Fibronectin Improves Transduction of Reconstituting Hematopoietic Stem Cells by Retroviral Vectors: Evidence of Direct Viral Binding to Chymotryptic Carboxy-Terminal Fragments

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Efficient transduction of reconstituting hematopoietic stem cells (HSC) is currently only possible by cocultivation of target cells directly on producer cell lines, a method not applicable to human gene therapy protocols. Our laboratory has previously shown adhesion of primitive hematopoietic stem cells and progenitor cells to the carboxy-terminal 30/35-kD fragment of the extracellular matrix molecule fibronectin (FN 30/35) (Nature 352:438, 1991) and increased transduction of human hematopoietic progenitor cells via retroviral vectors while adherent to this fragment (J Clin Invest 93:1451, 1994). Here we report that (1) transduction of reconstituting murine HSC assayed 12 months after infection with retrovirus supernatant on FN 30/35 is as effective as cocultivation directly on producer cells; (2) recombinant retrovirus particles directly adhere to FN 30/35 in a quantitative and dose-dependent fashion; and (3) increased transduction efficiency on FN 30/35 does not appear to be associated with increased cell proliferation or activation of protein phosphorylation typically induced by integrin-fibronectin interactions. Therefore, we speculate that supernatant infection of HSC on FN 30/35 leads to colocalization of retrovirus particles and target cells on FN 30/35 molecule with a large increase in local virus titer presented to the cell. These findings have direct and important implications for the modification of current human gene therapy protocols. © 1996 by The American Society of Hematology.

HEMATOPOIETIC cell adhesion in the complex hematopoietic microenvironment contained in blood-forming organs is mediated both by integrin- and proteoglycan-mediated mechanisms.1-5 Primitive hematopoietic stem cells (HSC) that are capable of multilineage differentiation and long-term reconstitution in vivo adhere to the extracellular matrix molecule fibronectin (FN)2 and endothelial cell vascular-cell adhesion molecule (V-CAM)5 and cell-surface glycosaminoglycans4,12 which interact with heparin-binding sequences located in several extracellular matrix molecules, including FN.7 More differentiated hematopoietic progenitor and precursor cells, especially of the erythroid lineage, interact with the integrin α5/β1,9-11

FN is a complex extracellular matrix molecule with multiple cell adhesion sites (Fig 1). Cell adhesion to the central cell binding domain (Cell) is mediated through the tetrapeptide Arg-Gly-Asp-Ser (RGDS) located in the middle of the FN molecule.12 Cell adhesion to the carboxy-terminus of FN is mediated by sequences contained within peptides termed CS-1 and CS-5 located within the non-type III connecting segment (IIICS) in some, but not all, fibronectin peptides.13,14 Additionally, RGDS-independent cell adhesion occurs in this region of FN to a heparin-binding domain (Heparin, Fig 1) and is mediated by at least two sequences represented in synthetic peptides FN-C/H1 and FN-C/H15,16

Genetic transduction of more primitive long-term hematopoietic reconstituting cells has been one problematic goal for the use of retroviral-mediated gene transfer technology in human gene therapy protocols aimed at lifelong cure of genetic hematopoietic diseases. Previous work by Moore et al17 and recent work by Nolta et al18 have shown increased gene transfer into hematopoietic cells adherent to stroma using retroviral vectors, although the mechanism involved in this increased transduction was unclear. Our laboratory has shown that retroviral-mediated gene transfer into human hematopoietic progenitor cells is substantially increased using cell-free virus to infect these cells while adherent to the carboxy-terminal chymotryptic fragment of fibronectin, FN 30/35 (Fig 1), which contains both the CS-1 site and the heparin-binding domain.19 Transduction of more primitive long-term culture-initiating cell (LTC-IC) was significantly better on FN 30/35 versus intact FN19 and this result parallels differences in adhesion of primitive hematopoietic cells versus progenitor cells to FN 30/35.2 Ward et al20 have recently confirmed increased transduction of human progenitor cells using fibronectin-coated plates.

In this report we show that transduction of murine long-term reconstituting HSC is substantially increased when these targets are infected on the FN 30/35 fragment. In addition, we demonstrate that recombinant retroviral vector particles bind quantitatively and in a dose-dependent fashion to this same FN 30/35 fragment. These data suggest that the mechanism of increased genetic transduction of primitive HSC may be related, in part, to colocalization of stem cells adherent to CS-1 and virus adherent to FN 30/35.

MATERIALS AND METHODS

Retroviral vectors and cell lines. The retroviral vectors N2/ZipT-KNEO (TK Neo)21 packaged in GP+enAM122 and the vector

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ZipPGK-hADA (PGK-hADA)$^{23}$ packaged in GP+E86$^{24}$ have been previously reported. The TKNeo vector was constructed in the N2/Zip backbone with the neo phosphotransferase (Neo) sequences expressed in the sense orientation (relative to the 5' long terminal repeat [LTR]) via the herpes simplex thymidine kinase (TK) promoter. In the PGK-hADA vector, the human adenovirous deaminase (ADA) cDNA is expressed in the sense orientation relative to the 5'LTR via the human phosphoglycerate kinase (PGK) promoter. The GP+E86 producer clone expressing ZipPGK-hADA is designated EPHA-5.$^{25}$

GP+envAM12 (containing TKNeo) or GP+E86 (containing PGK-hADA) producer cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Gaithersburg, MD) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT) and 100 U/mL penicillin and 100 μg/mL streptomycin (P/S; GIBCO). Virus containing supernatant was collected by adding 10 mL of Iscove’s modified Dulbecco’s medium (IMDM; GIBCO) (for amphotropic virus) or α-minimal essential medium (αMEM; GIBCO) (for ecotropic virus) containing 20% FCS plus P/S to confluent plates overnight. Harvested medium was filtered through 0.45-μm filters (Gelman Sciences, Ann Arbor, MI) and was used fresh or stored at −80°C until used.

NIH/3T3 cells were cultured in DME supplemented with 10% calf serum (CS; Sigma, St Louis, MO) and P/S. MO7e cells$^{27}$ were cultured in RPMI supplemented with 20% FCS, P/S, 1% (vol/vol) glutamine, and 100 ng/mL recombinant human stem cell factor (SCF) (Amgen, Thousand Oaks, CA).

Preparation of fibronectin fragments and dishes. The FN 30/35 fragment of FN (Fig. 1) were prepared by chymotryptic digestion of plasma FN and gelatin affinity chromatography as previously described.$^{26,27}$ Heparin-binding fragments were isolated from carboxy-terminal FN chymotryptic fragments by heparin affinity chromatography.$^{28}$ To further purify these heparin-binding fragments, the 1 μmol/L NaCl eluate from the heparin-agarose column was passed over an anion exchange column (DEAE sepharose fast flow; Pharmacia Fine Chemicals, Uppsala, Sweden) after being dialyzed overnight at 4°C against 10 mmol/L Tris-HCl, pH 7.0. The FN 30/35-kD fragments were collected in the unbound fraction while the 42-kD fragment was eluted from the column with 100 mmol/L NaCl. For use in retrovirus transduction protocols, FN 30/35 fragments were immobilized on either 35- or 100-mm Petri dishes (Falcon, Lincoln Park, NJ) at a concentration of 75 pmoles/cm$^2$ dissolved in 1 or 5 mL (2.5 μg/mL) phosphate-buffered saline (PBS; GIBCO) as previously described.$^{29}$ After incubation for 1 hour at room temperature under UV light with the dish lid open and an additional hour with the lid closed, dishes were blocked with 2% bovine serum albumin (BSA fraction V, proteinase-free; Boehringer Mannheim, Indianapolis, IN) for 30 minutes at room temperature. Dishes were then washed once with Hanks’ balanced salt solution (HBSS) supplemented with 2.5% (vol/vol) 1 mol/L HEPES (both from GIBCO). Control plates were coated in analogous fashion with 2% BSA only.

Transduction protocols and hematopoietic assays. MO7e cells, 5 × 10$^5$, were incubated for 2 hours on FN 30/35 or BSA-coated 35-mm dishes with 1 mL TKNeo retrovirus containing supernatant supplemented with polybrene (7.5 μg/mL) (Aldrich Chemical, Milwaukee, WI) and recombinant (r) human (h) stem cell factor (SCF) (100 ng/mL). Thereafter, retrovirus supernatant was exchanged once. Nonadherent cells were re-added to the cultures, and cells were incubated overnight in the continued presence of rhSCF and polybrene. Subsequently infected MO7e cells were seeded into colony assays in methylcellulose with or without G418. For these cultures 10$^3$ to 10$^4$ MO7e cells were plated in 1 mL of 2.4% IMDM methylcellulose (Fluka, Ronkonkoma, NY) containing 20% FCS, P/S, and rhSCF (100 ng/mL) in 35-mm gridded tissue culture dishes (Falcon). The cultures were incubated at 37°C in 5% CO$_2$ for 8 days and single cell-derived colonies were scored under an inverted microscope. Efficiency of infection with the TKNeo virus was analyzed by determining the percent of colonies resistant to 1.5 mg/mL G418 (dry powder; GIBCO). Controls with nontransduced mock-infected cells were performed in each experiment and culture of these mock infected cells with 1.5 mg/mL G418 consistently demonstrated less than 1% background colonies.

Transduction of human hematopoietic cells was performed as previously described.$^{30}$ Briefly, bone marrow (BM) cells from healthy adult donors were collected in tubes containing sterile, preservative-free sodium sulfate heparin according to protocols approved by the Institutional Review Board of Indiana University School of Medicine. Adherent-negative, low-density mononuclear cells were prepared and prestimulated as described$^{30}$ and incubated for 6 hours on plates coated with BSA (control plates) or FN 30/35 and then infected with TKNeo virus supernatant. Virus supernatant was replaced with fresh virus after 2 hours and cells were incubated for an additional 12 to 24 hours. Any nonadherent cells were re-added with the fresh virus supernatant.

Methylcellulose cultures containing 1 × 10$^5$ human BM cells were cultured with or without 1.5 mg/mL G418 for 12 to 14 days at 37°C in 5% CO$_2$ and colonies of greater than 50 cells were scored under an inverted microscope.$^{31}$ Efficiency of transduction with TKNeo virus was determined by the percent of methylcellulose colonies resistant to G418. Mock infections were performed in each experiment by incubating BM with supernatant conditioned by the GP+EnvAM12 packaging line containing no recombinant virus. Culture of these mock infected cells with 1.5 mg/mL G418 consistently showed less than 1% background colony growth.

Transduction of murine hematopoietic cells was performed as previously described.$^{30}$ Briefly, BM was obtained from femurs and tibia of 6- to 8-week-old C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) 2 days after administration of 150 mg/kg 5-fluorouracil (SoloPack Laboratories, Franklin Park, IL). Cells were prestimulated at a concentration of 1 × 10$^6$ cells/mL in αMEM/20% FCS plus P/S with 100 ng/mL recombinant rat(r)SCF (rSCF; Amgen, Thousand Oaks, CA) and 100 μL/mL recombinant human interleukin-6 (rhIL-6; Peppro Tech Inc., Rock Hill, NJ) for 48 hours. Subsequently, gene transfer efficiency with the PGK-hADA vector produced by EPHA-5 producer cells was compared using three different infection protocols: (1) cocultivation on EPHA-5 producer cells; (2) supernatant infection on FN 30/35; (3) supernatant infection (on BSA). For coculture, EPHA-5 cells in 4 mL medium were incubated with 10 μg/mL mitomycin C (Bristol-Meyer Squibb, Princeton, NJ) for 2 hours at 37°C, washed, trypsinized, and seeded on 10-cm tissue culture dishes at a concentration of 3 × 10$^4$ cells in 10 mL αMEM/20% FCS plus P/S. The next day, 5 × 10$^5$ prestimulated BM cells with 100 U/mL rhIL-6, 100 ng/mL rhSCF, and 1 μg/mL polybrene
were added for 48 hours. Following the infection protocol, nonadherent cells were decanted and adherent hematopoietic cells were collected from the cultures using Cell Dissociation Buffer (CDB; enzyme-free/PBS-based; Gibco) according to the manufacturer's instructions. The adherent cells were added to the nonadherent fraction, washed twice with PBS, and resuspended in approximately 1 mL of HBSS/2.5% (vol/vol) HEPES for transfection or clonogenic assays (see below).

For supernatant infection on FN 30/35, 5 × 10^6 prestimulated (as above) BM cells were incubated with 10 mL of virus supernatant obtained from EPHA-5 cells supplemented with 100 U/mL rhIL-6, 100 ng/mL rSCF, and 5 μg/mL polybrene on FN 30/35-coated dishes. For control supernatant infection, dishes were coated with BSA only. Two hours later cells were decanted, resuspended in 10 mL fresh virus-containing supernatant, and re-added including growth factors and 5 μg/mL polybrene. The next day, the procedure was repeated and the cells incubated overnight.

For transplantation studies, all cells obtained from infection of 5 × 10^6 prestimulated BM cells were injected into the tail veins of individual recipient mice that had been subjected to lethal total body irradiation (700 plus 400 cGy with a 3-hour interval, 173Cs-source).29 The transduction of HSC was analyzed by examining peripheral blood cells of reconstituted mice for the presence of the introduced hADA protein by cellulose acetate in situ enzyme analysis.32 Enzyme analysis was performed beginning at 3 months and was repeated for up to 1 year posttransplant.

Proliferation, cell cycle, and phosphorylation studies. M07e cells, 1 × 10^5 were seeded in 24-well tissue culture plates coated with FN 30/35 or BSA in IMDM supplemented with 20% FCS and 100 ng/mL rhSCF at 37°C in 5% CO2. Cell counts from 3 to 6 wells were performed after 2 and 4 days using a hemocytometer. For cell-cycle analysis, tritiated thymidine suicide studies were performed according to the method described by Byron30 with some modifications. Aliquots of 10 × 10^5 M07e cells were incubated on BSA or FN 30/35 in 10 mL IMDM supplemented with 20% FCS. Six hours later either "cold" (nonradioactive) thymidine or 0.1 μCi/mL of high specific activity (~80 Ci/mmol/L) [methyl-3H]-thymidine (New England Nuclear [NEN], Boston, MA) was added. After a 20-minute incubation period, the plates were washed three times with 10 mL ice-cold IMDM/20% FCS containing an excess (100 μg/mL) "cold" thymidine. The remaining cells were removed from the plates using CDB. Cells from all washes and CDB treatment were combined, washed twice, and placed in clonogenic methylcellulose assays as described above. The %S-phase was determined as the fraction of clonogenic cells killed by high specific activity thymidine.

Antiphosphotyrosine immunoblotting and mitogen activated protein (MAP) kinase assays were performed on M07e cells that had been starved of SCF overnight before placing on dishes with and without FN 30/35 coating. Aliquots of cells were obtained at selected time points, then lysates were prepared and assayed for MAP kinase activity or immunoblotted with antiphosphotyrosine antibody as previously described.31

Retrovirus binding to FN 30/35. The viral titer of supernatant was determined using NIH3T3 cells according to standard procedures.32 Briefly, NIH3T3 cells were plated at a concentration of 1,000 cells/well in a 6-well tissue culture plate and grown overnight. Serial dilutions of virus supernatant in 2 mL of medium were added to each well with 7.5 μg/mL polybrene and incubated for 2.5 hours at 37°C after which 2 mL of fresh medium was added. After 24 hours, the wells were fed with medium containing G418 (0.75 mg/mL, dry powder; Gibco) and the plates incubated for 10 to 12 days and stained with methylene blue. The number of G418^+ colonies/well × the dilution of virus supernatant divided by 2 was used as the infectious particles (cfu)/mL of supernatant.

We assessed ("titered") the amount of retroviral particles remaining on FN 30/35- or BSA-coated plates after incubation with virus supernatant and extensive washing with PBS. Serial dilutions of virus supernatant were added for 30 minutes to 35-mm bacteriologic dishes coated with FN 30/35 or BSA. After removal of the virus supernatant, each well was washed three times with PBS and 1,000 NIH/3T3 cells were added per 35-mm bacteriologic dish in DMEM/10% CS. Twenty-four hours later, cultures were fed with medium containing 0.75 mg/mL G418 and the cells were incubated for 10 to 12 days with one medium change. The presence of adherent virus was quantitated by enumerating G418^+ NIH/3T3 colonies. To assess whether virus binding to FN 30/35 occurs in a dose-dependent fashion, the experiments were repeated by coating 35-mm bacteriologic dishes with 1, 4, 10, and 20 μg/cm² FN 30/35 as described above. A 1:50 dilution of frozen TKNeo virus stock previously titered at 1 × 10^6 infectious particles/mL was incubated on FN 30/35-coated plates for 30 minutes. After intensive washing, 2,000 NIH/3T3 cells were added to each well. Selection was performed as above and G418^+ NIH/3T3 colonies counted after 10 days of selection.

Antibody experiments. 35-mm bacteriologic dishes were coated with 1 mL of PBS (pH adjusted to 9.0) containing 15 μg of murine monoclonal antibodies (MoAbs) and 1% BSA at 4°C for 4 hours. Antibodies used included antihuman Very Late Antigen (VLA)-4 (clone 44H6), antihuman CD44 (clone B-G15), antihuman CD34 (clone QBEND/10; all from Biosource International, Camarillo, CA). Thereafter, plates were washed three times with HBSS (GIBCO). Human BM was obtained by aspiration from volunteers and adherence-negative, low-density mononuclear human BM cells were prepared and prestimulated as previously described33 and 5 × 10^6 cells were incubated for 6 hours on plates coated with the MoAbs, BSA (control plates), or FN 30/35 (as described above) and then infected with TKNeo virus supernatant. Virus supernatant was replaced after 2 hours with fresh virus supernatant and cells were incubated an additional 12 to 24 hours. Following the infection protocol, nonadherent cells were decanted and adherent hematopoietic cells were collected using CDB. The adherent cells were added to the nonadherent fraction, washed twice with medium, and counted. Harvested cells were plated in progenitor assays as described above and the percent of G418^+ colonies determined.

Statistical analysis. All experiments were performed in triplicate unless otherwise noted. Results are shown as mean ± SEM unless indicated. One-way analysis of variance was used to compare gene transfer efficiency on FN 30/35 versus BSA (control).

RESULTS

Transduction of murine long-term reconstituting HSC using FN 30/35. We have previously shown that supernatant infection of human BM cells was greatly improved when the infections were performed on chymotryptic-generated 30/35-kD fragments of fibronectin (FN 30/35) (Fig 1).16 These studies used in vitro assays for hematopoietic cells, and thus could not demonstrate the effect of FN 30/35 on transduction of long-term repopulating (reconstituting) stem cells. To test whether transduction of reconstituting hematopoietic stem cells was improved by infection on FN 30/35, we infected murine hematopoietic stem cells with identical retrovirus vectors in three different protocols: by cocultivation; with supernatant virus on FN 30/35; or by supernatant infection without FN 30/35. Infected cells were then injected into lethally irradiated mice in a long-term reconstitution BM transplant model. Long-term and stable BM reconstitution of mice with genetically manipulated hematopoietic stem cells

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is generally accepted as adequate to determine the efficiency of stem cell transduction after a period of 4 months posttransplant. Analysis of recipients of BM transduced with the PGK-hADA retrovirus 12 months after transplantation by ADA isoenzyme analysis (Fig 2) showed that: (1) human ADA cDNA expression was present in mice transplanted with BM cells transduced either by the coculture protocol or by supernatant infection on FN 30/35 but absent in the mice transplanted after supernatant infection without FN 30/35; and that (2) the levels of expression of introduced hADA were comparable for the coculture and FN 30/35 groups. Results from 12 months are shown in Fig 2 where three mice transplanted with cells infected on FN 30/35. Data from additional experiments, all analyzed 6 months after reconstitution, are very similar. In total, 17/23 mice transplanted with cells transduced by supernatant on either FN 30/35 or recombinant FN peptide containing analogous domains of FN (see Discussion) demonstrate human ADA expression in peripheral blood cells. Of the 6 negative animals in these experiments, 3 animals were transplanted with cells infected on FN 30/35 that had been repeatedly frozen/thawed and, therefore, we suspect were not optimal for technical reasons. In concurrent experiments, 0/12 mice transplanted with cells transduced by supernatant alone were positive, whereas 12/12 mice transplanted with cocultured cells were positive.

Mechanism of increased retroviral-mediated transduction of hematopoietic cells on FN 30/35. One potential mechanism for increased transduction of cells on FN 30/35 is the effect of target cell adhesion on virus particle uptake, because HSC are known to adhere to FN 30/35. We first analyzed the role of non–FN 30/35 mediated adhesion on retroviral transduction of human hematopoietic progenitor cells. For this purpose, we studied the effects of immobilization of target cells with various MoAbs recognizing surface antigens and compared this to FN 30/35. VLA-4 antibody recognizes the integrin receptor on hematopoietic cells which mediates adhesion of stem cells to the CS-1 site in FN 30/35. VLA-4 antibody not only stimulates but also up-regulates the expression of hADA, whereas CD44 stimulates the expression of hADA but does not enhance its expression. As seen in Fig 3, transduction of human progenitor cells by TKNeo, analyzed by the number of G418-progenitor colonies, was unaffected by immobilization via antibodies to VLA-4, CD44, or the combination of antibodies to VLA-4 and CD44. In the same experiment transduction of hematopoietic progenitor cells was increased greater than fivefold over BSA control on FN 30/35. Adhesion to antibody-coated plates was equivalent between FN 30/35, 36.7% ± 1.5% (mean ± SEM); CD49, 31.1 ± 4.5; CD44, 28.3 ± 1.5; CD49 + CD44, 32.9 ± 2.7% versus BSA, 8.8 ± 3.6. Adhesion to CD34 plated was less than other antibody coated plates, 20.0 ± 1.1%. Thus, specific interaction with FN 30/35 is critical for increased transduction of hematopoietic cells. Immobilization, even via the same receptor (VLA-4) implicated in CS-1 (FN 30/35) adhesion, has no effect. In an effort to further characterize the role of FN in the...
increased transduction of cells, we assessed the capacity of FN 30/35 to bind retroviral particles. In initial experiments FN 30/35–coated plates were incubated with TKNeo virus containing supernatant for 30 minutes. After removal of supernatant and vigorous washing with PBS, primary hematopoietic cells were added. Transduction of hematopoietic progenitors was nearly as efficient on washed plates as when cells were exposed directly to virus supernatant in the presence of FN 30/35 (27% ± 3% vs 36% ± 3% G418 colonies/plate, respectively; mean ± SD of three experiments; \( P > .05 \)). Concurrent BSA controls demonstrated 5% ± 2% infection of progenitors. The data were highly reproducible. For instance, after removal of supernatant and washing of plates 34/137, 29/109, and 37/121 of added progenitors were transduced using G-418–resistant colony growth as the assay for transduction. These data suggested that retroviral particles directly adhere to FN 30/35.

To quantitatively assess retroviral binding to FN 30/35, serial dilutions of TKNeo virus were incubated on FN 30/35– (or BSA) coated bacteriologic dishes as above. Subsequently, NIH/3T3 cells were added to immobilized virus and analyzed for transduction using G418' (Fig 4 and Table 1).

Adherent retrovirus measured by the growth of G418' NIH/3T3 colonies ("apparent titer") was reduced by more than 3 logs (4 × 10^3 to 0) on BSA-coated plates, whereas apparent titer of bound virus was reduced only 1 log on plates coated with FN 30/35. These data were highly consistent in each of three independent experiments (Table 1). These data show that retrovirus quantitatively binds to FN 30/35 but does not bind to dishes coated with BSA (control dishes). These experiments were repeated with increasing concentrations of FN 30/35 (1, 4, 10, and 20 \( \mu \)g/cm^2) coating the dishes. Figure 5 shows that the numbers of G418' NIH/3T3 colonies increased as the amount of FN 30/35 coating the plate increased. In these experiments, in which ~90 cfu were plated/well, 7.0% ± 1.7% to 37.1% ± 2.8%, respectively (1 \( \mu \)g/cm^2 to 20 \( \mu \)g/cm^2) of added NIH/3T3 cells were transduced. Concentrations of FN 30/35 above 20 \( \mu \)g/cm^2 did not demonstrate any additional viral binding; ie, the virus binding to FN 30/35 was saturable. Therefore, virus binding to FN 30/35 occurs in a dose-dependent fashion. This dose response was still present when 10× higher concentrations of virus was used (data not shown).

To explore an additional potential role of FN on gene transfer, we sought to correlate increased transduction on FN 30/35 with changes in cell proliferation using the MO7e myeloid cell line. Transduction of MO7e cells incubated on FN 30/35 led to increased gene transfer efficiency by threefold to fourfold (Fig 6A) with supernatant containing a retroviral vector encoding neo phosphotransferase (TKNeo, titer 1 to 2 × 10^5 G418' cfu/mL). However, no increase in cell proliferation could be shown when MO7e cells were incubated on FN 30/35 versus control either by thymidine suicide (Fig 6B) or by increases in cell number when analyzed either after 2 or 4 days of incubation (data not shown). Therefore, increased transduction of MO7e on FN 30/35 could not be correlated with changes in cell proliferation.

**DISCUSSION**

Efficient transduction of primitive (reconstituting) HSC remains an important goal of somatic gene therapy protocols.
and is a prerequisite for successful lifelong cure of severe genetic diseases of the BM.\textsuperscript{35-37} Although transduction of murine reconstituting HSC has been accomplished by a number of investigators, efficient transduction of these cells using retroviral vectors has required direct incubation of the target stem cells with producer cell lines and most frequently involved the use of BM obtained from animals pretreated with 5-fluorouracil (5-FU).\textsuperscript{17,19} This approach has obvious limitations with respect to human gene therapy applications.

Because infection of human HSC appears inefficient, alternative approaches to transduction of these cells have been developed with limited success. These include the use of mobilized peripheral blood stem cells,\textsuperscript{38} umbilical cord stem cells,\textsuperscript{3} and the infection of target cells while incubated on allogeneic stroma.\textsuperscript{37,38,39} Although retroviral infection on stroma appears to increase transduction of hematopoietic cells, manipulation of human stroma in gene therapy protocols may prove technically difficult. Our laboratory has previously demonstrated efficient infection of human hematopoietic cells, including progenitors and primitive long-term culture-initiating cells (LTC-IC) on the carboxy-terminal FN 30/35 fragment of FN.\textsuperscript{35} Transduction of long-term reconstituting HSC was not evaluated in this study. Although the mechanism(s) of improved transduction efficiency afforded by incubation on FN 30/35 were not clearly elucidated, this study did demonstrate the need for adhesion of target cells specifically to FN 30/35 versus other cell binding domains in FN for efficient transduction of more primitive populations.

We have now investigated the effect of incubation of reconstituting HSC on FN 30/35 during retroviral infection on transduction of these primitive cells. Analyzing expression of introduced sequences 1 year after BM transplantation of transduced cells we show that infection of HSC on FN 30/35 was highly efficient and was comparable with cocultivation directly on the producer cells. These data suggest that specific adhesion of HSC to FN 30/35 is required for increased transduction efficiency, since our laboratory and other investigators have implicated CS-1 and Hep II in HSC and primitive progenitor adhesion.\textsuperscript{5,4} We have examined multiple hematopoietic cell lines that adhere to FN via the three adhesion domains described above. Transduction of HEL, HL-60, and MO7e cells are all significantly increased by the use of FN during transduction protocols (Hanenberg et al, manuscript submitted).

Although cell adhesion via integrin receptors is known to affect diverse cellular processes, such as spreading, signal transduction and proliferation,\textsuperscript{12,46} we have been unable to correlate increased gene transfer into cells adherent to FN 30/35 and increased cell proliferation in data reported here. This is in agreement with previous data from our laboratory examining cell cycle of primary human hematopoietic progenitor cells during retroviral infection protocols on FN 30/35 by thymidine suicide assay.\textsuperscript{46} These results reflect the assays used to detect proliferative changes or may be due to the specific culture conditions used in our experiments since these were optimized for the retroviral infection protocol. Indeed, we have recently shown that adhesion of primitive murine purified BM populations to FN 30/35 in the presence of concentrations of growth factors suboptimal for proliferation does lead to differences in survival of hematopoietic stem cells ex vivo (P.D., M. Yoder, D.A.W., V. Patel, unpublished results, June 1995).

Increased transduction of HSC could also be related to increased viral titer at the cell surface due to colocalization of virus and receptor. Quantitative adhesion of virus particles is shown in data presented here. Adhesion is dose-dependent and transduction of both NIH/3T3 and hematopoietic cells can be demonstrated by virus remaining bound to FN 30/35 after vigorous washing of plates. These data show that considerable amounts of virus have been immobilized on FN 30/35 from the supernatant. Increased virus particle presentation at the cell surface could also augment internalization of occupied receptors. Such viral internalization has been shown in other viral and bacterial uptake systems.\textsuperscript{41}

The use of FN fragments to mediate high-efficiency gene transfer may be a useful modification of human gene transfer protocols if transduction efficiency approaching cocultivation can be achieved in reconstituting human HSC. Use of FN fragments would also be an important improvement in current gene transfer protocols if the use of 5-FU could be avoided. We are currently examining optimal and simplified protocols using recombinant FN fragments expressing the high-affinity heparin binding site and CS-1 sequence and human cells transplanted into SCID/NOD mice after transduction and primate cells in autologous transplants. In preliminary experiments we have shown successful transduction of primate BM cells responsible for in vivo reconstitution using virus supernatant on FN 30/35 (D. Bodine, D.A.W., unpublished results, July 1995) at a level similar to previous results using genetically modified stromal cells expressing FN/CS-1 and human SCF.\textsuperscript{42} Further, the use of recombinant fragments will add additional safety to the protocols and may clarify viral-specific binding sequences that could be used for targeting of vectors to specific receptors in mammalian cell populations.
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Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments

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