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The stem cell antigen CD34 functions as a regulator of hemopoietic cell adhesion

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ABSTRACT **Although the CD34 antigen is widely used in the identification and purification of hemopoietic stem and progenitor cells, its function within hemopoiesis is unknown. We have investigated this issue by ectopically expressing human (hu) CD34 on the surface of murine hemopoietic cells. Forced expression of hu-CD34 in the thymocytes of transgenic mice did not appear to affect the development, maturation, or distribution of murine T cells but did significantly increase their ability to adhere to bone marrow stromal layers of human but not mouse origin. Ectopic expression of hu-CD34 on murine 416B cells, a multipotential progenitor that expresses murine CD34, yielded similar results. In both cases hu-CD34-dependent adhesion was enhanced by molecular engagement of the hu-CD34 protein using anti-CD34 antibodies. These results provide evidence that CD34 promotes the adhesive interactions of hemopoietic cells with the stromal microenvironment of the bone marrow thereby implicating CD34 in regulation and compartmentalization of stem cells. We propose that CD34 regulates these processes in part via an indirect mechanism, signaling changes in cellular adhesion in response to molecular recognition of an as yet unidentified stromal CD34 counterreceptor or ligand.**

Expression of the stem cell antigen CD34 is a defining hallmark of hemopoietic stem cells and progenitors and CD34 is therefore widely used as a tag for the enumeration, isolation, and manipulation of these cells (1–3). Despite this extensive use, the normal function of the CD34 molecule within hemopoiesis has remained enigmatic. Human (hu) and murine CD34 homologues are highly conserved in their protein coding regions, which predict a type 1 transmembrane protein (4, 5). The cytoplasmic domains of the human and mouse proteins share 90% amino acid identity; the transmembrane and C-terminal regions of the extracellular domains are also well conserved with 73–82% amino acid identity. The N-terminal portions of the extracellular domains are the least well conserved regions of the molecule (45% amino acid identity). Both hu-CD34 and mouse CD34 are extensively modified posttranslationally by carbohydrate and are highly sialylated (6, 7). This pattern of processing provides different epitopes for the various CD34 monoclonal antibodies and may well be relevant to the function of the molecule (8). The expression pattern of CD34 is also conserved between human and mouse. Thus, in addition to being expressed selectively during human (1–3, 8) and murine (4, 7) hemopoiesis on stem cells and progenitors, both mouse CD34 and hu-CD34 are expressed outside the hemopoietic system on vascular endothelium (9, 10), high endothelial venules (HEVs) (8, 11), and some fibroblasts (4, 12). This distribution of CD34 implies a function outside of hemopoiesis. CD34 on HEVs appears to be processed differently from that on hemopoietic cells and vascular endothelium (8) and may provide an adhesive binding site recognized by L-selectin on circulating lymphocytes (11). In this report, we have investigated the function of CD34 during hemopoiesis by ectopically expressing hu-CD34 in murine hemopoietic cells.

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MATERIALS AND METHODS

Hu-CD34-Expressing Transgenic Mice. pCD2-hu-CD34; a 1.5-kb *Not* I/*Hin*dIII fragment containing the entire hu-CD34 coding region (5), was isolated from πH3M-CD34 and inserted into the *Bam*HI site of pβG-CD2 using *Bgl* II synthetic linkers. The *Kpn* I/*Not* I fragment was used to produce the three lines of transgenic mice used in this study (13).

Hu-CD34-Expressing 416B Cells. pMT-hu-CD34; the *Kpn* I/*Not* I fragment described above, was inserted via *Bgl* II synthetic linkers into the *Bgl* II site of MTIIaΔCAT, which contains promoter sequences from the human metallothionein IIa gene from −302 to +76 bp, with the AP-1 binding site at −106 to −97 mutated (14). A neomycin-resistance gene driven by the herpes simplex virus thymidine kinase promoter was inserted downstream of the simian virus 40 splice and polyadenylylation sequences in the same orientation as the CD34 cDNA. Stably transfected clones of 416B cells were obtained by electroporation followed by drug selection as described (15).

Immunophenotypic Analysis. Single cell suspensions of thymocytes were obtained from the thymuses of 8- to 10-week-old sex-matched mice. Thymocytes or 416B cells were incubated with an Fcγ blocking reagent, CD16/CD32 (Pharmingen), before staining with antibodies directly conjugated to either fluorescein isothiocyanate (FITC) or R-phycoerythrin (R-PE); subclass-specific antibody controls were included. Flow cytometric analyses were carried out with a FACScan (Becton Dickinson).

Adhesion Assays. Human stromal cultures were grown for 4–6 weeks from the mononuclear fraction of bone marrow originally plated in a 25-cm^2 tissue culture flask at 10^7 cells per 10 ml in α medium 10% fetal calf serum (FCS), 10% horse serum, and 2 μM methylprenisolone (Upjohn) and fed weekly. The preformed mouse stromas were also 4–6 weeks old. These were established as follows: femurs were removed from 8- to 10-week-old BD F_1 mice and flushed into Fischer's medium (Life Technologies, Grand Island, NY), cells were centrifuged and resuspended in Fischer's medium supplemented with 20% horse serum and 1% hydrocortisone succinate (Sigma) to a final concentration of one femur equivalent per 10 ml of medium, and 10-ml aliquots were dispensed into 25-cm^2 tissue culture flasks. Cultures were fed at weekly intervals by demidepopulation. The MS5 cell line (16) was cultured in flasks precoated with 1% gelatin (Sigma) in the same medium used to grow human bone marrow cultures. For adhesion assays,

Abbreviations: hu, human; HEV, high endothelial venule; FITC, fluorescein isothiocyanate; R-PE, R-phycoerythrin; CAM, cell adhesion molecule.

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hemopoietic cells were suspended in α medium (Life Technologies), containing 15% FCS (PAA Laboratories, Durham, U.K.) at a concentration of 1×10^6 cells per ml and incubated on the preformed stromal layers for 2 h at 37°C before washing three times with Hanks' buffered saline solution (HBSS; Life Technologies) to remove nonadherent cells. The latter were centrifuged, resuspended in phosphate-buffered saline (PBS), and counted with a hemocytometer. Preliminary experiments showed that adhesion was maximal at ≈1 h. A 2-h end point was used in all subsequent experiments, as in our previous studies on progenitor cell adhesion to bone marrow stroma (17).

Antibody Experiments. Cells were washed in PBS/0.1% bovine serum albumin (Sigma), centrifuged, and resuspended at 1×10^6 cells per 100 μl. Cells were then incubated with 15 μg each of unlabeled anti-hu-CD34 antibodies, HPCA-2, QBend 10, and Immu 133 (8) or with 45 μg of control mouse IgG1 for 30 min on ice. At the end of this incubation period, 4.75 ml of α-MEM medium supplemented with 15% FCS was added to the cells. The cells were then added to preformed primary human bone marrow stromas or to the MS5 cell line and incubated at 37°C for 2 h. Nonadherent cells were washed off using HBSS, and the cells were counted with a hemocytometer.

RESULTS

Targeting CD34 Expression to T Lymphocytes. Hu-CD34 expression was targeted to the T-cell compartment of transgenic mice using an expression vector containing the CD2 locus control region (18) (Fig. 1*A*). Greater than 95% of CD2-reactive T cells expressed hu-CD34 (Fig. 1*B*) and processed it similarly to human hemopoietic cells (Fig. 1*C*) as judged by reactivity to a panel of monoclonal antibodies whose recognition of CD34 varies according to its modification by carbohydrate. Class I epitopes are sensitive to sialidase (neuraminidase), class II epitopes are destroyed by glycoprotease (*Pasteurella haemolytica*), while class III epitopes are resistant to these enzymes. CD34 antibodies selectively binding to these three classes of epitopes react equivalently with transgenic mouse thymocytes and human KG-1A cells (Fig. 1*C*); KG-1A is a human leukemia cell line considered to represent the lymphomyeloid stem cell compartment (19). We therefore infer that mouse thymocytes process the hu-CD34 molecule similarly to human hemopoietic cells. The ectopic expression of hu-CD34 did not alter either the intrathymic development of $CD3^+$ T cells (Fig. 1*D*) or the relative distribution of thymic T-cell subsets as defined by CD4 and CD8 expression (Fig. 1*E*). Numbers of $CD3^+$ T cells and $CD4^+$ or $CD8^+$ T-cell

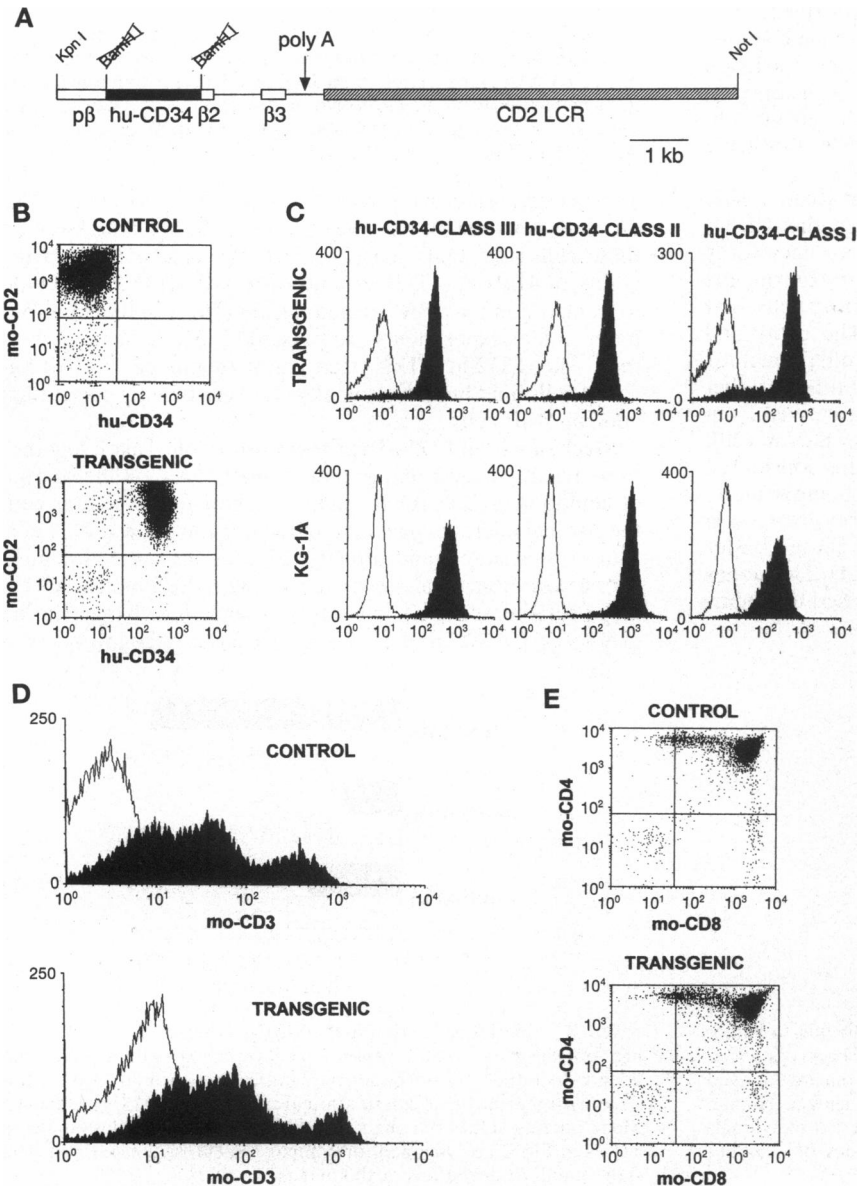

FIG. 1. Targeting hu-CD34 expression to the murine T-cell compartment. (*A*) pCD2-hu-CD34 used for murine transgenesis. A hu-CD34 cDNA (solid box) encoding its own translational initiation and termination codons is transcribed and processed using human β-globin promoter, splice, and polyadenylylation sequences (open boxes); high level T-cell-specific expression is directed by the human CD2 LCR (hatched box). (*B*) Flow cytometric analysis of hu-CD34 expression. Thymocytes were simultaneously labeled with anti-hu-CD34 [FITC-conjugated-HPCA-2(8G12); Becton Dickinson] and anti-mouse CD2 (R-PE-conjugated-RM2-5; Pharmingen) antibodies. Note that nearly all the $CD2^+$ transgenic thymocytes express hu-CD34. (*C*) Post-translational modification analysis of hu-CD34 in transgenic thymocytes. Thymocytes from CD2-huCD34 transgenic mice were labeled with class I (Immu 133-R-PE; Immunotech, Luminy, France), class II (Qbend 10-FITC; Quantum Biosciences, Cambridge, U.K.), and class III (HPCA-2-FITC) anti-hu-CD34 antibodies; CD34 epitopes are classified according to neuraminidase and glycoprotease sensitivity (see text for details). Similar flow cytometric profiles are obtained from CD2-hu-CD34 thymocytes and human hemopoietic progenitor cells (KG-1A). (*D*) Cell surface expression of the CD3 component of the T-cell receptor. Thymocytes from control and transgenic mice display similar flow cytometric profiles when vitally stained with anti-mouse CD3 (biotin-conjugated 145-2C11; PharMingen) antibodies. Antibody labeling was visualized using R-PE-conjugated streptavidin (PharMingen). (*E*) Analysis of thymic T-cell subsets. Transgenic and control thymocytes were simultaneously labeled with anti-mouse CD4 (L3T4-R-PE; PharMingen) and CD8 (53-6.7-FITC; PharMingen). Flow cytometry analysis showed no differences in the relative numbers of double-negative, single-positive, and double-positive thymocytes.

subsets in blood, spleen, and lymph nodes of transgenic mice were also in the normal range (data not shown).

CD34 Promotes Adhesion of Thymocytes to Bone Marrow Stroma. We next examined the potential involvement of CD34 in the adherence of hemopoietic cells within the stromal microenvironment of the bone marrow by testing whether the expression of human CD34 by murine thymocytes could alter their adherence to bone marrow stromal layers *in vitro*. First, we cocultured known numbers of thymocytes from transgenic or control animals with stromal layers prepared from normal human bone marrow. Nonadherent thymocytes were recovered by sequential washing of the stromal layers and the percentages of adherent vs. nonadherent thymocytes were determined. The results obtained from six independent experiments using stromal layers derived from different individuals are presented in Fig. 2; the percentages of thymocytes bound to human bone marrow stroma are considerably higher in the transgenic animals (42–58%; mean, 50%; SD, 6%) than in the nontransgenic littermates (4–30%; mean, 13%; SD, 10%). Hu-CD34 transgenic thymocytes adhere predominantly to fibroblast-like stromal cells, whereas most of the adhesion observed with control thymocytes is instead to large blanket-like cells (Fig. 3, arrow); the frequency of these cells in different stromal layers varies considerably and may underlie the variation in background levels of adhesion observed with control thymocytes. Note that hu-CD34 transgenic thymocytes do not exhibit any enhanced adhesion to murine bone marrow-derived stromal cells (Fig. 4); this apparent species restriction in the ability of hu-CD34 to recognize its cognate cell adhesion molecule (CAM) ligand presumably explains the normal development and distribution of T cells in hu-CD34 transgenic mice.

CD34-Dependent Adhesion of Multipotential Hemopoietic Progenitor Cells. We next examined whether the CD34-dependent stromal adhesion we observed was necessarily dependent on the T-cell background used in our experiments or was also a feature of hemopoietic progenitor cells that characteristically express the CD34 antigen. The rarity and heterogeneity of normal hemopoietic stem and progenitor cells preclude their straightforward use in quantitative adhesion assays. The murine cell line 416B has been derived from long-term cultures of virally infected mouse bone marrow (20), expresses the murine CD34 antigen (4, 15), and is considered representative of the multimyeloid progenitor compartment. We transfected murine 416B cells with a eukaryotic expression vector in which the hu-CD34 cDNA had been placed under control of the metallothionein promoter (Fig. 5*A*). The clones obtained (416B-hu-CD34) constitutively expressed appropriately posttranslationally modified hu-CD34 molecules (Fig. 5*B*; data not shown); the level of murine CD34 expression on these cells was unaltered (Fig. 5*B*). We next compared the ability of 416B-hu-CD34 vs. untransfected 416B cells to bind to human bone marrow stromal layers. The results from four independent experiments are presented in Fig. 5*C* and show that, like CD2-hu-CD34 transgenic thymocytes, 416B-hu-CD34 cells bind human stromal layers significantly better than control 416B cells.

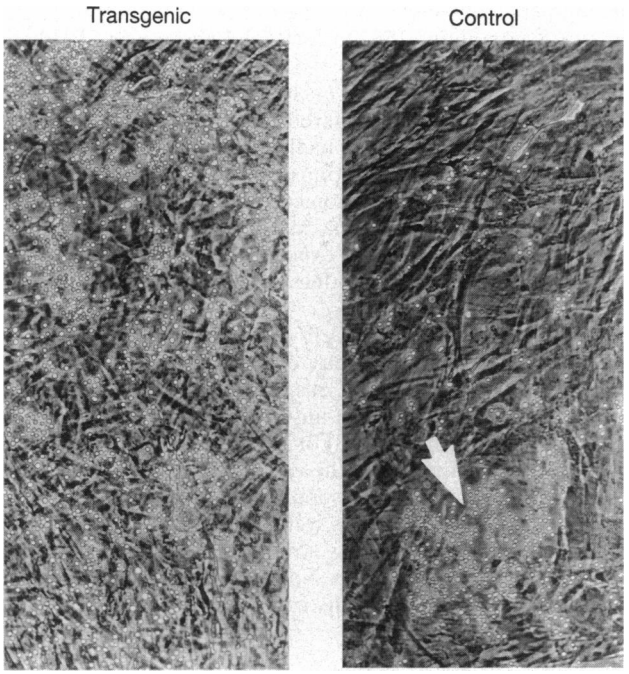

FIG. 3. Photomicrographs of transgenic and control thymocytes (small bright cells) adhered to human bone marrow stroma (fibroblast-like cells). Visual inspection of stromal layers reveals microheterogeneity in thymocyte binding in both transgenic and control experiments; fields shown are typical of the general patterns in binding observed. Arrow identifies a large blanket cell.

Mechanisms of CD34-Dependent Adhesion. Taken together these results suggest that the function of CD34 on the surface of hemopoietic cells relates to their adhesive interactions with the stromal microenvironment of the bone marrow. CD34 may achieve this simply and directly (Fig. 6*A*, model 1) by interacting with a stromal-associated CAM. Alternatively, CD34 may promote adhesion (model 2) by signaling changes in the profile of CAMs at the cell surface after recognition of a

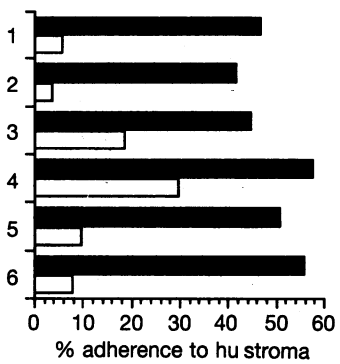

FIG. 2. Adherence of transgenic and control thymocytes to hemopoietic stromal layers. Percentages of transgenic (solid bars) or control (open bars) thymocytes that adhere to human bone marrow-derived stromal layers in six independent experiments are shown. Human stromal layers used in each experiment are from different individuals and results were obtained with two independent lines of CD2-hu-CD34 transgenic mice.

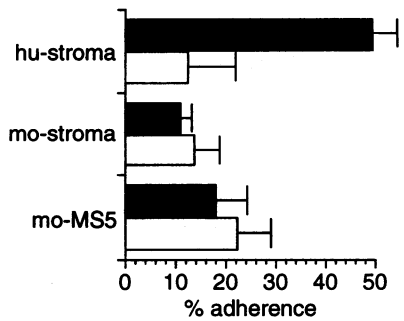

FIG. 4. Species restriction in CD34-dependent adhesion. Percentages of transgenic (solid bars) or control (open bars) thymocytes that adhere to either mouse bone marrow-derived stromal layers (mo-stroma) or murine hemopoietic stromal cell lines (mo-MS5) are shown. Mean binding values obtained in the human stromal adhesion assays shown in Fig. 2 are included for comparison (hu-stroma). Error bars show standard deviations of the means.

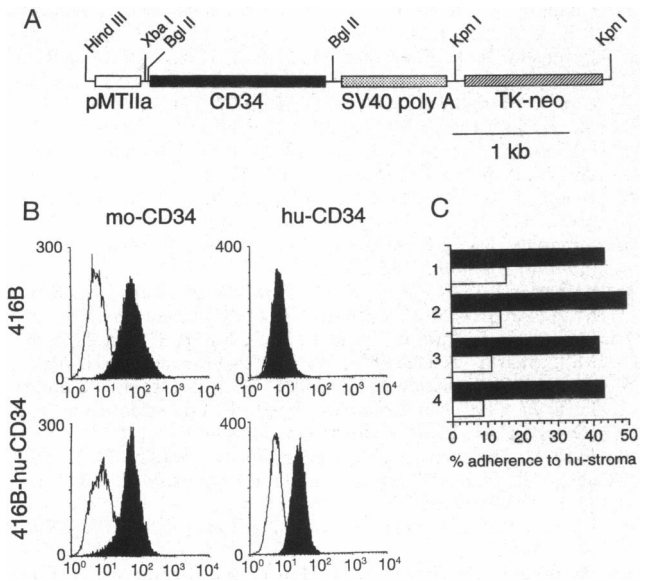

FIG. 5. Ectopic expression of hu-CD34 in murine hemopoietic progenitors. (*A*) pMT-hu-CD34. The hu-CD34 cDNA (see Fig. 1) was placed under the control of the human metallothionein IIa promoter (open box) and simian virus 40 (SV40) splice and polyadenylylation (polyA) signals (stippled box). Drug resistance was provided by a TKneo cassette (shaded box). (*B*) Flow cytometric analysis. Transfected and control 416B cells were labeled indirectly with an anti-mouse CD34 rabbit polyclonal antibody (*Left*) or an anti-hu-CD34 antibody (HPCA-2-FITC) (*Right*). Note that expression of murine CD34 was unaffected in the 416B-hu-CD34 cells. (*C*) Adherence to human bone marrow stroma. Percentages of 416B-hu-CD34 (solid bars) and control 416B (open bars) cells that adhere are shown.

stromally associated CD34 ligand or receptor. We have investigated these possibilities by analyzing the effect of anti-CD34 antibodies on the binding capacity of transgenic thymocytes. The results presented in Fig. 6*B* show that pretreatment of transgenic mouse thymocytes with a mixture of class 1, 2, and 3 anti-hu-CD34 antibodies does not abrogate their binding capacity but instead significantly increases it; increased binding was not observed when cells were pretreated with isotype-matched control antibodies. Increased adhesion was also observed when hu-CD34-transfected 416B cells were treated with anti-hu-CD34 antibodies (data not shown). In this regard, it is interesting to note that the homotypic adhesion of CD34-expressing KG-1 cells has recently been reported to also increase as a result of the engagement of anti-CD34 antibodies (21, 22). Taken together, our results are most easily reconciled with the second of the two models and raise the possibility that the stromal adhesion observed in transgenic thymocytes is mediated by modulation of murine CAMs in response to CD34-mediated signaling. These murine CAMs should presumably facilitate binding to mouse as well as human stroma, and we therefore asked whether hu-CD34 transgenic thymocytes, pretreated with anti-hu-CD34 antibodies, had acquired the ability to bind to mouse stromal layers. The results presented in Fig. 6*C* show that antibody treatment causes an increase in binding of transgenic thymocytes to murine stroma, in accord with our hypothesis.

DISCUSSION

Adherence of hemopoietic stem cells and progenitors to the stromal cell bone marrow microenvironment is considered to be a key component of the developmental sequestration and regulation of these cells (23–25). Several different molecules are likely to be involved in what is probably a multicomponent or cascading adhesive interaction. The selective expression of CD34 on the surface of hemopoietic progenitors has led to the speculation that it may be involved in this process, although, paradoxically, most speculation has centered around the notion that stem cell CD34 functions as an antiadhesion molecule. This hypothesis has stemmed from both the rapid downregulation of CD34 that accompanies the cytokine-mediated upregulation of vascular endothelial adhesion molecules *in vitro* (26) and *in vivo* (27) and from the structural and biochemical features of the N terminus of the CD34 molecule, which is extensively glycosylated with both N- and O-linked carbohydrates including sialic acid [reviewed in ref. 9]. This region is therefore predicted to adopt the conformation of an extended rod, protruding a considerable distance above the cell membrane and presenting a net negative charge. By analogy with N-CAM (28) and CD43/leukosialin (29), a hemopoietic sialomucin with some structural similarities to CD34, a cellular halo of sialic acid-dependent negative charge was postulated to function as a barrier to cell–cell interaction (26). In such a scheme, CD34 function is presumed to be primarily dependent on the overall conformation and charge of the N terminus rather than its precise amino acid sequence.

Adhesive functions of molecules are likely to be very context dependent and our results demonstrate a pro adhesive role for the CD34 sialoglycoprotein in the interaction of hemopoietic and stromal cells. Furthermore, our observation that ectopic expression of the hu-CD34 molecule confers increased binding to human stromal cells and cell lines but not to their murine counterparts implies that the adhesive function of CD34 is not simply attributable to the negatively charged rod-like conformation of its N terminus but rather involves specific recognition of the CD34 molecule, presumably by a stromally associated counterreceptor or ligand. A likely explanation for the

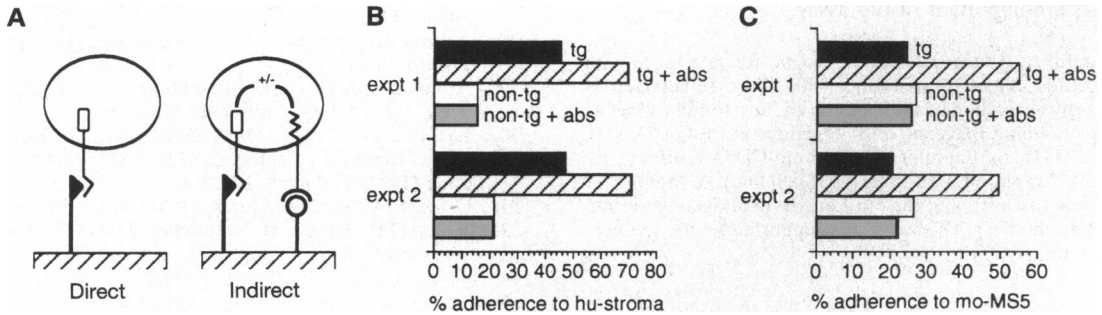

FIG. 6. Models for CD34-mediated cellular adhesion. (*A*) (*Left*) Model 1: CD34 functions simply and directly as an avid CAM. (*Right*) Model 2: adhesion is promoted via modulations in the cell surface profile of murine adhesion molecules (either upregulation of adhesive CAMs or downregulation of antiadhesion molecules) in response to hu-CD34-mediated intracellular signaling; the initial CD34 ligand–receptor interaction may itself be an adhesive interaction. (*B*) Effect of anti-hu-CD34 antibodies (abs) on adherence of transgenic (tg) and control (non-tg) thymocytes to human bone marrow stroma. A mixture of class I, II, and III anti-hu-CD34 antibodies was used and the results of two independent experiments are shown. (*C*) Effect of anti-hu-CD34 antibody mixture on adherence of thymocytes to murine hemopoietic stromal cell line MS5.

failure of hu-CD34 to increase binding of hemopoietic cells to murine stroma is that the hu-CD34 molecule is unable to recognize the murine CD34 counterreceptor ligand. This would provide a plausible explanation for the apparent normality of the T-cell compartment in CD2-hu-CD34 transgenic mice and would be consistent with the observation that hu-CD34 and mouse CD34 diverge the most in the N-terminal portions of their extracellular domains where they share only 45% amino acid identity (4, 5). Our observation that engagement of the hu-CD34 molecule by anti-CD34 antibodies facilitates the binding of hemopoietic cells to bone marrow-derived stromal layers also implicates molecular recognition of the extracellular domain of CD34 as a critical component of CD34-dependent adhesion. The fact that anti-hu-CD34 antibodies enhance the ability of hu-CD34-expressing murine hemopoietic cells to bind to murine stromal layers (in the absence of antibody addition, these cells show no increased binding relative to non-hu-CD34-expressing controls) leads us to speculate that CD34 promotes adhesion in this context by signaling changes in the cell surface profile of murine CAMs in response to molecular recognition of its extracellular domain. The high degree of evolutionary conservation in the intracellular domains of hu-CD34 and mouse CD34 (90% amino acid identity) (4, 5) as well as recently reported experimental evidence that anti-CD34 antibodies promote the homotypic adhesion of the CD34-expressing KG-1 cells (22, 23) also support a role for CD34 in adhesive signaling. However, CD34 molecules may also possess direct CAM-like activity since preliminary experiments suggest that CD34 lacking an intracellular C-terminal tail still facilitates adhesion to stroma (L.H. and G.M., unpublished observations).

Identifying the molecule in human bone marrow stroma that is normally recognized by CD34 may suggest strategies for stem cell manipulation and mobilization. Some biochemical evidence has indicated that L-selectin can bind sulfated CD34 derived from HEVs (11), suggesting that leukocyte adherence to, and/or migration across, HEVs might involve CD34 as well as the major L-selectin ligand on HEVs provided by Sgp50 (Gly CAM1) (30). Several lines of evidence rule out L-selectin as the likely bone marrow stromal ligand for CD34. Firstly, L-selectin does not bind to hemopoietic CD34 on KG-1 cells (21, 31). Secondly, the antibody MECA79 recognizes a sulfated carbohydrate on L-selectin ligands and blocks binding (32), but we find that it does not influence the adhesion of hu-CD34 transgene-expressing murine cells to human bone marrow stroma (L.H., unpublished observation). Finally, CD34 molecules on HEVs appear to be glycosylated differently from those on vascular and hemopoietic cells (8, 10). These differences in carbohydrate modification may well influence ligand recognition, in which case glycosylation-dependent recognition would have to be accommodated in any strategy for cloning the CD34 counterreceptor which uses CD34 itself as a component of the assay.

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