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Expression and function of cell adhesion molecules on fetal liver, cord blood and bone marrow hematopoietic progenitors: Implications for anatomical localization and developmental stage specific regulation of hematopoiesis

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The mechanism of localization, migration, and regulation of hematopoiesis at different stages of ontogeny is not well understood, but may relate to the specific adhesive interactions between hematopoietic stem cells and their microenvironment at different ontogenic stages. We studied the expression of cell adhesion molecules (CAM) on fetal liver (FL), umbilical cord blood (UCB) and adult bone marrow (ABM) CD34$^+$ cells, and the adhesion of committed progenitors (CFC) from all three sources to ABM stromal layers and purified extracellular matrix proteins. Compared to ABM CFC, significantly more UCB CFC and fewer FL CFC adhered to ABM stroma. Adhesion of FL CFC to fibronectin (FN), the 75 kD RGD containing FN fragment and the 33–66 kD COOH-terminal heparin binding FN fragment was also significantly less than that of ABM CFC. Like ABM CFC, the adhesion of FL CFC was mediated through α4β1 and α5β1 integrins. Of note, more FL CD34$^+$ cells expressed α5 integrins and the number of α4, α5 and β1 integins per cell (mean channel frequency) was similar or higher for FL CD34$^+$ cells than ABM CD34$^+$ cells. Further, treatment of FL CFC with a β1 integrin activating antibody (8A2), increased adhesion of FL CFC to FN to the same level as that of 8A2 treated ABM CFC. This suggests that the α4β1 and α5β1 integrins on FL CD34$^+$ cells may be present in a low avidity/affinity state. We also show that unlike ABM, FL CD34$^+$ cells expressed α2 and that approximately 20% FL CFC adhered to collagen IV. Further, α2β1 integrin on FL CFC was functional since their engagement, either by adhesion to collagen IV or through blocking α2 antibodies, transmitted proliferation inhibitory signals. In contrast to α4b and α5β1 integrin dependent adhesion, α2β1 dependent adhesion of FL CFC to collagen IV was not enhanced after treatment with 8A2. The reason for this is not clear but suggests that α2 integrins on FL CFC are maximally activated. This novel adhesive interaction with collagen IV, reminiscent of that described for CML progenitors, may have a role in the extramedullary localization of FL hematopoiesis or its developmental stage-specific regulation by its microenvironment. Studies to evaluate these possibilities are underway.

Keywords: **Hematopoiesis—Fetal liver—Stem cells—Integrins—Microenvironment**

Introduction

During fetal life, hematopoiesis takes place sequentially in the yolk sac, fetal liver (FL) and spleen, and the bone marrow. In the fetal liver, erythropoiesis predominates whereas hematopoiesis in the bone marrow is predominantly granulopoietic [1–3]. During fetal life and immediately after birth, a large number of hematopoietic progenitor and stem cells can also be found in the circulation. However, within 24–48 hours after birth, circulating progenitors disappear from the circulation, presumably because of lodgment in the marrow cavity. In the adult, hematopoiesis takes place chiefly in the bone marrow. The reasons for this migratory behavior or the mechanisms underlying the localization, migration and homing of the hematopoietic stem cells are not well understood. However, changes in sites of hematopoiesis are thought to be due to differences in the ability of hematopoietic stem and progenitors cells at different stages of ontogenic differentiation to migrate and home to different hematopoietic organ [1,4] as well as differences in the capacity of the hematopoietic microenvironment of a specific hematopoietic organ to support the development of progenitors from different ontogenic age [5–7]. We hypothesized that differences between the adhesive characteristics of he-

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matopoietic progenitors in the maturing fetus, cord blood, and adult marrow may underlie the migratory behavior of hematopoiesis.

Progenitors from normal adult bone marrow (BM) adhere in a lineage-specific and differentiation stage-specific manner to several ECM components, including heparan-sulfate, thrombospondin, and fibronectin [8–13]. We and others have demonstrated that primitive progenitors, functionally defined as long-term culture initiating cells (LTC-IC) and more mature colony forming cells (CFC) adhere to vascular cell adhesion molecule (V-CAM) and fibronectin present in stroma through the α4β1 and α5β1 integrins [10–13]. In addition, progenitors express β2-integrins and selectins, both of which may allow them to interact with endothelial cells [14] and CD44, which may allow them to adhere to fibronectin and hyaluronate [12,15].

Aside from these in vitro studies demonstrating an important role of integrins in progenitor-stroma interaction, several in vivo studies demonstrate a dominant role for the β1-integrin family in the homing and retention of stem cell in the BM microenvironment. Engraftment of murine or baboon stem cells can be inhibited by anti-α4 antibodies [16,17]. Likewise, homing of human stem cells to a xenogeneic ovine marrow microenvironment can be inhibited by antihuman α4 antibodies [18]. Intravenous administration of anti-α4 antibodies in baboons or mice results in peripheralization of CFU-GM and stem cells [19,20]. β1-defective embryonic stem cells cannot successfully compete with wild type stem cells in competitive engraftment experiments, due to their inability to migrate to the fetal liver [21]. All these studies indicate that β1-dependent interactions are needed to localize stem cells in the marrow and to allow their migration and adhesion to the marrow.

The microenvironment is essential for retaining progenitors in a specific organ and for regulating their normal proliferation and differentiation. We and others have shown that specific adhesive interactions between adult bone marrow (ABM) hematopoietic progenitors and the ABM microenvironment play a crucial role not only in the homing of ABM progenitors but also in the regulation of their growth and differentiation [22,23]. Hematopoietic progenitors as well as the microenvironments of hematopoietic organs at different stages of ontogeny have different anatomic and functional characteristics. Thus, it is likely that specific progenitor-microenvironment interactions are important for regulation of hematopoiesis at each stage of ontogeny. Since hematopoietic progenitors are found in the FL, umbilical cord blood (UCB) and ABM at different stages of ontogeny, we examined the possibility that the expression and function of cell adhesion molecules (CAM) on FL, UCB and ABM progenitors might be different.

We compared the CAM expression profile on FL, UCB, and ABM $CD34^+$ cells and the adhesion of committed progenitors (CFC) from all three sources to BM stromal layers and purified extracellular matrix proteins. We describe a previously unrecognized adhesive interaction between FL CFC and collage type IV, and present evidence that it may have growth regulatory influence on FL CFC.

Materials and methods

Cell source

Fetal liver. Human FL tissue (gestational age 7–14 weeks) was obtained from the Central Laboratory for Human Embryology, University of Washington (Seattle, WA) or from Advanced Bioscience Resources, Inc. (Alameda, CA) following informed consent according to guidelines of their institutional Committee on the Use of Human Subjects in Research. FL tissue was disaggregated in a single cell suspension as described previously [24].

Umbilical cord blood. UCB (umbilical cord blood) was collected after informed consent, using guidelines approved by the Committee on the Use of Human Subjects for Research at the University of Minnesota.

Adult bone marrow. The ABM was obtained from the posterior iliac crest of normal, healthy volunteer donors after informed consent, using guidelines approved by the Committee on the Use of Human Subjects for Research at the University of Minnesota. UCB mononuclear (CBMNC) cells and BMMNC were isolated by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient separation (specific gravity 1.077) for 30 minutes at 37°C and 400*g*.

CD34 cell selection

FL and CBMN cells were enriched for $CD34^+$ cells using the MACS® CD34 isolation kit (Miltenyi Biotec Inc., Sunnyvale, CA) as described previously [10]. A CD34-enriched population was obtained from BMMNC using a biotinylated anti-CD34 monoclonal antibody and a Ceprate® avidin-biotin immunoadsorption column (CellPro Inc., Bothell, WA) as described [10].

Fluorescence-activated cell
sorting analysis of cell surface receptors

The following antibodies coupled to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used: mouse antibodies directed to CD11a (LFA-1), CD11b (Mac-1), CD11c, CD18 (β2 integrin), CD29 (β1 integrin), CD34, CD44, CD49b (α2 integrin), CD49d (α4 integrin), CD49e (α5 integrin), CD49f (α6 integrin), CD62P (P-selectin), CD62E (E-selectin) and CD62L (L-selectin). Antibodies purchased from Becton Dickinson (San Jose, CA) were anti-CD11c, CD44, CD62L, CD62P, and CD34; from Immunotech (Marseilles, France), anti-CD49b, CD49d, CD49e, CD18, and CD11b; from Southern Biotech (Birmingham, AL), anti-CD49e, CD49f, and CD62E; and from Pharmingen (San Diego, CA), CD29. For dual color analysis 100,000 $CD34^+$ enriched cells were suspended in 100 mL of PBS + 0.3% bovine serum albumin (BSA) (Sigma), labeled with 20 ng anti-adhesion receptor antibodies in conjunction with 20 ng of anti-CD34 antibodies, incubated for 30 minutes at 4°C, washed, and then analyzed on a fluorescence-activated cell sorting (FACS) Star Plus flow cytometer (Becton-Dickinson). FITC and PE conjugated isotype-matched mouse Igs (Becton-Dickinson) were used as controls.

Adhesive ligands

Bone marrow stroma. Stromal layers were generated by plating adult bone marrow mononuclear cells in long-term culture (LTC) medium as described [9]. Once confluent, stroma was irradiated with 2000 Rads and subcultured in 24 or 6 well plates with LTC medium.

Glutaraldehyde fixed stromal feeders. Irradiated ABM stromal layers were treated with 2% glutaraldehyde (Sigma) in 0.1M Sorenson's buffer for 5 minutes and washed four times with IMDM as described [25]. We have shown that adhesive ligands are still recognized by specific monoclonal antibodies, but that the fixed stromal cells are no longer metabolically active [25].

Purified extracellular matrix components and fragments. Human plasma fibronectin, a 75 kD tryptic fragment containing the RGD cell binding domain and the 33kD-66 kD COOH-terminal heparin binding fragments of fibronectin were obtained as previously described [26,27]. Collagen type IV was isolated from the Engelbreth-Holm-Swarm tumor grown subcutaneously in Swiss Webster mice using previously described techniques [27,28]. Collagen I and Laminin were purchased from Gibco-BRL (Rockville, MD). Fibronectin (100 μg/mL), 75 kD fibronectin fragment (60 μg/mL), 33–66 kD fibronectin fragment (60 μg/mL), collagen I (60 μg/mL), collagen IV (60 μg/mL), and laminin (60 μg/mL) were diluted at their appropriate concentrations in Voller's carbonate buffer [23]. Bovine serum albumin at 1 mg/mL (BSA, fatty acid free; Miles Naperville, IL) and poly L-lysine (10 μg/mL) (Sigma) were used as negative and positive controls. Proteins and controls were adsorbed to wells of 48 well plates (Costar, Cambridge, MA) overnight in a humidified atmosphere at 37°C and 5% CO_2. Nonspecific sites were blocked the following day with 1 mg/mL BSA in PBS for 1 hour prior to adhesion assays.

Cell adhesion assays

Adhesion to ABM stroma. $CD34^+$ cells from ABM, UCB, or FL were suspended in IMDM and plated in contact with human ABM stromal layers in 24 well plates and incubated for 2 hours in a humidified atmosphere at 37°C. Nonadherent cells were removed by four standardized washes using warm IMDM and adherent cells by trypsinization [23]. Both adherent and nonadherent fractions were cultured in short-term methylcellulose culture to enumerate CFC. The percent of adherent CFC was calculated as: (number of CFC in the adherent fraction):(number of CFC in adherent + nonadherent fraction) × 100.

Adhesion to ligands. Two to five × 10^3 cells suspended in 200 μl IMDM were plated on ligand coated or control wells and incubated in a humidified atmosphere at 37°C and 5% CO_2 for 4 hours. Nonadherent cells were removed by four washings with warm IMDM after standardized shaking of the plates (30 horizontal excursion in 30 seconds) and adherent cells after trypsin treatment [23]. Adherent and nonadherent fractions were cultured in short-term methylcellulose culture. The percent of adherent CFC was calculated as: (CFC in adherent fraction)/(CFC in adherent + nonadherent fraction) × 100.

In some experiments the adherent and nonadherent cell populations were subjected to Thymidine suicide assay to determine the fraction of CFC in S-phase.

Adhesion blocking experiments. In some experiments, FL and ABM $CD34^+$ cells were incubated with an activating anti-β1 antibody, 8A2 [29] (1:10,000 dilution), or blocking antibodies against the α5 (P1D6, 1:400 dilution), α4 (P4C2, 1:400 dilution), α2 (P1E6, 1:400 dilution), β1 (P4C10, 1:400 dilution), or control mouse IgG (Sigma) for 30 minutes before adhesion assays [30,31]; 8A2 was a kind gift from Dr. Nicolas Kovach (University of Washington, Seattle, WA). The integrin-blocking monoclonal antibodies P4C2, P1D6, P4C10, and P1E6 were purchased from GIBCO-BRL. We have previously demonstrated that addition of these antibodies to methylcellulose progenitor assays of $CD34^+$ cells does not increase or decrease the number of BFU-E or CFU-GM compared with cultures to which either no antibodies were added or to which control mouse IgG was added [12,30].

Assessment of proliferation

Induction of proliferation. Since < 20% of CFC in ABM and no CFC present in fetal tissue shipped overnight from Seattle or Alameda to the University of Minnesota were in S-phase, $CD34^+$ cells were induced to proliferate by incubating in IMDM based defined media (serum free media) containing BSA (20 mg/mL) (GIBCO), insulin (10 mg/mL), transferrin (200 mg/mL) (Sigma, St. Louis, MO), 10^{-4} M 2-mercapto-ethanol, penicillin 100 U/mL, and streptomycin 100 U/mL (GIBCO), GM-CSF (200 pg/mL), G-CSF (0–100 ng/mL), SCF (200 pg/mL) (Amgen, Thousand Oaks, CA), LIF (50 pg/mL) (R&D Systems, Minneapolis, MN), MIP-1α (200 pg/mL) (R&D) and IL-6 (1 mg/mL) (Genetics Institute). The concentration of the different cytokines corresponds to the levels at which they are present in Dexter-type culture [32]. We have previously shown that as for stroma conditioned medium, this defined medium induces proliferation of 40%–50% of ABM CFC and does not affect the ability of ABM CFC to adhere to stromal feeders or purified extracellular matrix components [33]. After 4 days, cells were harvested and used in proliferation inhibition assays.

Adhesion mediated proliferation inhibition. Proliferating ABM or FL CD34+ cells were plated for 4 hours in contact with stroma or purified adhesive ligands. Adherent and nonadherent cells were collected separately and subjected to thymidine suicide assay to determine the effect of adhesion on their proliferation. Coculture of cells with BSA or poly-l-lysine served as control. That recovery of the adherent cells with trypsin did not affect progenitor proliferation was shown in experiments in which cells adherent to poly-l-lysine collected with trypsin proliferated to the same extent as the nonadherent population or cells cocultured with BSA.

Receptor engagement by antibodies. Proliferating ABM or FL $CD34^+$ cells were incubated at 4°C with adhesion-blocking anti-α2, α4, α5, and β1 (GIBCO) antibodies (all at 1:400 dilution) or mouse IgG (Sigma). After 30 minutes, cells were washed in cold IMDM and incubated with goat antimouse IgG (10 μg/mL) (Biosource International, Camarillo, CA) for 4 hours at 37°C to induce receptor clustering. Cells were then subjected to thymidine suicide assay to determine the fraction in S-phase.

Thymidine suicide assay. Proliferation of CFC was assessed as previously described [25,34]. Briefly, cells recovered in the adherent and nonadherent fractions of adhesion assay or after engagement of receptors were washed and resuspended in 200 μl serum free warm IMDM and then incubated at 37°C for 20 minutes with or without 20 μCi/mL of [^{3}H] thymidine (DuPont, Boston, MA). Cells were washed with 10 mL of excess cold thymidine (100 μg/

mL) followed by one wash with cold IMDM and plated in methylcellulose assay.

%progenitors in S-phase = %CFC kill = (CFC in untreated population) − (CFC in treated population) × 100/(CFC in untreated population)

Short-term methylcellulose progenitor culture

Cells recovered from adhesion assays or after exposure to thymidine were cultured in methylcellulose culture supplemented with 3 IU erythropoietin (Epotein, Amgen) and SCF (Amgen), GM-CSF (Immunex, Seattle, WA) and IL-3 (R & D) each at a final concentration of 5 ng/mL as described [25]. The cultures were assessed at days 12 to 14 for the presence of BFU-E and CFU-GM as previously described [25].

Statistical Analysis

Results of experimental points obtained from multiple experiments were reported as the mean ± 1 SEM. Significance levels were determined by two-sided Student's *t*-test analysis.

Results

Expression of various cell adhesion molecules of the β1 and β2 integrin family, selectins, and CD44 on $CD34^+$ cells from FL, UCB, and ABM was determined by dual color FACS analyses. Compared to ABM, UCB $CD34^+$ cells expressed significantly more CD44 and CD49f, but less CD11a and CD18 ($p < 0.05$) (Fig. 1). Expression of CD11b, CD11c, CD29, CD49b, CD49d, CD49e, CD62E, CD62L, and CD62P on ABM or UCB $CD34^+$ cells was not different (not shown). Compared with ABM, FL $CD34^+$ cells expressed more CD29 and CD49e ($p = 0.01$) and CD49b ($p < 0.05$) but less CD11a, CD18, CD44 ($p < 0.01$) and CD62L ($p < 0.05$) (Fig. 1). The expression of CD11b, CD11c, CD49b, CD49d, CD49e, CD49f, CD62E, and CD62P on FL or ABM $CD34^+$ cells was not different. The number of α4, α5, and β1 integrins on per cell (mean channel frequency) was similar or higher on FL $CD34^+$ cells than ABM $CD34^+$ cells, although the difference was not statistically significant (Fig. 1).

We next investigated the in vitro adhesion of $CD34^+$ cells from FL, UCB, and ABM to ABM stromal layers and purified extracellular matrix components, including fibronectin (FN), the RGD containing, α5β1 binding 75 kD fragment of FN (75 kD FN), the CS1 containing C-terminal 33–66 fragment of FN that allows cell binding through the α4β1 integrin and cell surface proteoglycans (33–66 FN), laminin, collagen type I and collagen type IV. Significantly more UCB CFC (46.6% ± 1.9%) than ABM CFC (36.6% ± 0.9%) ($p = 0.001$) adhered to ABM stromal feeders. In contrast, FL CFC were significantly less adherent (19.4% ± 2.5%) to ABM stroma than ABM CFC (29% ± 1.4%, $p = 0.03$) (Fig. 2). Adhesion of FL CFC to stroma was tested using glutaraldehyde fixed ABM stromal feeders to avoid inhibition of FL BFU-E growth by soluble factor(s) secreted by ABM stromal layers [35]. We have previously demonstrated that glutaraldehyde fixation metabolically inhibits stromal feeders but preserves adhesive ligands [25] and prevents inhibition of FL BFU-E growth [35]. Adhesion of ABM and UCB CFC to FN, 75 kD FN and 33/66 kD FN was similar. No adhesion of UCB and ABM CFC was ob-

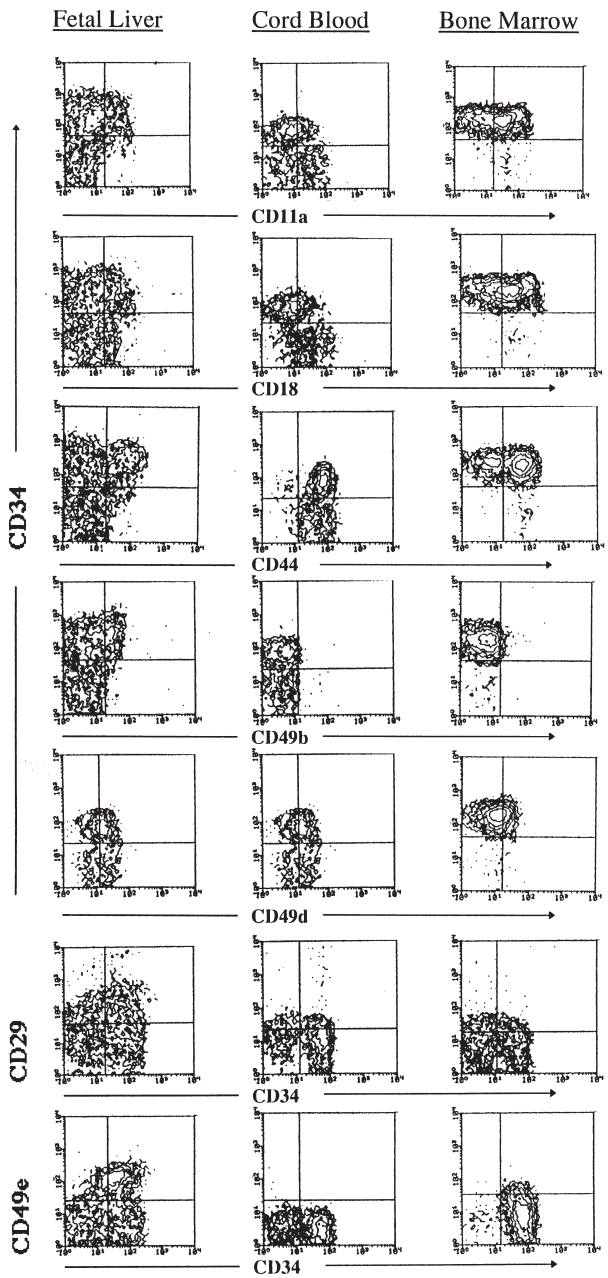

Figure 1. Umbilical cord blood (UCB) $CD34^+$ cells express more CD44 and CD49f, while fetal liver (FL) $CD34^+$ cells have higher expression of CD29, CD49b, and CD49e than adult bone marrow (ABM) $CD34^+$ cells. Expression of various cell adhesion molecules of the β1 and β2 integrin family, selectins and CD44 on FL, UCB, and ABM derived $CD34^+$ cells was studied by dual color flow cytometric analyses. Compared to ABM, UCB $CD34^+$ cells expressed significantly more CD44 and CD49f but less CD11a and CD18 ($p < 0.05$). Compared with ABM, FL $CD34^+$ cells expressed more CD29 and CD49e ($p = 0.01$) and CD49b ($p < 0.05$) but less CD11a, CD18, CD44 ($p < 0.01$) and CD62L ($p < 0.05$).

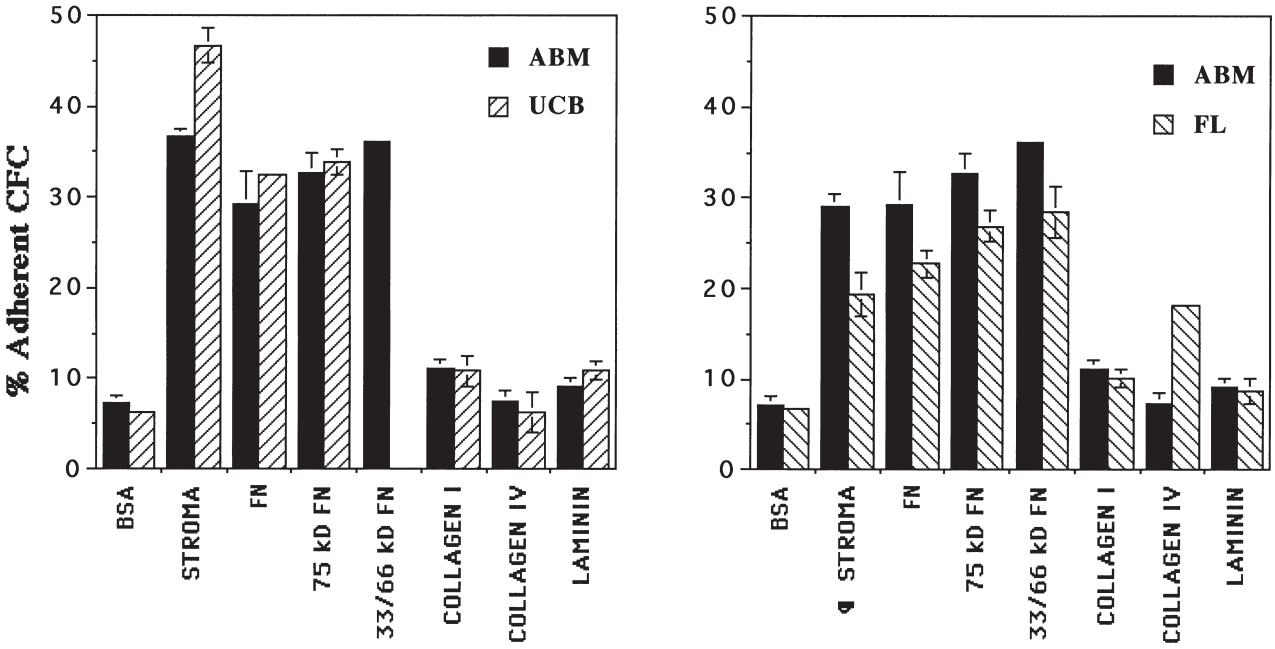

Figure 2. CFC derived from fetal liver (FL), umbilical cord blood (UCB), and adult bone marrow (ABM) demonstrate significant differences in their adhesion to ABM stroma or purified extracellular matrix components. $CD34^+$ cells from ABM, UCB or FL were plated in IMDM in contact with ABM stromal layers or extracellular matrix protein-coated wells for 2 hours. Nonadherent and adherent cells were recovered as described in the Methods section. Both adherent and nonadherent fractions were cultured in short-term methylcellulose culture to enumerate CFC. Significantly more UCB than ABM CFC adhered to ABM stroma ($p = 0.001$) while FL CFC were significantly less adherent ($p = 0.03$). Adhesion of FL CFC to fibronectin ($p = 0.04$), 75 kD fibronectin ($p = 0.06$), and 33–66 kD fibronectin ($p = 0.035$) was significantly lower than that seen for ABM CFC. In contrast to ABM or UCB CFC, a significant number of FL CFC ($p = 0.02$) adhered to collagen type IV. ($^*p < 0.05$). ¶Since soluble factor(s) secreted by ABM stromal layers inhibit growth of FL BFU-E [22], adhesion of FL CFC to stroma was tested using glutaraldehyde fixed stromal feeders. Results in the right-hand panel for adult marrow are also using glutaraldehyde fixed stroma.

served to laminin, collagen type I or IV. In contrast, adhesion of FL CFC to FN (22.8% ± 1.5% vs. 29.2% ± 3.7%, $p = 0.04$), 75 kD FN (26.9% ± 1.7% vs. 32.7% ± 2.2%; $p = 0.06$) and 33–66 FN (28.5% ± 2.9% vs. 36.1% ± 0.6%, $p = 0.035$) was lower than that seen for ABM CFC (Fig. 2). No adhesion was observed to laminin or collagen type I. In contrast to ABM or UCB CFC, a significant number of FL CFC (18.6% ± 0.6%, $p = 0.02$ vs. BSA control) adhered to collagen type IV (Fig. 2).

We then studied the effect of blocking anti-integrin antibodies (α2, α4, α5, and β1) and the activating anti-β1 antibody, 8A2, on CFC adhesion to FN and collagen IV. As we have previously reported, anti-β1 antibodies significantly inhibited the adhesion of ABM CFC to FN. As would be expected, blocking anti-α4 and anti-α5 antibodies but not anti-α2 antibodies inhibited adhesion of ABM CFC to FN. In addition, preincubation of ABM $CD34^+$ cells with the activating anti-β1 antibody, 8A2, significantly increased adhesion of ABM CFC to FN (Fig. 3). None of the antibodies increased or decreased adhesion of ABM CFC to Collagen type IV. As was seen for ABM, adhesion of FL CFC to FN was significantly reduced by prior incubation with anti-α4, α5 and β1 antibodies, unaffected by anti-α2 antibodies and increased by the activating anti-β1 antibody (Fig. 3). Furthermore, anti-α2 and anti-β1 antibodies significantly reduced the adhesion of FL CFC to collagen type IV, while anti-α4 and anti-α5 antibodies had no effect (Fig. 4). This demonstrates that the adhesive interaction of ABM and FL CFC with FN is mediated by α4β1 and α5β1 integrins and that of FL CFC with collagen type IV is mediated largely by α2β1 integrin. In contrast to the effect of 8A2 on the α4β1 and α5β1 mediated adhesion of ABM and FL CFC to FN, α2β1 mediated adhesion of FL CFC to collagen type IV was only minimally enhanced by treatment with the activating anti-β1 antibody, 8A2.

We have previously shown that adhesion of ABM CFC to stroma or FN via the α4β1 and α5β1 integrins inhibits their proliferation [25]. To determine if a similar growth regulatory effect is mediated by adhesion of FL CFC to FN or collagen type IV, we examined the proliferative status of ABM or FL CFC after coculture for 4 hours with FN or collagen type IV by thymidine suicide assay. A significantly smaller fraction of ABM CFC and FL CFC adherent to FN or 75 kD FN were in S-phase than progenitors recovered in the adherent fraction of poly-l-lysine coated dishes or progenitors present in the nonadherent fraction of FN, 75 kD

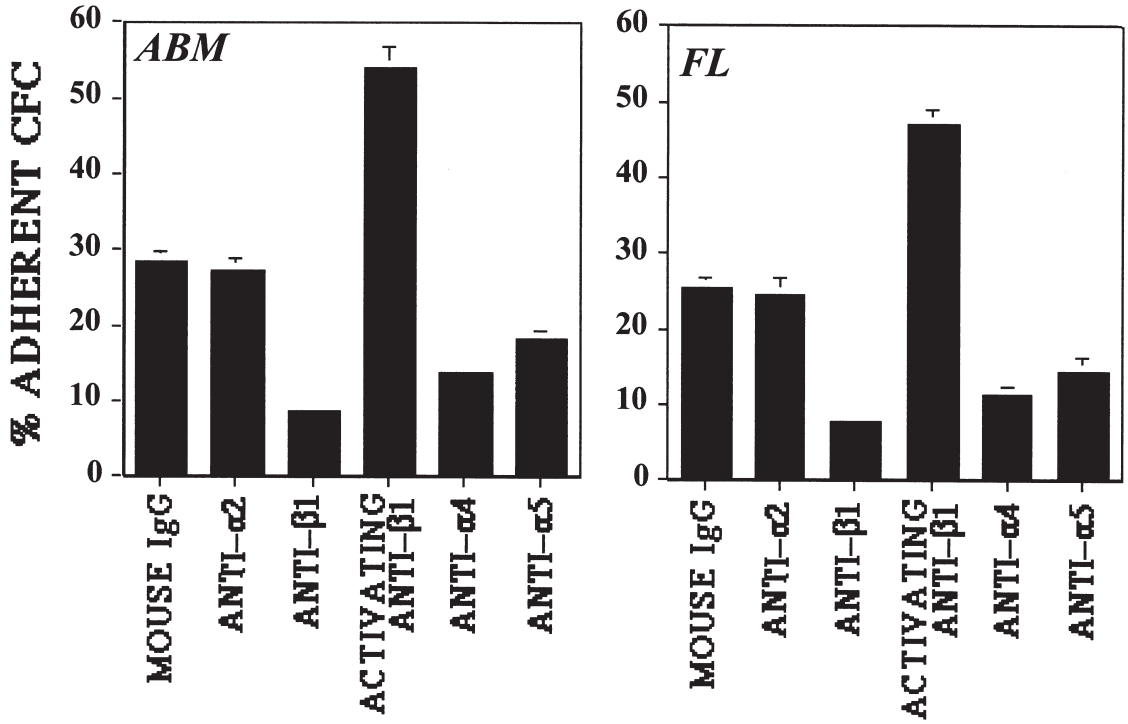

Figure 3. Adhesion of adult bone marrow (ABM) and fetal liver (FL) CFC to fibronectin is mediated by α4β1 and α5β1 integrins and is enhanced by pre-incubation with an activating anti-β1 antibody (8A2). FL and ABM CD34$^+$ cells were incubated with blocking antibodies against α5, α4, α2, β1, or an activating anti-β1 antibody, 8A2, or control mouse IgG for 30 minutes followed by adhesion assays. Adhesion of both ABM (left panel) and FL (right panel) CFC to fibronectin was inhibited by incubation with antibodies against α4, α5, and β1 but not affected by incubation with antibodies against α2. Pre-incubation with activating anti-β1 antibody, 8A2, increased the adhesion of both ABM and FL CFC to fibronectin.

FN or poly-l-lysine coated dishes ($p = 0.02$). Although, the inhibition of proliferation in the adherent FL CFC population was significant, the degree of inhibition was somewhat lower than that seen for adherent ABM CFC. Since the fraction of CFC in S-phase in the nonadherent fraction of FN, 75 kD FN or poly-l-lysine coated wells was similar, the decreased proliferation of CFC adherent to FN or 75 kD FN cannot be attributed to selective adhesion of nonproliferating CFC. Consistent with the lack of adhesion of ABM CFC to collagen type IV, coculture of ABM CD34$^+$ cells with collagen type IV did not affect ABM CFC proliferation (Fig. 5). However, proliferation of FL CFC adherent to collagen type IV was significantly lower than that of FL CFC adherent to poly-l-lysine or FL CFC recovered in the nonadherent fraction of collagen type IV coated dishes (Fig. 5), suggesting that as for α4β1 and α5β1, engagement of the α2β1 integrins on FL CFC can transmit growth regulatory signals.

To confirm these results, we also studied the effect of direct antibody mediated engagement of integrin receptors on ABM and FL CFC on their proliferation. Proliferating ABM or FL CD34$^+$ cells were incubated with anti-integrin antibodies (α2, α4, α5, β1 or mouse IgG) followed by goat anti-mouse IgG to induce receptor clustering. The proliferation status of control mouse IgG and anti-integrin antibody treated CFC was determined by thymidine suicide assay. Engagement of α4, α5 and β1 integrins inhibited proliferation of ABM CFC significantly ($p < 0.005$) while incubation of ABM CD34$^+$ cells with anti-α2 integrin antibodies did not affect ABM CFC proliferation (Fig. 6). As was seen for ABM CFC, engagement of α4, α5 and β1 integrins on FL CFC inhibited their proliferation significantly ($p < 0.01$), even though the inhibition of proliferation was less for FL CFC than ABM CFC. However, in contrast to ABM, incubation of FL CD34$^+$ cells with anti-α2 integrin antibodies also significantly inhibited the proliferation of FL CFC ($p = 0.012$) (Fig. 6).

Discussion

In this article, we describe differences in the adhesive phenotype and adhesive behavior of CD34$^+$ clonogenic cells derived from ABM, UCB, and FL. We demonstrate that aside from numerical differences in adhesion receptor expression between these ontogenically different cell populations, the adhesive behavior of FL, UCB, and ABM CFC differs considerably. UCB CFC adhered significantly better to ABM stromal feeders than their counterparts in ABM.

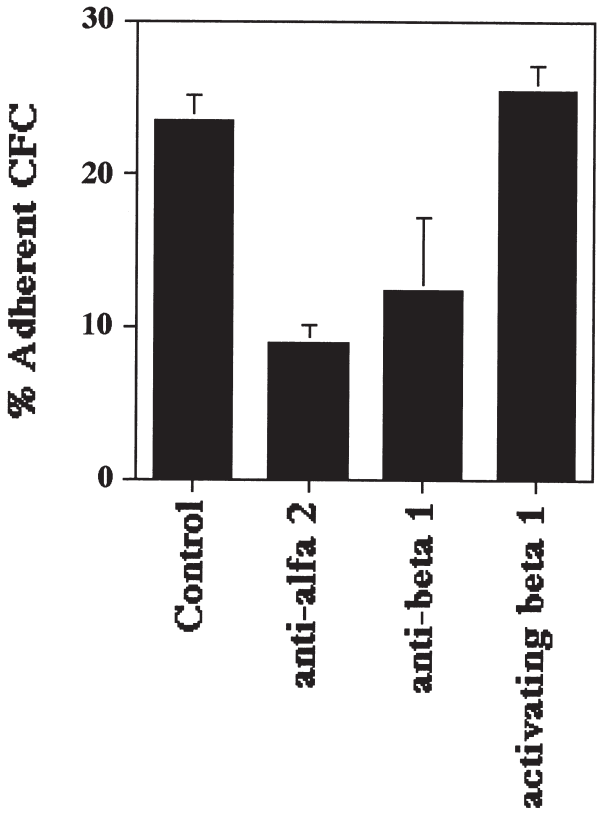

Figure 4. Adhesion of fetal liver (FL) CFC to collagen type IV is mediated by the α2β1 integrin but is not enhanced by pre-incubation with the activating anti-β1 antibody (8A2). FL $CD34^+$ cells were incubated with blocking antibodies against α2, β1, or an activating anti-β1 antibody, 8A2, or control mouse IgG for 30 minutes followed by adhesion assays. Adhesion of FL CFC to collagen type IV was inhibited by incubation with antibodies against α2 or β1. However, in contrast to its effect on fibronectin adhesion, pre-incubation with activating anti-β1 antibody did not enhance the adhesion of FL CFC to collagen type IV.

Likewise, UCB CFC may adhere somewhat better to the ECM component fibronectin than what we and others have seen for adhesion of ABM CFC, even though these differences did not reach statistical significance. At least two other conditions are characterized by an "aberrant" circulation of $CD34^+$ cells and CFC in the peripheral blood (PB). CFC recovered from the PB of individuals treated with cytokines to mobilize progenitor cells express less α4 integrins, which may be related to their inability to adhere to marrow stroma and fibronectin and may underlie their localization in the blood [36]. In patients with chronic myelogenous leukemia, Ph^+ CFC circulate prematurely in the blood not because of lack or adhesion receptor expression but because of functional defects of the β1-integrin family that disallows adhesion of CML CFC to stroma and fibronectin [27,31]. In contrast to CFC in cytokine-mediated mobilized PBPC and CML, we demonstrate here that $CD34^+$ CFC recovered from the peripheral blood circulation immediately after birth adhere significantly better to stroma and possibly better to the ECM component, FN, than their ABM counterparts. Thus, the abnormal localization in the peripheral blood of UCB $CD34^+$ cells and CFC cannot readily be explained by decreased adhesion of UCB progenitors to ABM microenvironment components. UCB $CD34^+$ cells and progenitors disappear from the PB within 24 hours after birth, presumably by homing to the marrow microenvironment. We hypothesize that the increased adhesive behavior of UCB progenitors may be acquired at the time of birth, allowing homing to the ABM microenvironment while progenitors circulating in the blood of the fetus before birth may have a decreased tendency to interact with marrow stromal components causing their aberrant circulation.

Compared to ABM CFC, FL CFC adhered significantly less to ABM stromal feeders. Further, adhesion of FL CFC to FN, and the 75 kD FN and 33–66 kD FN fragments was also lower than that of ABM CFC. As for ABM CFC, adhesion of FL CFC to FN, although reduced, was mediated by the α4β1 and α5β1 integrins. We have previously demonstrated that aside from anchoring progenitors in the bone marrow microenvironment, integrins on human hematopoietic progenitors may also regulate progenitor proliferation [25]. Engagement of β1 integrins by their adhesive ligand or by receptor clustering with adhesion-blocking anti-integrin antibodies decreases the fraction of ABM CFC that is in S-phase. Compared with ABM CFC, engagement of the α4, α5 and β1 integrin on FL CFC by FN or by monoclonal adhesion blocking anti-integrin antibodies also inhibited their proliferation less. However, more FL $CD34^+$ cells express the α5 and β1 integrins than ABM $CD34^+$ cells and the number of α4, α5, and β1 integrins per FL $CD34^+$ cells is similar or higher than that on ABM $CD34^+$ cells (mean channel fluorescence). Thus, the differences in adhesive behavior between FL and ABM CFC to FN nor the differences in adhesion mediated proliferation inhibition between these two progenitor populations are readily explained by differences in adhesion receptor expression. We demonstrate also that treatment of FL CFC with the activating anti-β1 integrin antibody, 8A2, increased FL CFC adhesion to FN to the same level as that seen for 8A2 treated ABM CFC. Although no formal assessment of integrin function was done, these studies suggest that the decreased adhesion of FL CFC to FN and β1-integrin-mediated regulation of proliferation of FL CFC is due to functional and not numerical differences in α4β1 and α5β1 integrins. It is well known that adhesion of cells depends on the recognition and binding of integrins to their ligand as well as on postreceptor events, such as cytoskeletal rearrangement and formation of focal contacts/adhesions required to establish firm cell adhesion [37,38]. The ability of a receptor to bind to its ligand and induce firm adhesions can be altered by other stimuli, including cytokines and engagement of other adhesion receptors on the cell surface [39]. We have demonstrated that adhesion of ABM $CD34^+$ cells to stroma and FN depends on the

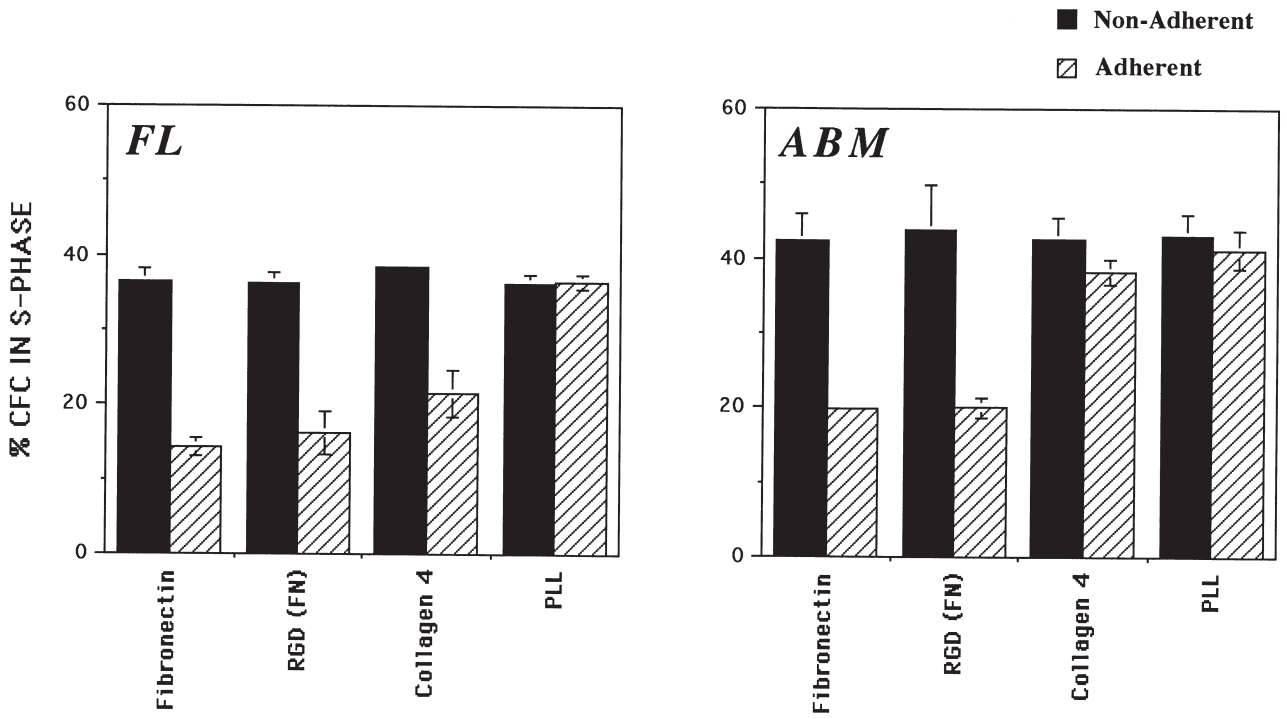

Figure 5. Proliferation of adult bone marrow (ABM) and fetal liver (FL) CFC was inhibited by adhesion to fibronectin or 75 kD fibronectin fragment. In contrast to ABM, proliferation of FL CFC was inhibited by adhesion to collagen type IV. Proliferation status of ABM and FL CFC in the adherent and nonadherent fractions after coculture for 4 hours with fibronectin or collagen type IV was determined by thymidine suicide assay. Significantly less ABM or FL CFC adherent to fibronectin or 75 kD fragment of fibronectin were in S-phase ($p = 0.02$). Proliferation of FL CFC was also inhibited by adhesion to collagen IV. Consistent with lack of adhesion, coculture with collagen IV did not inhibit ABM CFC proliferation. (PLL = poly-L-lysin; RGD = 75 kD RGD containing FN fragment).

coordinated action of β1-integrins and the cell surface proteoglycan, CD44 [12]. It is, thus, possible that the significantly decreased expression of CD44 receptors on FL $CD34^+$ cells compared with ABM $CD34^+$ cells may be responsible for the decreased function of the β1 integrins on FL CFC. A number of studies have also shown that stimulation through L-selectin upregulates the function of β1 integrins [40,41]. Decreased expression of L-selectin on FL CFC could also contribute to the decreased ability of these progenitors to adhere to ABM stroma. These possibilities are currently being evaluated.

Somewhat surprisingly, we show that more FL than ABM $CD34^+$ cells express the α2 integrin and that approximately 20% of FL CFC, but no ABM CFC adhere to the basement membrane protein collagen type IV via the α2β1 integrin. Not only does the α2β1 integrin mediate adhesion of FL CFC to collagen, engagement of the α2β1 integrin either by the collagen type IV or by blocking anti-α2 antibodies transmits growth regulatory signals. This is a novel adhesive interaction not previously described for normal hematopoietic progenitors and suggests a possible physiologic role for α2β1 integrin mediated interactions between FL progenitors and their microenvironment. In contrast to ABM hematopoiesis, FL hematopoiesis takes place in the extravascular space of the FL [42,43]. Interaction of progenitors with and regulation by microenvironments outside of the ABM may require that cells can interact with ECM components different from FN. This adhesive behavior is reminiscent of that of progenitors found in CML [27]. As for FL CFC, CML CFC adhere less well to FN, as a result of a functional impairment of the α4β1 and α5β1 integrins but adhere to some degree to the basement membrane protein collagen IV [27]. It is noteworthy that in CML, hematopoietic progenitors also thrive in microenvironments other than the marrow microenvironment, such as spleen and liver. Thus the ability of progenitors to interact with collagen type IV may be required for their development in extramedullary locations. Further, for hematopoietic progenitors to access the vascular space, they have to traverse the basement membrane lining of the capillaries and sinusoids, which is rich in collagen type IV [44]. Adhesive interactions of FL and CML progenitors with collagen may, therefore, help their egress into the circulation. It will be of interest to also examine the adhesive behavior of fetal bone marrow derived CFC. If interaction with extramedullary microenvironments requires expression and functional activity of integrins different than the α4β1 and α5β1 integrins that interact with FN and ABM stromal ligands, then one would anticipate

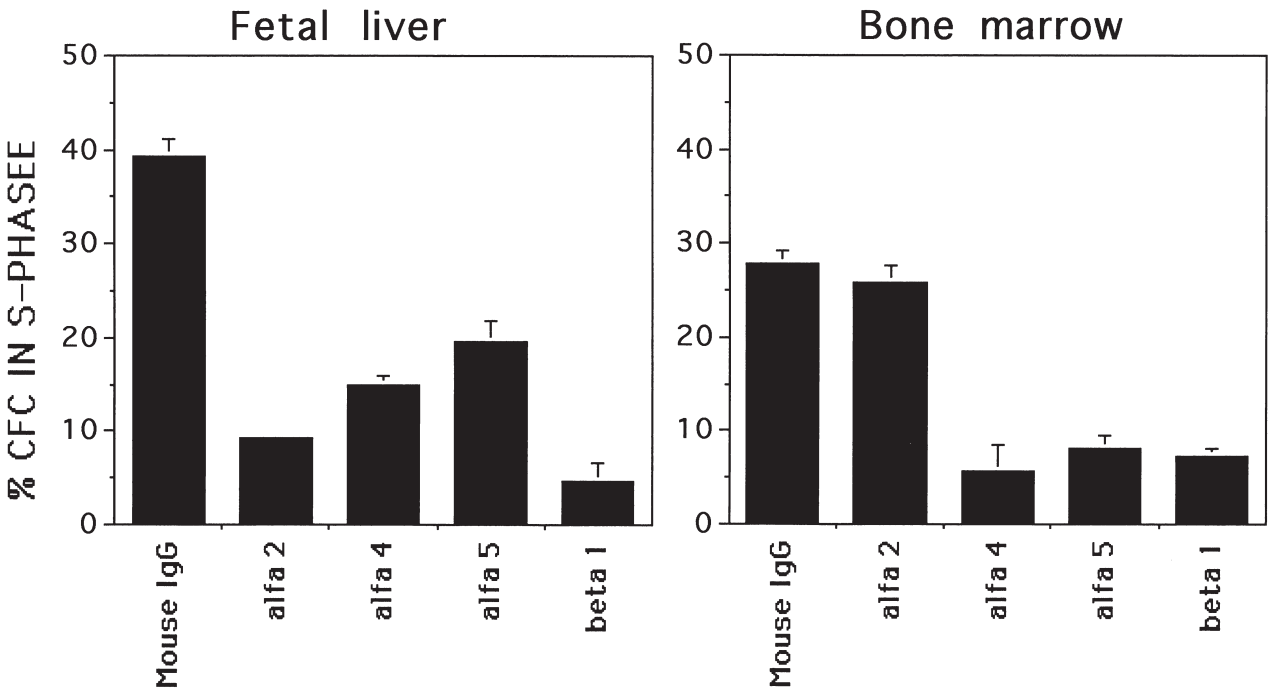

Figure 6. Engagement of α4, α5 and β1 integrins on adult bone marrow (ABM) CFC and α2, α4, α5 and β1 integrins on fetal liver (FL) CFC inhibits their proliferation. ABM or FL CD34$^+$ cells were incubated with anti-integrin antibodies (α2, α4, α5, β1 or mouse IgG) followed by goat anti-mouse IgG to induce receptor clustering and their proliferation status determined by thymidine suicide assay. Engagement of α4, α5 and β1 integrins significantly ($p < 0.01$) inhibited proliferation of ABM and FL CFC. In contrast to ABM CFC, engagement of α2 integrin also inhibited ($p = 0.01$) FL CFC proliferation.

that fetal BM CFC do not have the ability to adhere to and be regulated by collagen type IV.

The α2β1 integrin mediated adhesion of FL CFC to collagen type IV could not be upregulated by the activating anti-β1 integrin antibody, 8A2, while adhesion through the α4β1 and α5β1 integrins could be increased after treatment with 8A2. Since only 20% of FL CFC express the α2β1 integrin heterodimer, it is possible that the α2β1 but not the α4β1 and α5β1 integrin on FL CFC is already maximally activated. Although it is commonly accepted that the β1 integrin intracellular domain is responsible for transmitting signals that increase the affinity of the receptor and transfer signals to allow firm adhesion and regulation of other cellular functions, some studies have suggested that the type of a chain present in the integrin heterodimer may affect not only ligand specificity but also the functional status of the integrin heterodimer [45]. Thus, it is possible that the α2, but not α4 and α5, integrin chain modulates the functional status of the β1-integrin. A similar phenomenon is seen in CML. The α4β1 and α5β1 integrins, although present on CML CFC, are functionally impaired and support adhesion and adhesion mediated proliferation inhibition significantly less than the same integrin heterodimers present on normal ABM CFC. This can be overcome by preincubating CML CFC with the activating anti-β1 antibody, 8A2. However, adhesion to collagen type IV occurs in CML. Although we have not yet examined the effect of 8A2 on CML CFC adhesion to collagen type IV, the finding that <20% of CML CFC express α2 integrins while approximately 20% of CML CFC adhere to collagen type IV, suggest that, as we show here for FL CFC, the α2 integrin may activate the β1 integrin chain in CML.

The physiologic significance of the differences in expression and function of adhesion receptors on FL, UCB, and ABM CD34$^+$ cells is not clear. We can only speculate at this point that, for example, these differences may underlie the localization of the progenitors in their respective microenvironments. Several differences have been reported in the anatomic and ultrastructural features of FL and ABM microenvironments [46–48]. However, the relative contribution of microenvironmental differences versus differences in expression and function of adhesion molecules on FL progenitors in regulation of FL hematopoiesis can only be speculated at this time. To confirm these findings, in vivo studies in which for instance FL, CB or ABM CD34$^+$ cells are infused in NOD/SCID mice and the role of the different adhesion receptors in early "homing" evaluated, will be needed.

Our characterization of adhesion receptor expression and function on hematopoietic progenitors at different ontogenic developmental stages will help in defining the receptors responsible for differences in anatomic localization of hematopoiesis at different stages of ontogeny and in studying the mechanisms underlying migration, homing and develop-

mental stage-specific contact mediated regulation of hematopoiesis.

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