Surface fucosylation of human cord blood cells augments binding to P-selectin and E-selectin and enhances engraftment in bone marrow

Lijun Xia, J. Michael McDaniel, Tadayuki Yago, Andrea Doeden, and Rodger P. McEver

Mouse hematopoietic stem and progenitor cells (HSPCs) home to bone marrow in part by rolling on P-selectin and E-selectin expressed on endothelial cells. Human adult CD34+ cells, which are enriched in HSPCs, roll on endothelial selectins in bone marrow vessels of nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice. Many human umbilical cord blood (CB) CD34+ cells do not roll in these vessels, in part because of an uncharacterized defect in binding to P-selectin. Selectin ligands must be α1-3 fucosylated to form glycan determinants such as sialyl Lewis x (sLex). We found that inadequate α1-3 fucosylation of CB CD34+ cells, particularly CD34+CD38low cells that are highly enriched in HSPCs, caused them to bind poorly to E-selectin as well as to P-selectin. Treatment of CB CD34+ cells with guanosine diphosphate (GDP) fucose and exogenous α1-3 fucosyltransferase VI increased cell-surface sLex determinants, augmented binding to fluid-phase P- and E-selectin, and improved cell rolling on P- and E-selectin under flow. Similar treatment of CB mononuclear cells enhanced engraftment of human hematopoietic cells in bone marrows of irradiated NOD/SCID mice. These observations suggest that α1-3 fucosylation of CB cells might be a simple and effective method to improve hematopoietic cell homing to and engraftment in bone marrows of patients receiving CB transplants. (Blood. 2004;104:3091-3096)

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Introduction

When expressed on activated endothelial cells, P-selectin and E-selectin cooperatively mediate leukocyte rolling, the first step in leukocyte recruitment into sites of inflammation.1-3 P- and E-selectin are membrane-bound C-type lectins that bind to cell-surface glycoconjugate ligands. Physiologically relevant selectin ligands must be α1-3 fucosylated to form terminal glycan determinants such as sialyl Lewis x (sLeα). We found that inadequate α1-3 fucosylation of CB CD34+ cells, particularly CD34+CD38low cells that are highly enriched in HSPCs, caused them to bind poorly to E-selectin as well as to P-selectin. Treatment of CB CD34+ cells with guanosine diphosphate (GDP) fucose and exogenous α1-3 fucosyltransferase VI increased cell-surface sLeα determinants, augmented binding to fluid-phase P- and E-selectin, and improved cell rolling on P- and E-selectin under flow. Similar treatment of CB mononuclear cells enhanced engraftment of human hematopoietic cells in bone marrows of irradiated NOD/SCID mice. These observations suggest that α1-3 fucosylation of CB cells might be a simple and effective method to improve hematopoietic cell homing to and engraftment in bone marrows of patients receiving CB transplants. (Blood. 2004;104:3091-3096)

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marrow of NOD/SCID mice that lack P- and E-selectin and-by diminution rolling in bone marrow microvessels of mice after injection of blocking monoclonal antibodies (mAbs) to P-selectin or PSGL-1.14 A subset (~30%) of CB CD34+ cells does not bind to P-selectin, and many of these are CD34+/CD38−/low cells that constitute an enriched population of multipotent hematopoietic stem cells.14 These data imply that diminished homing of CB cells might result in part from defective interactions of CD34+ cells with P-selectin in bone marrow vessels. CD34+ CB cells express the PSGL-1 polypeptide, suggesting that defective posttranslational modification of PSGL-1 might account for defective binding to P-selectin.14 However, the putative defect in posttranslational modification has not been identified. It is also not known whether CB CD34+ cells have defective binding to E-selectin, which might further impair homing to bone marrow.

Compared with bone marrow or mobilized peripheral blood, CB cells have a higher potential to reconstitute recipient bone marrow because they contain 3 to 6 times more repopulating cells, a small subset of cells critical for long-term engraftment.15 Despite this advantage, the small number of cells obtained from each umbilical cord limits the utility of CB as a source of hematopoietic cells for transplantation. Correction of the homing defect in CB cells might overcome this limitation. In this study, we demonstrate that inadequate α1-3 fucosylation of CB CD34+ cells caused defective binding to E-selectin as well as to P-selectin. Transient fucosylation of CB cells with guanosine diphosphate (GDP) fucose and exogenous α1-3 fucosyltransferase increased binding to both P- and E-selectin, particularly to E-selectin, and enhanced engraftment in bone marrows of irradiated NOD/SCID mice.

Materials and methods

CB cells

CB was obtained from umbilical cords from healthy full-term newborns. Informed consent was obtained according to a protocol approved by the Institutional Review Board of the Oklahoma Medical Research Foundation. Sodium citrate or heparin was used as anticoagulant. CB was first mixed with an equal volume of 6% dextran 70 in 0.9% sodium chloride (McGraw, Irvine, CA). After sedimentation, nucleated cells were collected and washed once in Hanks balanced salt solution (HBSS; Mediatech, Herndon, VA). Sodium citrate or heparin was used as anticoagulant. CB was first mixed with fluorescein isothiocyanate (FITC)–labeled goat anti–rat IgM (BD Biosciences). Data were collected and analyzed using the CellQuest program (BD Biosciences).

Surface fucosylation

To introduce α1-3–linked fucose to cell-surface glycans, 10^6 CB mononuclear cells were treated with 1 mM GDP fucose (EMD Biosciences, San Diego, CA), 20 mM/L α1-3 fucosyltransferase VI (FTVI; EMD Biosciences), and 10 mM MnCl2 in 0.5 mL HBSS containing 1% human serum albumin (HSA) for 30 minutes at 37°C in a humidified atmosphere containing 5% CO2.16 These conditions resulted in minimum toxicity to CD34+ cells as tested by propidium iodide staining measured by flow cytometry (data not shown). Sham-treated cells were treated identically except that GDP fucose was not added.

Flow cytometry

For all flow cytometry analyses, Fc receptors on CB cells were first blocked with human immunoglobulin G (IgG; BD Biosciences, San Diego, CA).

To measure sLex determinants, CB mononuclear cells were incubated with phycoerythrin (PE)–conjugated anti-human CD34 mAb or control murine IgG (IgG1; BD Biosciences) and with rat anti-sLex mAb HECA-452 (IgM; hybridoma from American Type Culture Collection, Manassas, VA)17 or control rat IgM (BD Biosciences). Bound HECA-452 was detected with fluorescein isothiocyanate (FITC)–labeled goat anti–rat IgM (EMD Biosciences).

For the P-selectin–binding assay, CB mononuclear cells were incubated with human P-selectin/human IgG chimera (P-selectin/IgG), which was purified from conditioned medium of stably transfected human 293 cells (a generous gift from Dr Ajit Varki, University of California at San Diego). The bound P-selectin was then detected by biotinylated protein A and streptavidin-FITC (BD Biosciences).14 Incubations were performed for 20 minutes at 4°C for each step. In some experiments, P-selectin isolated from human platelets was used.18 P-selectin binding was then detected with FITC-labeled S12, a nonblocking mAb to human P-selectin.19 A saturating amount of P-selectin, determined by measuring binding over a range of P-selectin concentrations, was used in all experiments. In control experiments, binding was measured in the presence of G1, a blocking mAb to P-selectin20; PL1, a blocking mAb to PSGL-1;21 or 10 mM EDTA (ethylenediaminetetraacetic acid), which eliminates Ca2+–dependent selectin–ligand interactions. For E-selectin binding, murine soluble E-selectin/human IgM chimera (E-selectin/IgM) was used.3 CD34+ human IgM chimera (CD34/5fGm) was used as negative control. The chimeras were obtained from conditioned medium of COS-7 cells that were transfected, respectively, with CD38 vectors encoding each molecule (generous gifts from Dr John B. Lowe, University of Michigan Medical Center, Ann Arbor). E-selectin binding was detected with FITC-labeled goat anti–human IgM (Chemicon International, Temecula, CA). Incubations were performed at 4°C for 30 minutes. A saturating amount of E-selectin, determined by serial titration, was used in each experiment. In control experiments, binding was conducted in the presence of 9A9, a blocking mAb to murine E-selectin22 (a generous gift from Dr Barry Wolitzky, Roche Research Center, Hoffmann-La Roche, Basel, Switzerland), or 10 mM EDTA.

To triple-colored staining, CB mononuclear cells were incubated with PE–cy5–conjugated anti-CD34 mAb with one of the following combinations: anti-sLex mAb HECA-452 followed by either PE–conjugated anti-CD38 mAb or PE–cyanine 5 (PE-Cy5)–conjugated anti-CD34 mAb with one of the following combinations: anti-sLex mAb HECA-452 followed by either PE–conjugated anti-CD38 mAb or PE-Cy5–conjugated anti-CD34 mAb with one of the following combinations: anti-sLex mAb HECA-452 followed by either PE–conjugated anti-CD38 mAb or PE-Cy5–conjugated anti-CD34 mAb with one of the following combinations: anti-sLex mAb HECA-452 followed by either PE–conjugated anti-CD38 mAb or PE-Cy5–conjugated anti-CD34 mAb with one of the following combinations: anti-sLex mAb HECA-452 followed by either PE–conjugated anti-CD38 mAb or PE-Cy5–conjugated anti-CD34 mAb.
saline without CB mononuclear cells. Six weeks after transplantation, the mice were bled and killed. Bone marrow cells were isolated from both femora and filtered through a 100-μm mesh cell strainer to remove debris. After lysis of erythrocytes, the bone marrow nucleated cells from each mouse were resuspended in HBSS at a concentration of 1 × 10^7/mL. Engraftment of human cells was analyzed by human hematopoietic progenitor assays and by flow cytometry.

For assays of human clonogenic hematopoietic progenitor cells, 1 × 10^6 bone marrow cells were plated in duplicate on 35-mm culture dishes in MethoCult H4433 medium (Stem Cell Technologies, Vancouver, BC, Canada) and incubated at 37°C, 5% CO2 in a humidified chamber. This medium only supports the growth of human hematopoietic cells. Total colonies, erythroid burst-forming units (BFU-Es), granulocyte-macrophage colony-forming units (CFU-GMs), and granulocyte-erythroid-megakaryocyte-macrophage colony-forming units (CFU-GEMMs) were enumerated on day 14 of culture and analyzed. The human origin of the colonies was confirmed by flow cytometry of cells collected from different colonies that were stained with FITC-conjugated mAb to human CD45 (BD Biosciences) to identify leukocytes or with FITC-conjugated mAb to glycophorin A (BD Biosciences) to identify erythroid cells.

Flow cytometry, equal numbers of nucleated cells from peripheral blood or bone marrow were incubated with FITC-conjugated anti-human CD45 mAb and analyzed for the percentages of human CB-derived leukocytes.

**Results**

**CB CD34+ cells that lack SLex epitopes bind poorly to both P-selectin and E-selectin**

We asked whether CB CD34+ cells might have impaired binding to E-selectin as well as P-selectin and whether defective selectin binding might correlate with inadequate α-1,3 fucosylation of cell-surface ligands. Flow cytometric analysis demonstrated that approximately 25% of CB CD34+ cells did not express SLex as detected by binding of anti-SLex mAb HECA-452 (Figure 1A). Triple-colored staining showed that 88% ± 2% (n = 3) of CB CD34+ cells without SLex did not bind to P-selectin, whereas only 35% ± 11% of CB CD34+ cells with SLex failed to bind to P-selectin (Figure 1B). Significantly, 67% ± 9% of CB CD34+ cells without SLex also did not bind to E-selectin, whereas only 10% ± 5% of CB CD34+ cells with SLex failed to bind to E-selectin (Figure 1C). Consistent with published data, approximately 30% of CB CD34+ cells were CD34+CD38low, which are enriched in HSPCs (Figure 1D). Triple-colored staining revealed that 76% ± 12% of CB CD34+CD38low cells did not bind to P-selectin (Figure 1E), and that 74% ± 13% of CB CD34+CD38low cells did not bind to E-selectin (Figure 1F). Furthermore, 63% ± 9% of CB CD34+CD38low cells did not express SLex (data not shown). These data demonstrate that most CB CD34+CD38low cells lack SLex epitopes and fail to bind to E-selectin as well as to P-selectin. Thus, low SLex expression strongly correlates with defective selectin binding, suggesting a causal relationship.

**Transient fucosylation of CB CD34+ cell surfaces augments binding to fluid-phase P-selectin and E-selectin and enhances rolling on P-selectin and E-selectin**

We previously developed a technique to add SLex epitopes to cell surfaces by treating intact cells with GDP fucose and an exogenous α-1,3 fucosyltransferase, FTVI.16 This short-term treatment transiently increases cell-surface fucosylated glycan, which then decline as glycoproteins and glycolipids turn over and as cells divide. Using the same method, we tested whether forced fucosylation would increase functional selectin ligands on CB CD34+ cells. Treatment with both FTVI and GDP fucose created many new SLex epitopes on CB CD34+ cells, as measured by binding of HECA-452 (Figure 2).

Compared with sham-treated CB CD34+ cells, more FTVI-treated CB CD34+ cells bound to P- and E-selectin, and they expressed more binding sites, especially for E-selectin (Figure 3). Binding of P-selectin and E-selectin was specific because it was eliminated by addition of a blocking mAb to the respective selectin (Figure 3) or by EDTA (data not shown). Incubation with control CD45/IgM yielded the same staining profile as with cells incubated with E-selectin/IgM in the presence of EDTA or anti–E-selectin mAb, further demonstrating specificity (data not shown). These results demonstrate that forced fucosylation of CB CD34+ cells increases binding to P-selectin and markedly increases binding to E-selectin.

To determine whether forced fucosylation of CB CD34+ cells enhanced interactions with selectins in a more physiologically relevant setting, we compared rolling of FTVI-treated and sham-treated cells on immobilized human P- or E-selectin under flow conditions. FTVI treatment increased the number of cells rolling on P-selectin by 1.9-fold (Figure 4A) and on E-selectin by 2.9-fold.
No CD45<sup>+</sup> cells were detected in marrow or blood of mice that received only control saline, documenting the specificity of the mAb for human CD45 (Figure 5B).

**Discussion**

We have demonstrated that insufficient α1-3 fucosylation of selectin ligands on CB CD34<sup>+</sup> cells causes defective binding to both P-selectin and E-selectin. Transient α1-3 fucosylation of CB cells increases selectin binding and augments engraftment of CB cells in bone marrows of irradiated NOD/SCID mice. These findings suggest that in vitro fucosylation of CB cells might provide a simple method to improve hematopoietic cell homing to and engraftment in bone marrows of patients receiving CB transplants.

We observed a strong correlation between impaired selectin binding and low or absent expression of sLe<sup>x</sup> determinants on CB CD34<sup>+</sup> cells. This was particularly true for CD34<sup>+</sup>CD38<sup>−</sup>CD90<sup>+</sup> cells, which are significantly enriched in multipotent stem cells. Most of these cells lacked sLe<sup>x</sup> determinants, and approximately 75% of them failed to bind to P-selectin and E-selectin. Forced fucosylation of CD34<sup>+</sup> cells with exogenous GDP fucose and FTVI increased expression of sLe<sup>x</sup> and selectin ligands in parallel. The enzymes FTVI and FTVII are responsible for α1-3 fucosyltransferase activity in hematopoietic cells of mice and humans.26,31 CD34<sup>+</sup> cells may not express sufficient levels of these enzymes to fucosylate physiologically relevant selectin ligands or to express measurable levels of sLe<sup>x</sup> epitopes on the cell surface. There is also a correlation between expression of sLe<sup>x</sup> determinants and selectin ligands on mature human leukocytes, particularly on T cells, which alter expression of FTVII to regulate the parallel display of selectin ligands and sLe<sup>x</sup> epitopes.29,32 Thus, expression of sLe<sup>x</sup> determinants is a useful tool to assess the fucosylation of selectin ligands on human hematopoietic cells.

Fucosylation of CB CD34<sup>+</sup> cells augmented binding to P-selectin in a PSGL-1–dependent manner. This is consistent with previous observations that CB CD34<sup>+</sup> cells express a form of PSGL-1 that does not bind to P-selectin.14 The current data suggest that this defective binding is due to inadequate fucosylation of PSGL-1, most likely at an essential core 2 O-glycan linked to a specific threonine in the N-terminal PSGL-1–binding site.2,3 PL1, a mAb to the P-selectin–binding region of PSGL-1, did not inhibit fucosylation-augmented binding of CB CD34<sup>+</sup> cells to fluid-phase P-selectin and E-selectin under flow.

**Surface fucosylation of CB mononuclear cells enhances engraftment in bone marrow of NOD/SCID mice**

To examine whether transient surface fucosylation of CB cells improves engraftment in bone marrow, sublethally irradiated NOD/SCID mice received transplants of control saline or of FTVI-treated or sham-treated CB mononuclear cells. After 6 weeks, the mice were killed, and the number of human-derived hematopoietic cells in bone marrow and peripheral blood was analyzed. The bone marrows of mice that received FTVI-treated CB mononuclear cells had significantly improved engraftment of human hematopoietic progenitors of all lineages, as demonstrated by increases in BFU-Es, CFU-GMs, and CFU-GEMMs in colony-forming assays (Figure 5A). No colonies formed in bone marrow from irradiated mice that received only control saline, confirming the specificity of the colony-forming assay for human cells (Figure 5A). Of note, the sizes of the colonies were not significantly different in either recipient group (data not shown), indicating that short-term fucosylation did not change the growth rates of the CB hematopoietic cells. Consistent with the increased numbers of engrafted human cells, the mice that received FTVI-treated CB mononuclear cells had significantly more human CD45<sup>+</sup> cells in bone marrow and peripheral blood than mice that received sham-treated CB mononuclear cells (Figure 5B).

**Figure 4. Surface α1-3 fucosylation of CB CD34<sup>+</sup> cells augments rolling on P-selectin and E-selectin under flow.** (A) Sham-treated or FTVI-treated CB CD34<sup>+</sup> cells were perfused over immobilized human P-selectin or over a control surface coated only with HSA in the presence or absence of a blocking mAb to P-selectin (G1) or PSGL-1 (PL1). (B) Sham-treated or FTVI-treated CB CD34<sup>+</sup> cells were perfused over immobilized human E-selectin or over a control surface coated only with HSA in the presence or absence of a blocking mAb to E-selectin (ES1) or PSGL-1 (PL1). The wall shear stress was 0.5 dyne/cm<sup>2</sup>. After 4 minutes, the number of rolling cells was quantified. The data represent the mean ± SEM of 3 independent experiments.
E-selectin (L.X., J.M.M., and R.P.M, unpublished observation, February 2004). This suggests that surface fucosylation created additional binding sites for E-selectin on other regions of PSGL-1 or on other glycoproteins or glycolipids. Indeed, surface fucosylation created many more new binding sites for E-selectin than for P-selectin as measured by flow cytometry. Forced fucosylation increased physiologically relevant rolling of CB CD34+ cells on both P-selectin and E-selectin under flow. Rolling was particularly improved on E-selectin, consistent with the large increase in E-selectin ligands. PLI did not inhibit rolling on E-selectin, further implying that fucosylation created binding sites for E-selectin on glycoproteins other than PSGL-1. Although we did not determine whether multipotent stem cells actually rolled on selectins, fucosylation enabled virtually all CD34+ cells, including the CD34+CD38low cells, to bind to fluid-phase P-selectin and E-selectin. This suggests that fucosylation also improved the ability of CB HSPCs to roll on P-selectin and E-selectin.

Like recruitment of mature leukocytes to sites of inflammation, homing of HSPCs to bone marrow requires the combinatorial engagements of selectins, integrins, and chemokines with their respective receptors.5,9,33 These collective interactions enable HSPCs to roll and arrest on vessel surfaces and then emigrate into the bone marrow. Forced fucosylation of CB HSPCs might improve rolling on selectins but not increase entry into the bone marrow if the cells also lack integrins, chemokine receptors, or other molecules essential for homing. In a recent preliminary description, a subset of CB CD34+ cells was independently found to express few or no sLea determinants, in agreement with our findings.35 These workers demonstrated that forced fucosylation of CB CD34+ cells augmented rolling in bone marrow vessels of NOD/SCID mice, in agreement with our observations of enhanced rolling of treated cells on P-selectin and E-selectin in vitro. Furthermore, more treated cells transitioned from rolling adhesion to firm adhesion on bone marrow vessels, an important prerequisite for transendothelial emigration.35 Importantly, we observed that fucosylation of CB mononuclear cells significantly increased engraftment of human cells in bone marrows of irradiated NOD/SCID mice 6 weeks after transplantation, demonstrating that human hematopoietic cells could home to and function in the bone marrow microenvironment. The primitive human cells that proliferate and differentiate into multiple hematopoietic lineages in murine bone marrow are called SCID-repopulating cells (SRCs).15 SRCs are concentrated among CB CD34+ cells, especially the CD34+CD38− subpopulation, which supports the possibility that in vitro fucosylation of CB mononuclear cells enhances engraftment by improving selectin-dependent homing of CD34+ SRCs to bone marrow. However, human CD34+ cells have some SRCs as well as accessory cells that support survival and expansion of primitive hematopoietic cells.36-38 In addition, some human lineage-committed progenitors might contribute to hematopoietic recovery 6 weeks after transplantation, the end point studied in our experiments. Thus, further studies are required to determine the specific mechanisms by which in vitro fucosylation of unfractionated CB mononuclear cells improves engraftment.

In vitro, adhesion of human adult CD34+ cells to high densities of P-selectin or E-selectin induces growth inhibition and apoptosis.39,40 Although the physiologic relevance of these observations is unclear, our data suggest that surface fucosylation of CB cells did not adversely affect their ability to repopulate the bone marrow space after homing. In any event, short-term biochemical treatment with exogenous GDP fucose and FTVI only transiently increases fucosylated glycans on CB cells, which decline as glycoproteins and glycolipids turn over and as cells divide. Because the increased fucosylation is transient, it is less likely to affect the long-term functions of hematopoietic stem cells and accessory cells after they enter the bone marrows of conditioned recipients.

It is unclear whether P-selectin and E-selectin make equivalent contributions to HSPC homing to bone marrow of mice and humans. Murine hematopoietic cell lines roll cooperatively on both P- and E-selectin in murine bone marrow vessels.7 However, E-selectin appears to be used more than P-selectin for the actual bone marrow homing of murine adult hematopoietic cells.8 Adult human CB CD34+ cells apparently roll on both P- and E-selectin in bone marrow vessels of irradiated NOD/SCID mice, although the relative contributions of each selectin were not specifically tested.14 Defective P-selectin interactions account for most of the diminished rolling of CB CD34+ cells in NOD/SCID bone marrow vessels.14 Although the specific contributions of P- and E-selectin to homing of CB CD34+ cells to NOD/SCID bone marrow have not been examined, forced fucosylation of these cells might increase homing through more effective rolling interactions with P-selectin and especially with E-selectin in bone marrow vessels, as we observed with immobilized human P- and E-selectin in vitro. Human bone marrow endothelial cells constitutively express E-selectin and, like mice, they may constitutively express P-selectin.41 Thus, surface fucosylation of CB cells might also augment homing to and engraftment in human bone marrow.

CB is now a standard source of cells for pediatric hematopoietic cell transplantation.11,42 Unfortunately, the limited cell number per CB unit and the delayed engraftment after CB cell transplantation preclude wide use of CB as a donor source for adult recipients. Methods to increase cell dose by pooling multiple CB units and by ex vivo cell expansion are being pursued.11 However, multiple-unit transplantations may complicate matching of HLA antigens, and ex vivo cell expansion may risk altering the intrinsic properties of the HSPCs. In vitro fucosylation of CB cells might provide a simple and effective means to enhance hematopoietic cell homing to and engraftment in bone marrows of both pediatric and adult patients. Furthermore, it might significantly reduce the number of CB cells that must be administered, potentially expanding the utility of CB transplantations for adult recipients.
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