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Entry Kinetics and Cell-Cell Transmission of Surface-Bound Retroviral Vector Particles

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Abstract

Background—Transduction with recombinant Human Immunodeficiency Virus (HIV) -1 derived lentivirus vectors is a multi-step process initiated by surface attachment and subsequent receptor-directed uptake into the target cell. We previously reported the retention of vesicular stomatitis virus G protein (VSV-G) pseudotyped particles on murine progenitor cells and their delayed cell-cell transfer.

Methods—To examine the underlying mechanism in more detail we used a combination of approaches focused on investigating the role of receptor-independent factors in modulating attachment.

Results—Studies of synchronized transduction herein reveal cell-type specific rates of vector particle clearance with substantial delays during particle entry into murine hematopoietic progenitor cells. The observed uptake kinetics from the surface of the 1° cell correlate inversely with the magnitude of transfer to 2° targets, corresponding with our initial observation of preferential cell-cell transfer in the context of brief vector exposures. We further demonstrate that vector particle entry into cells is associated with the cell-type specific abundance of extracellular matrix fibronectin. Residual particle – ECM binding and 2° transfer can be competitively disrupted by heparin exposure without affecting murine progenitor homing and repopulation.

Conclusions—While cellular attachment factors, including fibronectin, aid gene transfer by colocalizing particles to cells and disfavoring early dissociation from targets, they also appear to stabilize particles on the cell surface. Our study highlights the inadvertent consequences for cell entry and cell-cell transfer.

Keywords

HIV-1; lentivirus vector; fibronectin matrix; cell-cell transfer

Introduction

Receptor-independent particle attachment through cellular and exogenous factors, including lectins and semen factor, plays an important role in HIV-1 infectivity [1,2]. Indeed, polybrene, a polycation that functions as an attachment factor, was recently shown to

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stabilize adsorbed HIV-1 virions on the cell surface, in turn shifting the kinetic balance in favor of cell entry, away from rapid dissociation [3]. Similarly, studies of HTLV-1 revealed an important role for the extracellular matrix in complexing particles on the surface of T-cells [4]. Conceptually, particles retained on the cell surface not only gain biological advantages of altered infectivity and reduced decay, but also improvements in tissue trafficking or diffusional mobility [5–7]. Enhancing particle-cell colocalization through physical means or attachment factors is also routinely used to improve gene transfer with recombinant retrovirus particles, largely by altering the adsorption of particles to the cell [7–12]. We became interested in the potential impact of attachment factors on cell entry and particle dissociation following our recent observation of lentivector persistence on murine hematopoietic progenitor cells after brief, 1–3 hour, transduction cultures [13]. While the rationale for rapid transduction in those studies was to minimize the risk of hematopoietic stem cell (HSC) loss or differentiation during *ex vivo* culture, we noticed that particles were inadvertently transmitted to 2° targets, despite saline wash procedures [14–16].

Others have previously shown that the non-specific surface attachment of virions is partly accounted for by cell surface proteoglycans [17–20]. More recently, Beer and colleagues demonstrated a novel role for extracellular fibronectin matrix (ECM) in modulating the transduction efficiency by γ -retrovirus vector pseudotyped with amphotropic or GALV envelope proteins [21,22]. We hypothesized that the wash-resistant retention and 2° particle transfer we previously observed might be linked to cellular attachment factors and reflect cell-specific clearance kinetics for lentivector particles [13]. To study productive particle entry while avoiding bias from defective virions contained in vector preparations, our experimental design relies on target cell infectivity as a surrogate measure. We also synchronized cell entry by binding particles to target cells at 4°C before initiating transduction at 37°C. Together, these widely used methods allowed us to experimentally distinguish attachment and uptake, in turn permitting the measurement of entry rates [3,5,23–25].

Results presented herein confirm that the time-dependent transfer of particles to 2° targets occurs from the cell surface and reflects cell-type specific entry kinetics. Comparatively, the synchronized uptake of lentivector particles into 1° murine hematopoietic progenitor cells is relatively slower than in several cell lines tested and is associated with the relative abundance of ECM fibronectin. Finally, particles that remain attached at the conclusion of transduction can be competitively displaced from the 1° target ECM by heparin treatment, in turn reducing 2° transmission.

Materials and Methods

Vector Production

Vector was produced using transient transfection of human embryonal kidney cells (293T) seeded at a density of 1.6×10^7 per 15 cm tissue culture dish pre-coated with 0.01% Poly-L-Lysine (Sigma), as previously described [26]. Lentiviral transfer vector: pWPXL-EGFP was kindly provided by D. Trono, Geneva, Switzerland. Four-plasmid transfection with packaging (pMD-Lg/p-RRE, pRSV-Rev) and envelope (pMD2.G) helper plasmids was carried out followed by a media change 16 hours later with Dulbecco's Modified Essential Media (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin (Pen/Strep) (all Gibco, Carlsbad CA), and 20mM HEPES pH 7.05 (Sigma). GFP tagged vector was produced by adding vprGFP fusion protein plasmid to the transfection solution as described elsewhere [27]. Vector supernatant was harvested 24, 36 and 48 hours later, filtered through a 0.45 μ m filter, pooled and concentrated by ultracentrifugation. Vector was stored at -86°C .

Cell culture and lentiviral transduction

293T cells were propagated in DMEM medium supplemented with 10% FBS and 1% penicillin/1% streptomycin (Pen/Strep). SupT-1 and Jurkat (human lymphoblastic), as well as L1210 (murine lymphoblastic) cells were cultured in high glucose (4.5 g/L) RPMI 1640 supplemented with 10 mM Hepes pH 7.05, 10% FBS, 1 mM sodium pyruvate, and 1% Pen/Strep. Murine whole bone marrow (WBM) and lineage-depleted (lin-) cells were grown in Iscove's media supplemented with 10% FBS, 10% horse serum, 1% Pen/Strep, 50 ng/mL murine Stem Cell Factor (mSCF), and 50 ng/mL murine interleukin (IL)-3 (Peprotech, Rocky Hill, NJ). For *ex vivo* transduction culture, WBM or lin- cells were exposed to lentivirus vector at 37°C (non-synchronized) or 4°C (synchronized), in the presence of 4 µg/mL protamine sulfate on RetroNectin-coated (2 µg/cm²; Takara Mirus, Madison, WI) non-tissue culture-treated six-well plates at various multiplicities of infection (MOI) in a final well volume of 1 mL. After 2–3 hours, synchronized transduction cultures were transferred to 37°C to allow particle uptake. Following vector transduction, cells were subjected to one or more wash procedures. Heparin-washed cells were incubated in 0–1000 U/mL heparin for 10 minutes at 37°C, as indicated. Pronase (Roche) treated cells were incubated in 1 mg/mL (final concentration) for 10 minutes at 37°C, washed twice and resuspended in appropriate culture media. For co culture conditions, transduced and washed WBM or other non-adherent cell types were added to wells previously seeded with 1×10^5 293T adherent cells (1 mL final volume). Non-adherent cells and media were aspirated 24 hours later and wells were re-fed with complete DMEM media until flow-cytometric analysis. Retronectin (CH296) pre-coating of Jurkat cells was performed by incubating cells for 1 hour at room temperature at 10 µg/mL.

Flow-cytometry

Enhanced Green Fluorescent Protein (EGFP) expression was measured via flow-cytometry using a FACS-Calibur instrument (BD Biosciences) and data were processed using FlowJo software (Tree Star, Ashland, OR, USA). Prior to flow-cytometric analysis, cell samples were washed in PBS containing 2% FBS and subsequently resuspended in PBS (2% FBS) containing 1 µg/mL propidium iodide, thereby excluding dead cells. Cell samples were stained with mouse, or human, antibodies directed against CD45 surface epitopes (APC or PE labeled) (BD Biosciences, San Jose, CA and eBioscience, San Diego, CA, respectively). Antibody staining was performed according to the manufacturer's recommendation. For serial follow-up studies after transplantation peripheral blood cells underwent hemolysis; leukocytes were stained with anti-CD45.1 and anti-CD45.1 antibodies (BD Biosciences, PE or APC labels, respectively) at 4°C for 30-minutes, washed twice in 2% FBS-PBS, and analyzed. Murine leukocyte subsets were analyzed using antibodies specific for B-lymphocytes (anti- B220-APC), T-lymphocytes (anti- CD3-APC) and myeloid cells (combined anti- Gr-1/Mac-1-APC). For fibronectin studies 2×10^5 Jurkat, L1210, and murine lineage-depleted whole bone marrow cells (sca-1 and c-kit enriched) were fixed with 4% paraformaldehyde for 15 minutes at 4°C. Following fixation, samples were washed in phosphate buffered saline (PBS) plus 2% fetal bovine serum (FBS) and permeabilized in 1 mL NET buffer pH 7.07 (0.05 M Tri [pH 7.0], 0.15 M NaCl, 0.005 M EDTA) containing 0.5% Triton. Samples were successively stained with polyclonal rabbit anti-fibronectin antibody (Sigma-Aldrich, St. Louis, MO) and goat anti-rabbit IgG Alexa Fluoro®-647 (Invitrogen) for 30 minutes at 4°C. Samples were twice washed and analyzed by FACS.

Semi-quantitative PCR assay

Semi-quantitative PCR was performed after extraction of genomic DNA from peripheral blood cells and loading was standardized by equal input DNA concentrations. GFP sequence-specific primers (sense, 5'-GGC GAC GTA AAC GGC CAC AAG TTC AG -3' and antisense, 5'-TGC CCC AGG ATG TTG CCG TC -3') and actin sequence-specific

primers (sense, 5'-TGT GAT GGT GGG AAT GGG TCA G -3' and antisense, 5'-TTT GAT GTC AGC CAC GAT TTC C -3') were used with the following thermocycler (Gene Amp 9700, Applied Biosystems) conditions: denaturing 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 58°C for 0.5 min followed by 68°C for 1.5 min. Final extension at 68°C for 10 minutes. Amplified sequences were resolved on a 1% agarose gel and imaged under UV exposure after ethidium bromide staining. DNA extracted from cells containing a single genomic GFP gene copy and GFP plasmid DNA were used as positive controls.

RNA extraction and reverse transcription

Total RNA was extracted from samples using an RNeasy Mini Kit, according to the manufacturer's protocol (Qiagen Inc., Valencia, CA. USA). Residual heparin was removed by adding 180 µL of 5% Chelex (wt/vol) as described by Poli and colleagues [28]. Samples were then incubated (56°C, 20 min), vortexed, boiled (8 min) and centrifuged (3 min at 12,000 x g). Supernatant was collected and reverse transcribed using random hexamer primers and SuperScript™ III RT (Invitrogen), according to the manufacturer's protocol.

Quantitative real-time PCR assay

GFP gene expression in complementary DNA samples was assayed via quantitative real time PCR, using GFP primers (sense: 5'-GTG GTG CCC ATC CTG GTC GAG C -3' and anti-sense: 5'-CAC CAG GGT GTC GCC CTC GAA C -3'), GAPDH endogenous control primers (sense: 5'-AAA TAT GAC AAC TCA CTC AAG ATT GTC A -3' and anti-sense: 5'-CCC TTC CAC AAT GCC AAA GT -3'), and Power SYBR Green PCR Mastermix, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA. USA). Complementary DNA, from murine WBM cells (transgenic GFP: C57BL/6CrSlc-Tg(ACTb-EGFP)OsbC14-Y01-FM131 [29]) was used as a GFP-positive control. All PCR reactions were set up in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems), and run in triplicate. All threshold cycle (Ct) values of GFP were normalized by Ct values of GAPDH endogenous controls. Thermocycling was performed on the ABI StepOne Plus sequence detection system (Applied Biosystems) using the following thermal cycling conditions: 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. Melting curve conditions included 95°C for 15s, 60°C for 1 min, and 95°C for 15s. The fluorescence spectrum was analyzed using ABI StepOne Software v2.0.

Western Blotting

Log-phase Jurkat, L1210, and murine lineage-depleted whole bone marrow cells (sca-1 and c-kit enriched) were solubilized in lysis buffer containing 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM Tris-Cl, 5 mM DTT, and Halt™ Protease Inhibitor Cocktail (Thermo Scientific). The following antibodies were used: polyclonal rabbit anti- fibronectin (Sigma-Aldrich, St. Louis, MO), mouse anti-β-tubulin (Hybridoma Bank at the University of Iowa, Iowa City), donkey anti-rabbit IgG and anti-mouse IgG both linked to horseradish peroxidase (both GE Healthcare, Little Chalfont). Briefly, a 7.5% reducing acrylamide gel was loaded with cell lysate supernatant from 1.4×10^5 cells per sample, according to standard protocol. Following transfer to a PVDF membrane, the blot was blocked for 2 hours with PBS-3% bovine serum albumin (BSA) at room temperature while agitated. Primary anti-fibronectin and anti-β-tubulin antibody were diluted in PBS-3% BSA and allowed to incubate for 2 hours at room temperature while agitated. The blot was washed in PBS-Tween 20 (0.05% vol/vol). Chemiluminescence was produced with an ECL plus detection kit (Perkin Elmer, Boston, MA) and images acquired on a LAS-4000 imaging station (FujiFilm Lifescience, Valhalla, NJ).

Animal husbandry, bone marrow transduction and transplantation

C57BL/6 mice (CD45.2) and Boy J -B6.SJL- (CD45.1) were group housed and maintained at 23°C on a 12 hr light/dark cycle (0700–1900 hr light) with *ad libitum* access to standard chow pellets (Purina Laboratory Rodent Diet 5001, Ralston Purina Co., St. Louis, MO). Whole bone marrow (WBM) cells were collected by flushing femur and tibia from 6 to 12 week-old mice with Iscove's Modified Dulbecco's Media. Cells were lineage-depleted using an Easy Sep® Mouse Hematopoietic Progenitor Cell Enrichment kit according to the manufacturer's instructions (StemCell Technologies Inc., USA), and cultured overnight in Iscoves media supplemented with 10% FBS in the presence of murine stem cell factor (mSCF, 50 ng/mL, Peprotech) and Interleukin -3 (IL-3, 50 ng/mL, Peprotech). The following day, lineage-depleted CD45-mismatched cells were transduced with EGFP expressing lentivector (depending on experiment for 3–5 hours at MOI 15–20) in the presence of 4µg/ml protamine sulfate on plates precoated with CH296 fibronectin, 2 µg/cm²). Following transduction, cells were either washed three times in 200 U/mL Heparin or washed three times in PBS, and 5×10^5 cells/animal were injected into non-irradiated, or sublethally irradiated (500 or 600 cGy, depending on experimental replicate) CD45-mismatched recipients. Following transplantation, retro-orbital bleeds were performed, and white blood cells were analyzed for donor-*versus* host-CD45 isotype and EGFP expression by flow-cytometry. Additional transplantation studies were carried out to measure engraftment and repopulation of 1×10^5 lineage-depleted, CD45 isotype mismatched, washed cells (PBS *versus* 200 U/mL heparin, 10 minutes, 37 °C). These recipients received 500cGy irradiation and were sacrificed for harvest of marrow and spleen 36 hours later.

Immunofluorescent microscopy

Murine L1210 cells were exposed to vprGFP vector for 1 hour, followed by two sequential washes with PBS plus 2% FBS. Cells were placed in direct co-culture with preplated murine 3T3 fibroblasts for 18 hours. Co-cultured cells were stained with Hoechst 33342 (5 µg/ml), and fixed with 4% paraformaldehyde. Cells were then stained with anti-CD45-PE (red) to label L1210 cells, and CD29 followed by Alexa Fluor 647 secondary stain (magenta) to label 3T3 fibroblasts. Deconvolution microscopy was performed at the OHSU Department of Molecular Microbiology and Immunology Shared Resource. The Applied Precision Deltavision Image Restoration System™ includes a chassis with precision nano-motorized XYZ stage, an Olympus IX71 wide field microscope, a Nikon Coolpix HQ Camera; and DeltaVision SoftWoRx™ software. Deconvolution is performed with SoftWoRx software (Applied Precision), and additional image processing is performed with Bitplane Imaris™ software. Images were acquired using the 100× 1.4NA oil lens. Z stacks of 4 colors (Hoechst33342, GFP, anti-CD45 PE, and Alexa-Fluor 647) were collected at 0.5 µm for the complete depth of the cells (19–20 z-planes) and were deconvolved for 9 iterations with the appropriate experimentally determined point spread function (PSF). Histograms were adjusted to display the data as 24 bit RGB tiffs. To optimize visual clarity the PE channel (red) was pseudo-colored orange using Adobe Photoshop CS; the pseudo-color was applied to the entire image, in a manner consistent with accepted image processing guidelines. ECM Fibronectin imaging was performed on 2.5×10^4 Jurkat, L1210, and murine lineage-depleted sca-1 and c-kit enriched bone marrow cells fixed with 4% paraformaldehyde for 15 minutes at 4°C. After fixation, samples were washed in PBS plus 2% FBS and permeabilized in 1 mL NET buffer pH 7.07 (0.05 M Tri [pH 7.0], 0.15 M NaCl, 0.005 M EDTA) containing 0.5% Triton for 10 minutes at room temperature. Samples were successively stained with polyclonal rabbit anti-human fibronectin antibody (Sigma-Aldrich, St. Louis, MO) and goat anti-rabbit IgG Alexa Fluor®-647 (Invitrogen) for 30 minutes at 4°C. Samples were washed twice, cytospun at 500 rpm for 5 minutes, and mounted according to standard protocol.

Statistical analysis

Numerical results are expressed as average plus or minus standard deviation (SD). Data were analyzed using a 2-tailed, unpaired Student t-test. P values of less than 0.05 were considered significant.

Results

Lentivector carryover results in long-term multi-lineage host marking in non-irradiated recipients

We undertook transplantation experiments to provide direct evidence of cell-cell transfer and 2° transduction in long-lived host hematopoietic stem/progenitor cells after intravenous injection of lentivector-exposed, serially-washed hematopoietic cells. We injected a cohort of non-irradiated CD45.2 animals (n=5) with CD45.1, lineage-depleted, GFP lentivector-exposed (1 hour, MOI 5), saline-washed cells and analyzed CD45.2/GFP expression (anti-CD45.2 APC+, GFP+ cells) in recipient peripheral blood leukocytes at serial time points, up to 20 weeks after transplantation by flow-cytometry and proviral PCR from genomic DNA, Fig. 1A,B. We did not observe engraftment of CD45.1 cells in these non-irradiated animals, thereby excluding transduced donor cells as a source of GFP expression (data not shown). At twenty weeks after transplantation peripheral blood leukocytes from these animals were pooled and flow-cytometrically sorted for cells expressing GFP, Fig. 1C. DNA extracted from sorted, GFP expressing host leukocytes (anti-CD45.2 APC+, GFP+ cells) was found to contain proviral integrants, again detectable by semi-quantitative PCR, Fig. 1D. To validate proviral expression in multiple leukocyte subsets, eight additional animals were transplanted. To specifically exclude isotype antibody staining bias, we switched donor-host CD45 isotype assignment. Recipients (CD45.1) were given 500 cGy of irradiation and injected with 2×10^5 lineage depleted, vector exposed (MOI 20, 3-hour transduction culture) CD45.2 murine progenitor cells. Recipient-specific GFP marking was analyzed at 18 weeks after transplantation. Flow-cytometry plots from one of these animals illustrate proviral GFP expression in recipient (CD45.1 isotype) leukocyte subsets (B220, CD3, Mac1/Gr-1) for peripheral blood, spleen and bone marrow compartments, respectively Fig. 2A–C. Taken together, these transplantation experiments indicate GFP expression and proviral integration in host-specific hematopoietic cells.

Protease-sensitive, time-dependent cell-cell transfer of surface bound vector particles

To better understand the persistence of cell-associated replication-deficient particles and gain insight into their cellular location we performed a series of co-culture experiments with 293T cells as 2° targets. Experiments in a tissue culture model (SupT1 cells) confirm that simple saline washes after VSV-G lentivector exposure do not prevent particle transfer during subsequent direct co-culture with preplated stromal cells (293T). Primary flow-cytometric data from a representative example shows a mixed population of SupT1 cells (CD45 PE+) and 293T 2° targets (CD45 PE-) after simple co-culture in the absence of vector exposure (neg. control), Fig. 3A. By comparison, when SupT1 cells are first exposed to GFP lentivector, washed in saline at the end of transduction and subsequently co-cultured for 24-hours with 293T cells, substantial GFP marking is demonstrated in 2° 293T target cells, Fig. 3B. By contrast, transduction of 1° SupT-1 targets is abrogated when vector exposure occurs at 4° C (allowing binding while preventing cellular uptake [3,7]) followed by protease treatment, Fig. 3C (squares). Loss of GFP marking under these conditions is consistent with the predominant retention of residual particles on the cell surface. By contrast, a time-dependent increase in the level of transduction is seen at 37° C when uptake is allowed to occur before pronase wash (open circles). The time-dependent increase in transduction at 37° C after wash in standard media versus pronase (Fig. 3C, closed circles) on the other hand reflects the continued uptake of particles retained on the cell surface,

resulting in ongoing transduction of SupT-1 cells, that resists routine media wash procedures. To demonstrate that this was not strictly SupT-1 cell line dependent, experiments were repeated and showed similar results in Jurkat and (murine) L1210 cells, used interchangeably hereafter. A principal advantage in using VSV-G pseudotyped lentivector for transduction of hematopoietic progenitor and stem cells is the ability to reduce *ex vivo* culture time. We therefore tested the relationship between the transduction duration of murine bone marrow cells as 1° targets (*x*-axis, Fig. 3D) and 2° transfer rates in 293T cells (*y*-axis) resulting from carryover during subsequent co-culture. Results showed a systematic, time-dependent decrease in proviral expression of 293T 2° targets. To explore the potential connection with the uptake of lentivirus vector particles into 1° bone marrow cells, we next compared 1° gene transfer and carryover to 2° targets after an overnight transduction culture at room temperature (25 °C, to slow down entry) and 37°C. As shown, reducing the rate of uptake by lowering the transduction temperature amplified carryover to co-cultured 293T cells, Fig 3E. Similar results were found in Jurkat cells, where progressive changes in transduction culture temperature (vector exposure duration 1 hour) systematically modulated uptake of residual particles from the cell surface. These experiments not only confirm the known temperature-dependent infectivity in 1° target cells, but also reveal inversely related rates of transduction of 2° targets, Fig. 3F. In other words, in both bone marrow progenitors and Jurkat cells the proportional transduction of co-cultured 2° 293T cells is substantially increased when vector exposure of the 1° cell occurs at lower temperature. Finally, to image cell-cell surface transfer of particles directly, we used deconvolution immunofluorescent microscopy. L1210 cells (1° target) were exposed to vprGFP fusion protein-tagged lentivector particles, washed and co-cultured with NIH3T3 cells (2° target). Individual image layers and a composite overlay are shown and illustrate the transfer of vprGFP tagged particles to 2° targets, Fig. 3G. Together, these experiments indicate that vector particle transfer to 2° targets occurs from the cell surface of the 1° target and correlates with transduction culture duration (and temperature). Such a model is consistent with observations by Cole, but also recent reports showing how delays in AAV and HIV-1 infectivity correlate with the prolonged susceptibility to antibody-mediated neutralization [24,25,30].

Uptake of lentivector particles is delayed in primary murine marrow cells

Experiments above imply that modulating cellular particle uptake, using timed exposures and lowered (< 37°C) transduction temperatures, can be rate-limiting to gene transfer efficiency. Further, as particle surface retention competes with dissociation kinetics, rapid transduction of 1° lin- murine bone marrow target cells may amplify the transfer to 2° targets [26]. We next compared rates of particle uptake in a number of different cell types. Adapting an experimental design that uses infectivity as a surrogate measure for cell entry [25,31], we determined that 50% maximum infectivity of Jurkat cells in routine transduction culture is reached in less than 60 minutes, with rapid saturation and over a wide range of vector particle input, Fig. 4A. We also tested 'synchronized' cellular infectivity in Jurkat cells, prechilled and exposed to vector at 4° C (2 hours) before transfer to 37° C for variable time periods. Results confirmed 50%_{max} infectivity is reached within 45 minutes (Fig. 4B). Again, similar results were observed in SupT-1 cultures (not shown, but used interchangeably in subsequent experiments) and in nonsynchronized and synchronized murine L1210 cells, Fig. 4C and 4D, respectively. However, while uptake was rapid in these cell lines, we found strikingly different infectivity kinetics in 1° murine hematopoietic cells. Synchronized infectivity in lineage-depleted bone marrow cells reached 50%_{max} after 24 hours, with 12–16 hours in non-synchronized cultures, Fig. 4E,F. As might be expected, increases in either MOI, or vector concentration showed similar kinetics, but boosted infectivity rates after transduction at 37° C [32], Fig. 4A,E. Together, these findings point to

the involvement of a cell-specific surface property. We deliberately studied multiple cell lines (human and murine) to ascertain that this is not a cell line specific artifact.

Cell specific fibronectin expression and rate of particle uptake

A range of target cell proteoglycans has previously been shown to modulate receptor independent vector-cell binding, without fully accounting for it [17,18,20]. More recent studies emphasize the role of extracellular- and vector particle associated fibronectin in modulating cellular uptake and transduction efficiency of replication deficient γ -retrovirus particles [21,22]. To investigate a potential association with the delayed surface clearance of pseudotyped lentivector particles, we determined the matrix fibronectin expression on several cell lines. Cell-type specific histogram shifts reflect the differential binding of fibronectin- specific antibody and reveal increased expression of fibronectin matrix protein on 1° murine lineage-depleted sca-1 and c-kit progenitor cells, Fig. 5A. Experiments were repeated multiple times with similar results and we confirmed that fibronectin expression in these cells did not vary over time in culture up to 48-hours after harvest, but the differential ECM expression during cell cycle passage or activation status can not be excluded (data not shown). These findings were confirmed using deconvolution microscopy (Fig. 5B) and immunoblotting (Fig. 5C). To experimentally test the impact of cell-associated fibronectin on particle uptake directly, we pre-exposed Jurkat cells to recombinant fibronectin fragment CH296 (FN) before vector exposure. Results demonstrate substantial delays in synchronized (4° C) and non-synchronized (37° C) infectivity, Fig. 5D. Together, these studies provide a novel link between ECM fibronectin and surface clearance rates of vector particles.

Heparin exposure disrupts the binding of particles to the fibronectin matrix and decreases 2° transduction

Given the separable nature of vector particle attachment and cell entry, we wanted to explore the possibility that non specific surface retention and cell-cell transfer can be minimized without compromising gene transfer to the 1° target [11,19,33]. The attachment of lentivirus particles to the cellular fibronectin matrix occurs via particle-associated heparan-sulfate [21,34] and can be experimentally disrupted by heparin [35,36]. Accordingly, the direct reconstitution of cell-free particles in heparin greatly reduced binding and gene transfer to target cells during subsequent transduction, Fig. 6A [18,20,37]. By contrast, when heparin exposure follows transduction culture, gene transfer to 1° SupT-1 target cells is similar to that seen after saline washes, Fig. 6B. At the same time, serial heparin washes at the end of vector exposure of primary murine whole bone marrow cells produce a progressive decrease in cell-cell transfer and 2° transduction, Fig. 6C. Using a real-time PCR assay adapted from Lizee and colleagues to quantify vector particles (i.e. RNA genomes), we recovered cell surface bound particles in heparin-containing wash solutions [38]. Over a 100-fold range of dilutions, PCR results indicate that wash solutions contain particles in heparin dose-dependent numbers, Fig. 6D. Consistent with the competitive displacement, heparin washes after vector exposure appear to offer a potential strategy to remove residual surface-bound particles from cells without substantial decreases in gene transfer to 1° target cells. Moreover, since the initial cellular attachment is thought to occur via particle associated heparan-sulfate, these competitive experiments provide another line of evidence for the role of matrix fibronectin in the initial cell-vector interaction [34]. Several groups have reported heparin interference with CXCR4 – SDF-1 α signaling [39–41]. However, serial heparin washes (10 minutes each at 200 U/ml) had no significant impact on the CXCR4 directed migration of lineage-depleted murine marrow cells in a standard transwell migration assay over a range of concentrations (data not shown). We next investigated homing of cells after heparin wash. Utilizing an experimental design with a CD45 isotype donor - host mismatch we injected two cohorts of four sublethally irradiated animals (500cGy) each with cells (1×10^5 /animal) washed in heparin (200 U/mL for 10 minutes at 37°C), or PBS to evaluate

donor chimerism in marrow and spleen at sacrifice, 36 hours later. Flow-cytometric determination of donor chimerism revealed no significant differences between PBS and heparin cohorts, suggesting that post-transduction heparin exposure does not compromise homing, Fig. 6E. Two additional cohorts of animals were transplanted to evaluate subsequent donor engraftment and recipient CD45 isotype specific GFP expression (from carryover by donor cells) after transplantation of vector exposed, heparin (n=5) *versus* PBS (n=7) -washed cells. Results show that engraftment is not significantly compromised by heparin exposure, while host GFP marking after transplantation is higher, but not significantly, in animals that received PBS washed cells, Fig. 6F. These experiments suggest that the competitive particle removal by heparin provides an experimental platform to further systematically reduce carryover *in vivo*.

Discussion

Recombinant HIV-1 -derived lentivector particles pseudotyped with Vesicular Stomatitis Virus G (VSV-G) protein transduce non-dividing cells and combine wide tissue tropism with high gene transfer rates in highly enriched hematopoietic stem and progenitor cell populations [42–44]. With no apparent VSV-G receptor restrictions, the transduction efficiency is nonetheless directly impacted by endogenous and exogenous attachment factors, including polycations and recombinant fibronectin fragment [8,12,22]. Indeed, the adherence of particles to the cell surface prior to entry represents a unique aspect of the viral life cycle allowing for immune recognition and neutralization, or systemic spread, dissociation and cell-cell passage [13,25,31,45,46]. Yet, in a substantial body of literature, little attention has been focused on the impact attachment has on the balance between cell entry and particle dissociation by pseudotyped lentivector particles [11,47]. Our studies explore the underlying mechanisms that lead to cell specific vector particle retention and cell-cell transfer from *ex vivo* transduced hematopoietic progenitors to 2° targets.

Initial experiments herein show that genetically marked host leukocytes contain proviral integrants and illustrate the systemic dissemination of intact particles from washed hematopoietic progenitors after brief *ex vivo* transduction culture. In keeping with recent studies by others, our *in vitro* experiments indicate that vector particles remain susceptible to neutralization by protease, suggesting the cell surface as the predominant site of persistence, [25,31]. Remarkably, as gene transfer to 1° target cells in these experiments increases with extended vector exposure time, cell-cell transfer to 2° targets systematically decreases. This observation has important implications for protocols relying on accelerated transduction schedules to offset *ex vivo* effects on target stem cell populations [14–16,26,48]. Combined with recent reports of delayed AAV and HIV-1 surface neutralization, this led us to hypothesize that delayed surface clearance might also be involved in lentivector transfer among cells [24,25,31]. To avoid bias from defective particles and non-productive uptake, we measured entry kinetics as reflected by their infectivity, and synchronized transduction events by particle binding at low temperature, two strategies successfully used by others [3,5,24,25,49]. The results echo the kinetics of existing studies of wild-type and VSV-G pseudotyped HIV-1 entry into Jurkat and SupT-1 cells [7,24,49]. But, further experiments also revealed striking differences between the rapid entry seen in cell lines and the slower kinetics in lineage-depleted murine progenitors. Validating the link between uptake kinetics and cell-cell-transfer to 2° targets, the systematic manipulation of transduction culture temperatures delayed particle entry into lin- murine marrow (and Jurkat) cells while increasing carryover marking in 2° targets. The inverse correlation of decreased cell entry rate into the 1° target with increasing 2° transduction rates is in close accordance with similar observations by Cole and colleagues [30]. These experiments also argue against a potential impact from differential receptor expression levels in Jurkat, L1210 and lin- cells [18,50,51]. Because target cell specific rates of particle clearance from the surface correlate

inversely with carryover, cell-cell transfer is relatively amplified after brief vector exposures. Unlike reports of HIV-1 transfer between C-type lectin expressing dendritic cells and T-lymphocytes, this is not limited to specialized immune cells [52,53].

The initial virus particle attachment to ECM fibronectin on the cell surface is experimentally separable from subsequent receptor mediated cell entry [11,18,21,41]. Glycosaminoglycans, such as heparin, can interfere with particle attachment and dramatically reduce infectivity through direct binding to producer cell-derived vector envelope components, when exposure precedes target cell binding [18,20,36]. Such heparin washes at the conclusion of transduction lead to a negligible decrease in 1° gene transfer from particles that have progressed to receptor-mediated entry [21], but result in a substantial concurrent reduction in carryover to 2° targets *in vitro*. These findings are broadly consistent with the model proposed by Beer and colleagues for A-MLV particles delineating the role of fibronectin in immobilizing particles on the surface. The authors' observation that fibronectin complexity can boost endpoint infectivity is not in conflict with our studies that focus on the kinetics of this process. When tested in cell lines, we too find increased endpoint infectivity at matched MOI in cells pre-coated with recombinant fibronectin (data not shown). As the investigators predict, and our experiments confirm, the entry kinetics and "infectious half-lives" are indeed affected by ECM fibronectin [21]. Further, we demonstrate that prior exposure of target cells to fibronectin fragment CH296 slows particle uptake during subsequent exposure. Others have shown that ECM fibronectin can stabilize virus, enhance particle transmission to target cells and may account for the more efficient mode of spread for several replicating viruses, compared with cell-free infection [34,54,55]. Our findings are also consistent with recent findings by Platt and colleagues who used immunofluorescent microscopy to track HIV-1 virions and demonstrated reduced rates of uptake in the presence of polybrene, a polycation attachment factor[56]. Taken together, this raises the question if ECM fibronectin itself might be amenable to manipulation so as to regulate particle attachment and optimize uptake [22,57].

Polycations and CH 296 fibronectin fragment substantially enhance experimental gene transfer and we are not proposing to abandon their established role. But, by shifting the kinetic balance, cell-specific and exogenous attachment factors reduce otherwise rapid rates of dissociation [56]. Our experiments therefore focus on surface ECM-bound particles "at-risk" for carryover and reveal that those can be removed from the surface of 1° target cells. Without significant interference in ongoing receptor-mediated cell entry and infectivity, these studies also confirm the separable nature of attachment and cell entry [11]. The data clearly show, that particles competitively displaced from ECM binding sites by heparin can be quantitatively recovered, as indicated by dose-responsive amplification of vector genomes in wash solutions. Corroborative evidence comes from a report by Pais-Correio who recently demonstrated the heparin-responsive recovery of HTLV particles retained by the ECM on T-cells, and strikingly analogous to our findings here [4]. And while some studies have suggested that heparin exposure of cells for transplantation may interfere with CXCR4-SDF-1 α signaling and cellular homing, our experiments have specifically excluded significant alterations in migration capacity, short- term homing to bone marrow and spleen, or early repopulation [39–41].

In conclusion, our data indicate that particle retention, ECM complexation and delayed cellular uptake play a potentially important role in the systemic, cell-mediated dissemination of HIV-1 derived lentivector seen after brief *ex vivo* transduction cultures of 1° bone marrow cells. The mechanism underlying time-dependent cell-cell carryover of vector particles points to cell-type specific entry kinetics and the ECM fibronectin. Our studies reveal important receptor-independent aspects of cell-vector biology.

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Figure 1. Proviral integration and expression in recipient hematopoietic cells after injection of GFP vector-exposed, washed cells

(A) GFP expression over time in peripheral blood leukocytes from primary, non-irradiated recipients that received vector-exposed, PBS-washed, CD45.1 (CD45 isotype mismatched), lineage-depleted bone marrow cells (GFP vector, MOI 5, 1-hour exposure, 5×10^5 cells per animal). GFP expression in the host (CD45.2) isotype fraction is shown. (B) Semi-quantitative PCR with proviral GFP signature in bone marrow leukocytes from 4 animals sacrificed at 20 weeks after transplantation. NC, GFP-negative control; PC, GFP-positive plasmid control; A–D, individual animals. Bottom panel: actin control amplification with identical sample sequence. (C) Histogram overlay illustrating GFP expressing cells isolated after flow-cytometric sort of peripheral blood leukocytes (anti-CD45.2 APC+) pooled from multiple recipients in (A). (D) Amplification of proviral GFP sequence in DNA extracted from sorted cells in (C). NIH3T3/GFP, single GFP copy genomic control.

Figure 2. Proviral GFP marking in host isotype peripheral blood, spleen and bone marrow

FACS plots illustrating the transduction in host hematopoietic cells (here CD45.1) present in peripheral blood, spleen and bone marrow. **(A)** GFP expression in host peripheral blood B-lymphocytes (B220), T-lymphocytes (CD3) and macrophage/myeloid cells (Mac-1/Gr-1) **(B)** GFP expression in host bone marrow B-lymphocytes (B220) and macrophage/myeloid cells (combined Mac-1/Gr-1) **(C)** GFP expression in host spleen B-lymphocytes (B220), T-lymphocytes (CD3) and macrophage/myeloid cells (Mac-1/Gr-1). Representative GFP marking from one of eight animals studied. Quadrant Gates were adjusted for each cell population studied for viability, antibody isotype stain and untreated control cell populations.

Figure 3. Proviral integration and expression after cell-cell transfer of particles *in vitro*

FACS plots illustrating 1° transduction in SupT-1 cells (CD45-PE+) and 2° transduction in 293T cells (CD45-PE-), measured by GFP expression, illustrating carryover transduction after direct co-culture with mock (A) or GFP vector (B)-exposed and PBS-washed SupT-1 cells (3 hour exposure, MOI 3). (C) Vector transduction and proviral GFP expression in SupT-1 1° targets after vector exposure at 37°C or 4°C (to allow binding, but prevent uptake) for the indicated durations and wash in pronase (1 mg/ml), or media. Pronase treatment degrades both cell-free and cell-bound vector particles (D) Systematic decline in transduction of 293T cells (2° target) after direct co-culture with whole bone marrow cells (1° target) which were previously exposed to GFP vector for increasing lengths of time (x-axis) and washed in PBS. (E) Lineage depleted murine bone marrow cells were exposed overnight to GFP expressing lentivector particles, as described above (MOI 3) at room *versus* incubator temperature. At the end of transduction cells were resuspended and placed in direct co-culture with preplated 293T cells. GFP expression in bone marrow cells (1° target) and 293T cells (2° target) was determined by flow-cytometry 72 hours later. The ratio of GFP marking in 2°: 1° bone marrow cells is noted. The experiment was repeated with similar results. (F) Jurkat cells (1°) were exposed to vector for one hour at the indicated temperature (x-axis). At the conclusion of transduction, cells were co-cultured with 293T cells (2°) at 37 ° C for 24 hours. Gene transfer (% Transduction, as expressed by proviral GFP expression) to 1° Jurkat (CD45-PE+) and 2° 293T (CD45-PE-) cells was determined by flow-cytometry 72 hours later. Higher proviral gene expression in both 1° and 2° cells was observed at increased transduction temperatures, but proportionally greater particle transfer to 2° targets occurred after transduction at lower temperatures (2°:1° ratio). (G) Merged z-stack images from immunofluorescent (IF) deconvolution microscopy studies. Murine L1210 cells were exposed to vprGFP fusion protein tagged vector for 1 hour, twice washed with 2% FBS-PBS and placed atop preplated murine 3T3 fibroblasts. Co-cultured cells were first stained with Hoechst 33342 (5 µg/ml), and fixed with 4% paraformaldehyde. Cells were then stained with anti-CD45-PE (orange) to label L1210 cells, and anti-CD29 followed by Alexa Fluor 647 2° stain (magenta) to label NIH3T3 fibroblasts. IF layers were acquired separately, merged and processed as described in *Methods*. Note that vprGFP-tagged particles can be seen on both, inside and outside of the 2° 3T3 fibroblasts. This experiment is representative of five, performed in these and other cell types. Experiments presented in figures 3A, 3B, 3C were performed 5 times with similar results. Samples in Figure 3D, were scored in triplicate.

Figure 4. Synchronized and non-synchronized transduction by lentivirus particles

VSV-G/GFP vector particle infectivity in Jurkat, L1210 or lineage depleted bone marrow cells transduced at 37°C (nonsynchronized, left-hand panels), or 4°C (synchronized for 2 hours and then transferred to 37°C to initiate uptake, right-hand panels). To terminate uptake (transduction) at the time points shown, cells were washed in pronase (1 mg/ml), eliminating residual surface-bound particles. **(A)** Non-synchronized infectivity of Jurkat cells over time at variable multiplicities of infection (MOI). **(B)** Synchronized infectivity of Jurkat cells. **(C)** Non-synchronized infectivity of murine L1210 cells. **(D)** Synchronized infectivity of murine L1210 cells. **(E)** Non-synchronized infectivity in lineage-depleted bone marrow at different vector concentrations. **(F)** Synchronized infectivity in lineage-depleted cells at two vector concentrations (MOI 3 in 100 ml or 1 ml final volume). Experiments presented in Figures 4A,B,C and D were performed twice with similar results. Experiments presented in Figures 4E and 4F were performed three times with similar results.

Figure 5. Cell-type specific ECM fibronectin and transduction kinetics

(A) Fibronectin expression on L1210, Jurkat and murine lin- cells (sca-1 and c-kit enriched). Cells were fixed, permeabilized, and stained for fibronectin as described in *Materials and Methods*. Expression was measured by flow-cytometry using Alexa Fluoro[®]-647 fluorescence (2° antibody). Black curve reflects background fluorescence (2° antibody only). (B) Representative deconvolved immunofluorescent images of fibronectin distribution on identical cells assessed as Alexa Fluoro[®]-647 fluorescence (red) and Hoechst33342 nuclear stain (blue). (C) Immunoblot of whole cell lysates from L1210, Jurkat and murine lin- cells. Fibronectin (upper band) and β -tubulin loading control (lower band). (D) Delayed infectivity in Jurkat cells cultured with recombinant fibronectin CH296 (2 μ g/ml, 1 hour, 37 °C), or mock, before vector exposure. Vector transductions were performed for the indicated duration and cells were washed twice in pronase (1 mg/ml) containing solution at the conclusion, with subsequent propagation in media. Infectivity over time was measured by scoring GFP expression 72 hours later. Results were obtained after synchronized (4 °C, right hand panel) or non-synchronized (37 °C, left hand panel) transduction, respectively. Experiments were repeated at least twice with similar results. Described in detail in *Materials and Methods*.

Figure 6. Effect of post vector exposure heparin washes on target cells and secondary particle transfer *in vitro*

(A) Pre-exposure of vector particles to heparin reduces gene transfer (measured as proviral GFP expression) during 1° transduction of SupT-1 targets. (B) Post vector exposure treatment of SupT-1 cells with heparin across a range of MOI (single 1-hour exposure) does not markedly reduce gene transfer rates compared with PBS. (C) Sequential heparin washes (10 minutes at 100 U/mL) reduce cell-cell transfer and 2° transduction of 293T targets *in vitro* compared to PBS washes. Error bars denote standard deviation. (D) Log scale quantification of GFP vector RNA species in wash solution from Jurkat cells after vector exposure. Cells were exposed to vector particles at 4° C for 2 hours to synchronize binding and washed in PBS or log-dilutions of heparin that might otherwise interfere with the PCR reaction [28]. Wash solutions were filtered using 0.22 µm filters. RNA was extracted and treated with Chelex-100 to bind residual heparin. RNA samples were then reverse transcribed and GFP copies were measured in samples from each cDNA dilution series via qPCR. PCR results confirm that over a 2-log range of dilution, heparin wash resulted in a dose-dependent increase in recovered particles. The inset graph depicts the standard curve

generated from serial dilutions of RNA template used to calculate copy numbers. Experiments presented in Figures 6A,B,C and D were performed in duplicate with similar results. Figure 6E experimental samples were scored in triplicate. **(E)** Donor chimerism in marrow and spleen from submyeloablatively (500cGy) irradiated animals (n=4/cohort) 36 hours after injection of 1×10^5 lineage-depleted, CD45-mismatched, bone marrow cells incubated for 10 minutes at 37°C in heparin (200 U/mL), or PBS, before injection. **(F)** Left hand panel: Peripheral blood *donor* chimerism from submyeloablatively irradiated animals (n=5/7 per cohort) 7 weeks after transplantation of 1×10^5 lineage-depleted, CD45-mismatched, bone marrow cells incubated for 10 minutes at 37°C in heparin (200 U/mL, solid symbols), or PBS (open symbols), before injection. Right hand panel: Proviral GFP expression in *host* CD45 isotype peripheral blood leukocytes of the same experimental cohorts.