity of binding, the data indicate that 10–15% of mouse HSCs are capable of adhering to peptide C-274 using VLA-5. Together, these results support the conclusion that VLA-5 is involved in the adhesion of mouse hematopoietic progenitors as well as long-term repopulating stem cells to extracellular matrix protein FN in vitro.

Role of VLA-5 in adherence of human hematopoietic cells to FN. Similar to the studies with mouse hematopoietic cells, binding studies with human CD34$^+$ cells were performed in the presence or absence of anti–VLA-5 monoclonal antibody. Primitive human hematopoietic cells were measured by intravenous injection of adherent cells into sublethally irradiated immunodeficient NOD/SCID mice, as previously described (38). To generate enough cells for transplantation, CD34$^+$ cells were purified from G-CSF mobilized peripheral blood apheresis products. We have recently shown high-level and multilineage engraftment from such products in NOD/SCID mice (38). CD34$^+$ cells were found to adhere to BSA-coated plates at a low level, while ~ 30% of the cells adhered to peptide C-274, and 95% of the cells adhered to CH-296 (Fig. 7 *A*). Incubation with anti–VLA-5 monoclonal antibody reduced the binding of cells to peptide C-274 (Fig. 7 *A*). The reduction in BFU-E, CFU-GM, CFU-Mix (Fig. 7 *B*; for all $P < 0.001$), and NOD/SCID-repopulating cells (Fig. 7 *C*, $P = 0.02$ and Fig. 8, *C–F*) was statistically significant. In contrast with the difference in binding of CD34$^+$ cells to peptides CH-296 and C-274 (Fig. 7 *A*, $P < 0.0001$), no statistical difference was found between the binding of NOD/SCID-repopulating cells to peptides CH-296 and C-274 (Fig. 7 *C*, $P = 0.3$) suggesting that the majority of these cells express VLA-5. This conclusion was also supported by the finding that injection of the nonadherent cells from the anti–VLA-5–treated plate induced a 41.9±0.8% (mean±SEM, $n = 2$) engraftment, which is similar to the 32.3±13.4% (mean±SEM, $n = 2$; $P = 0.55$) engraftment induced by the adherent cells from the isotype-treated plate. In all engrafted animals, human cells could also be demonstrated in the spleen and were found to be of multiple lineages (not shown). In addition, when highly purified CD34$^+$38$^-$ cells were used, which represent a 10-fold enriched population of primitive human hematopoietic cells as compared with the total CD34$^+$ population, binding of CD34$^+$38$^-$ cells to C-274 (30.1±3.5%, mean±SEM, $n = 14$) was found to be similar ($P = 0.99$) to the binding of CD34$^+$ cells (Fig. 7 *A*, 30.2±2.9%, $n = 3$). Furthermore, there was a highly significant linear correlation ($r = 0.88$, $P < 0.0001$) between the number of CD34$^+$38$^-$ input cells and number of C-274–adherent cells recovered when measured over a range of input cells from 1×10^4 to 24×10^4 cells per dish, suggesting that the binding of these cells to C-274 was direct and not mediated by accessory cells. Finally, expression of RGD-specific integrin αvβ1 could not be detected on peripheral blood CD34$^+$ cells (not shown), although we did confirm expression of this receptor on HEL cells, as previously reported (39). Together, these results confirm that VLA-5 is expressed on primitive human hematopoie-

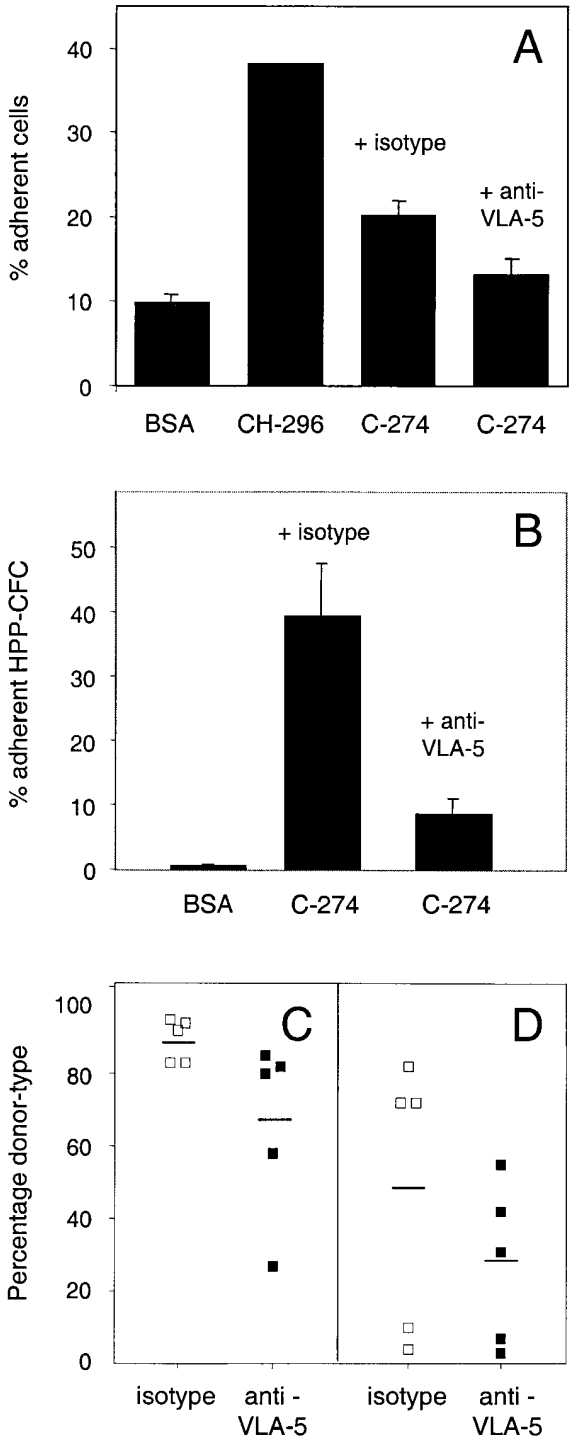

Figure 6. Role of VLA-5 in adhesion of mouse hematopoietic cells to RGD-containing peptide C-274. Low-density mouse hematopoietic cells were preincubated with either anti–mouse VLA-5 or isotype control monoclonal antibody and allowed to adhere to C-274–coated plates in the presence of antibody. Controls include plates coated with BSA and CH-296. Bars indicate mean (±SD). (*A*) Percentage of adherent cells; (*B*) percentage of adherent HPP-CFC; and (*C* and *D*) percentage of donor-type hemoglobin in recipient WBB6F1/J-Kit^{W}/Kit$^{W\text{-}V}$ mice transplanted with C-274–adherent BM cells from B6.*Hbb*^{d}/*Hbb*^{d}, *Gpi-1*^{a}/*Gpi-1*^{a} mice. Each animal received the adherent fraction of the equivalent of 1×10^5 (*C*) and 3×10^4 (*D*) total input low-density cells, respectively. Dots indicate percentage donor-type hemoglobin in individual animals as determined by densitometry; the horizontal line in each plot indicates the average. The difference in engraftment between control and anti–VLA-5 groups (*C* and *D*) was found to be statistically significant (ANOVA; $P = 0.0017$).

tic cells and can mediate binding to the CELL-binding domain of extracellular matrix protein FN.

Recombinant peptide CH-296 but not C-274 can interfere with engraftment. Since little data exists that validates in vitro adhesion assays with respect to physiological interactions important in stem cell engraftment in vivo, we used recombinant FN peptides to investigate the potential role of the different FN receptors in reconstitution of BM cells in irradiated recipient animals. Low-density BM cells were preincubated with FN peptides or with blocking anti–VLA-4 or anti–VLA-5 antibodies (or isotype controls) all at a saturating concentration of 100 μg/mL for 30 min at 4°C. Cells were washed once and injected into splenectomized irradiated syngeneic recipients (four animals/group). After 10 d, the number of CFU-C in the BM was enumerated in a progenitor assay. Compared with the BSA or isotype control, the number of CFU-C in groups transplanted with cells preincubated with peptide CH-296 but not C-274 was significantly reduced (Fig. 9 *A*; $P < 0.001$). On the other hand, preincubation with integrin VLA-4– and VLA-5–blocking antibodies, either combined (Fig. 9 *A*; $P < 0.001$) or used separately (not shown), also reduced the number of CFU-C. These data indicate that the receptors that play a role in the engraftment of hematopoietic cells to the BM of an irradiated recipient could be blocked by a recombinant peptide that contains both the VLA-5– and VLA-4–binding site as well as the high-affinity HEP-binding domain but not by a peptide exclusively containing the RGD site. Not surprisingly, this suggests that the effect of antibodies and peptides on engrafting cells in vivo is more complicated than on adhesion in vitro and implies that engraftment may require the cooperative interaction of multiple adhesion sites and receptors (see below).

Infusion of recombinant fragment CH-296 changes the number of progenitors in the spleen. Since peptide CH-296 inhibited engraftment in vivo of infused cells, we performed a series of experiments to assess the effect of intravenous injection of different recombinant peptides on steady state hematopoiesis. As a control, we examined animals that received a daily injection of recombinant human G-CSF (40). Injection of CH-296 (ranging from 0.5 to 7.5 mg/kg/day i.v. for 4 d) effected a dose-dependent increase in the number of HPP-CFC in the spleen (Fig. 9 *B*; $r = 0.98$, $P < 0.0001$), but not the bone marrow (not shown). At the highest dosage tested, a 6.5-fold increase in splenic HPP-CFC was noted ($P < 0.001$), which is comparable with the ninefold increase found in animals injected with G-CSF (Fig. 9 *B*). When compared with other recombinant FN fragments such as C-274, as is shown in Fig. 9 *C*, CH-296 but no other peptides were able to induce a significant increase in splenic HPP-CFC as compared with the BSA-injected animal ($P < 0.0001$). The fold increase between CH-296 and G-CSF treated animals was not significantly different ($P = 0.57$). These results indicate that recombinant FN peptide CH-296 can interfere with steady state hematopoiesis when injected intravenously. In addition, the lack of reactivity or low reactivity of peptides other than peptide CH-296, i.e., peptides containing only two of the three dominant FN-binding domains, suggests that in vivo interactions may be mediated by multiple binding domains, or that the domain structure

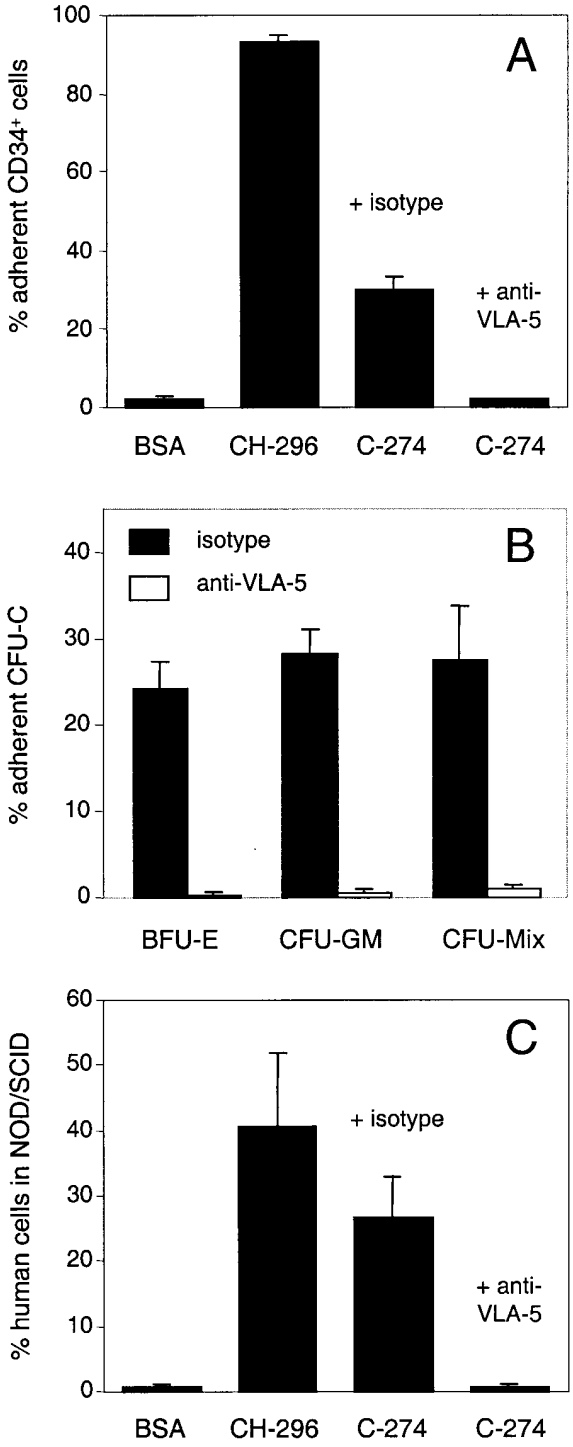

Figure 7. Adherence of human CD34$^+$ peripheral blood progenitor cells and NOD/SCID-repopulating cells to RGD-containing peptide C-274 is mediated by VLA-5. Nontissue culture 10-cm plates were coated with BSA or with peptides C-274 or CH-296 (both at 100 nmol/cm^2), as described in detail in Methods. Per plate, a total of 15–20 $\times$ 10^6 human peripheral blood CD34$^+$ cells was allowed to adhere, in some groups in the presence of anti–human VLA-5 or isotype control monoclonal antibody. Adherent cells were collected, tested in progenitor assays, and injected into sublethally irradiated NOD/SCID mice. (*A*) Percentage of CD34$^+$ adherent cells; (*B*) percentage of adherent BFU-E, CFU-GM, and CFU-Mix; (*C*) percentage of human cells in the BM of immunodeficient NOD/SCID mice at 2 mo after injection of cells adherent to BSA- or FN-coated plates (BSA group, n = 2; CH-296 group, n = 5; C-274 (+isotype antibody) group, n = 5; C-274 (+anti–VLA-5 antibody) group, n = 2). Individual animals received all adherent cells from one plate. Bars indicate mean (±SEM).

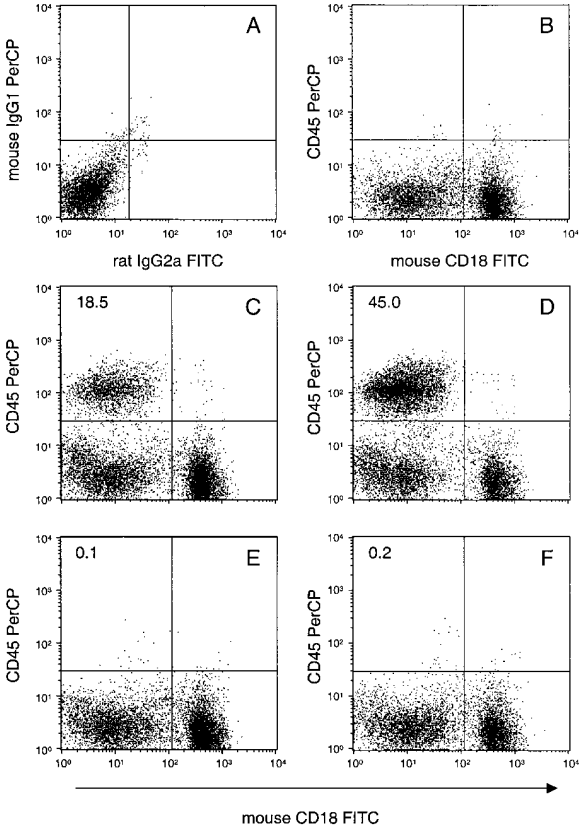

Figure 8. Flow cytometric analysis of transplanted NOD/SCID mice. Flow cytometric analyses of the BM of NOD/SCID nontransplanted control mice or mice transplanted with C-274–adherent human peripheral blood CD34$^+$ cells, at 2 mo after transplantation. CD34$^+$ cells (20 $\times$ 10^6 cells/plate) were allowed to adhere to C-274 in the presence of anti–human VLA-5 or isotype control monoclonal antibody and adherent and nonadherent cells were collected as described in Methods. (*A* and *B*) Dot plots of a nontransplanted animal; (*C* and *D*) two animals transplanted with isotype control antibody incubated C-274–adherent cells; (*E* and *F*) two animals transplanted with anti–VLA-5 incubated C-274–adherent cells. Individual animals received all adherent cells from one plate (*C–F*). Dot plots show the expression of the human common leukocyte antigen CD45 and mouse antigen CD18. The percentage of human (CD45$^+$ mCD18$^-$) cells has been indicated.

of soluble FN peptides may differ significantly from plastic-absorbed peptides.

Discussion

To study the adhesive properties of mouse and human hematopoietic cells, we used plastic-adhered recombinant peptides containing single domains or combinations of the three principle cell-binding domains of human plasma FN (29, 41). In agreement with previous studies (20, 27, 42, 43), we demonstrate the presence of both VLA-4 and VLA-5 on human hematopoietic progenitor cells. In addition, using peptide-adhesion assays and anti–VLA-5 blocking antibodies, we provide

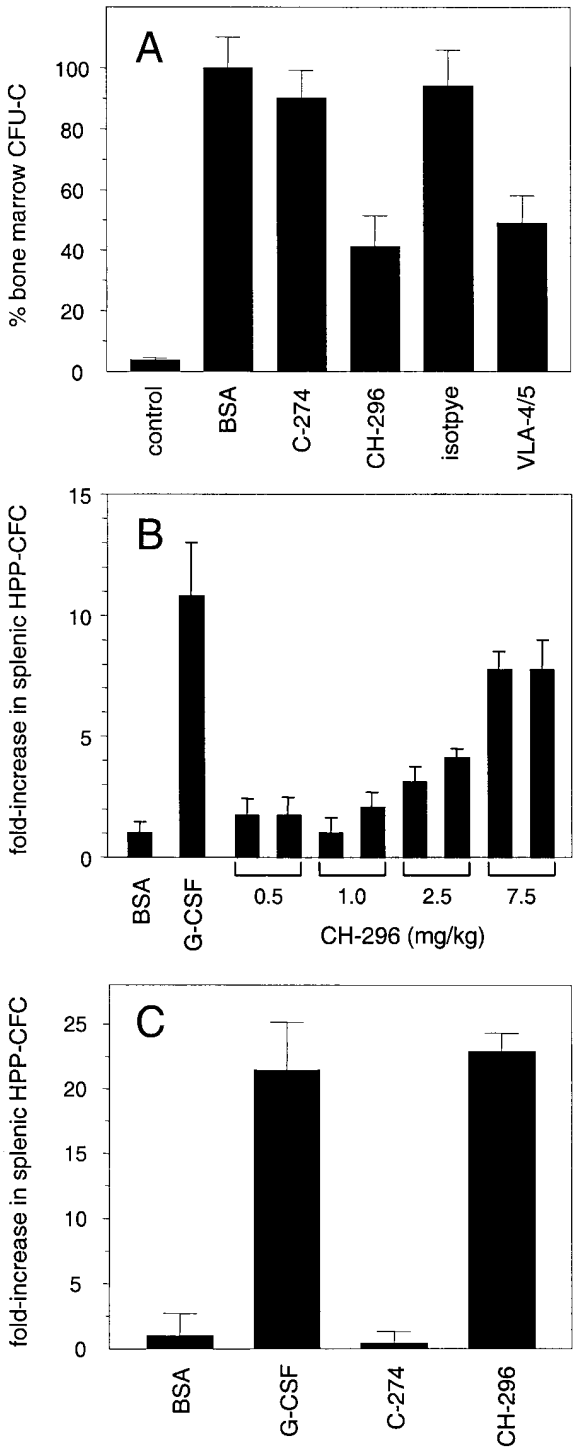

Figure 9. Effect of FN fragments on engraftment and distribution of hematopoietic cells in vivo. (*A*) Effect of preincubation of BM cells with recombinant FN fragments on BM engraftment. Low-density BM cells were preincubated with FN fragments or monoclonal antibodies (all at a saturating concentration of 0.1 mg/mL) for 1 h at 4°C and intravenously injected into splenectomized recipient animals preirradiated with 920 cGy (four animals/group, 4×10^5 cells/animal). After 10 d, BM was pooled per group and tested in methylcellulose cultures using triplicate dishes. Bars indicate mean percentage (±SD) of BM progenitors relative to the BSA-treated group. (*B*) Effect of intravenous injection of FN peptide CH-296 (0.5–7.5 mg/kg body weight per day for 4 d), human G-CSF (250 μg/kg/day), or vehicle (BSA) on the number of HPP-CFC in the spleen (one animal/group). (*C*) Effect of intravenous injection of peptides C-274 and CH-296 (both at 7.5 mg/kg/day), human G-CSF (250 μg/kg/day), or vehicle (BSA) of a representative experiment (one animal/group). The results are expressed as fold increase of HPP-CFC in the spleen relative to the BSA-injected control animal. Bars (in *B* and *C*) indicate average of triplicate cultures ±SD.

proof of VLA-5 receptors on primitive human hematopoietic cells, as identified by their ability to repopulate NOD/SCID mice with multiple lineages, and on mouse in vivo repopulating stem cells as measured by long-term repopulation in WBB6F1/J-Kit^W/Kit$^{W\text{-}V}$ mice. The data complement and extend previous findings of the expression of VLA-5 by primitive in vitro colony-forming cells (cobblestone area–forming cells, day 35; 28) and enhanced transfection of mouse HSC on FN fragment CH-271 (6). In apparent contrast with our current observations, others have used adhesion depletion of more mature progenitors on intact plasma FN as a method to purify hematopoietic stem cells from mouse fetal liver (44). However, it may be difficult to directly compare the binding of cells with intact FN versus enzymatically cleaved or recombinant FN peptides as evidence suggests that the domain structure may differ significantly between these molecules (45). Such differences may also explain the increased adhesion of HSC to the carboxy-terminal FN 30/35 fragment of plasma FN as compared with intact FN (19) and greater transduction efficiency of LTC-IC on FN 30/35 compared with intact FN (4). In addition, in the present study, we have used hematopoietic cells from adult mouse BM, which may differ in their adherent properties from fetal liver hematopoietic cells. Thus, within the hematopoietic microenvironment, not only the level of expression and activation state of integrin receptors on hematopoietic cells, but also the presence of enzymatically modified peptides within the extracellular matrix that present an enhanced but otherwise cryptic domain structure (45, 46) may be an important determinant of primitive hematopoietic cell adhesion.

When mouse BM cells were preincubated with FN peptide CH-296, which displayed the highest cell-binding activity of all peptides tested, the engraftment of progenitors into the BM of irradiated splenectomized animals was significantly decreased. This decrease was comparable with that obtained after preincubation with integrin-blocking antibodies. In addition, when peptide CH-296 was intravenously injected, the number of HPP-CFC in the spleen was significantly increased indicating that the peptide disturbed steady state hematopoiesis by changing the distribution of hematopoietic progenitor cells between BM and spleen. This effect was specific for peptide CH-296 and could not be observed with peptides that only contained one or two of the FN cell-binding sites. Supporting these data, others have found that pretreatment of transplanted BM cells or recipients with a synthetic CS1 peptide alone did not change the homing of transplanted CFU-S to the BM as compared with control nor did it change the number of CFU-C recovered from the BM, spleen, or peripheral blood (47, 48). Our data on the effects of peptide CH-296 suggest that there may be a significant additive or cooperative effect between VLA-5, VLA-4, and proteoglycan/CD44 in vivo, which has not previously been described. In addition, it seems clear from these data that interpretation of in vitro adhesion studies, and extrapolation of these results with respect to relationships between BM cells and the microenvironment in vivo, must be done cautiously.

Integrin VLA-4 and VLA-5 have both been described to play a role in the adhesion and/or migration of cells in other systems. For example, VLA-4 and VLA-5 have been implicated in the migration and differentiation of human thymocytes (24). While VLA-4 by itself was involved in adherence during all stages of differentiation, a coordinated action of both VLA-4 and VLA-5 was required for the persistence and directionality of migration. In addition, studies on neural crest cells, Chinese hamster ovary cells and tumor cells have suggested that VLA-5 may be involved in maintaining the direction of migration of cells, while VLA-4 has been thought to be required for the speed of migration (49–51). In fact, β1-integrin–deficient embryonic stem cells have been shown to be defective in migration in vivo but not in their proliferation or differentiation (52). For mast cells it has been shown that the VLA-5–dependent adhesion to immobilized FN could be enhanced by activation of the *c-kit* tyrosine kinase, by either the soluble or membrane-associated form of SCF (53, 54). Based on this observation, it has been suggested that the defect seen in migrational lineages in viable W mutants that express *c-kit* with a nonfunctional kinase domain may be linked to the inability of these cells to activate VLA-5 (53). VLA-4 has also been linked to the *c-kit* pathway by the observation that the anti–VLA-4 induced mobilization of progenitor cells from the BM into the peripheral blood required a cooperative signal involving *c-kit* (55). While in BM transplantation VLA-4 has been shown to be important for the initial steps of homing and engraftment (48), VLA-5 may be involved in the migration of HSCs in the BM microenvironment as suggested above or in different aspects of stem cell behavior. In this regard, in vitro assays may not entirely define cell and matrix interactions important for homing and engraftment in vivo, as demonstrated here for VLA-5. Nonetheless, future studies should examine in more detail the potential role(s) of VLA-5 in hematopoietic cell survival, cell cycling, and apoptosis (9, 10, 56–58) as they may be important for the manipulation of hematopoietic cells ex vivo, especially in retroviral infection protocols that use extracellular matrix proteins such as FN.

Acknowledgments

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