Brief Report

Foamy and Lentiviral Vectors Transduce Canine Long-Term Repopulating Cells at Similar Efficiency

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Abstract

Foamy viral vectors and lentiviral vectors are attractive gene transfer vectors for hematopoietic stem cell gene therapy because they both efficiently transduce stem cells using rapid *ex vivo* transduction protocols designed to maintain engraftment potential. Here we directly compared the ability of foamy and lentiviral vectors to transduce long-term hematopoietic repopulating cells in the dog model, using a competitive repopulation assay with vectors that express enhanced yellow or green fluorescent proteins (EY/GFP). Mobilized canine peripheral blood CD34⁺ cells were divided into two fractions and exposed to either foamy (EGFP) or lentiviral (EYFP) vectors at a multiplicity of infection of 5 in an 18-hr transduction protocol and then reinfused after conditioning with 920 cGy of total body irradiation. Both dogs studied had rapid neutrophil engraftment and multilineage engraftment of transduced cells. Marking was similar for both vectors, particularly at later time points, indicating that both vector types transduce long-term repopulating cells at similar frequencies.

Introduction

STEM CELL GENE THERAPY studies in patients with severe combined immunodeficiency (SCID-X1) (Hacein-Bey-Abina *et al.*, 2002; Gaspar *et al.*, 2004), adenosine deaminase deficiency (ADA) (Aiuti *et al.*, 2002; Gaspar *et al.*, 2006), and chronic granulomatous disease (CGD) (Ott *et al.*, 2006) have demonstrated the enormous potential of stem cell gene therapy. However, the vector-mediated leukemias observed in the French SCID-X1 study (Hacein-Bey-Abina *et al.*, 2003) and more recently in the British study have stimulated research to develop safer vector systems. Foamy viral vectors are derived from foamy or spumaretroviruses and have several properties that distinguish them from gammaretroviruses or lentiviruses, including the fact that they are nonpathogenic (Hooks and Gibbs, 1975; Falcone *et al.*, 2003). They integrate less frequently near promoters than do gammaretroviral vectors and integrate within transcripts less frequently than both gammaretroviral and lentiviral vectors in human cells (Trobridge

et al., 2006) and in canine repopulating cells (Beard *et al.*, 2007). Foamy viral vectors are also less likely to increase expression of adjacent genes in a transient transfection assay (Hendrie *et al.*, 2008). Both foamy and lentiviral vectors can be produced at high titers as self-inactivating (SIN) vectors with expression of transgenes from internal promoters (Dull *et al.*, 1998; Trobridge *et al.*, 2002) and allow for efficient long-term marking in the clinically relevant canine model (Kiem *et al.*, 2007). Foamy viral vectors have been used to successfully treat canine leukocyte adhesion deficiency, and integration analysis suggests they may be safer than gammaretroviral vectors (Bauer *et al.*, 2008). Dogs do not have a potent species-specific block to lentiviral infection mediated by tripartite motif-5 α (TRIM5 α) as observed in some nonhuman primates, so the canine model is an excellent preclinical model in which to compare foamy and lentiviral vectors. Here we directly compared the ability of foamy and lentiviral SIN vectors to transduce long-term canine repopulating cells at an identical multiplicity of infection (MOI), using a competitive repopulation assay. In this

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approach interanimal variability for engraftment and transduction is eliminated and the relative marking between vector types can be directly compared.

Materials and Methods

Animals

Dogs were housed at the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA) under conditions approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD). Study protocols were approved by the Institutional Review Board and the Institutional Animal Care and Use Committee of the FHCRC. In preparation for the harvest of stem/progenitor cells, the dogs received canine granulocyte colony-stimulating factor (cG-CSF, 5 μ g/kg body weight subcutaneously, twice daily) and canine stem cell factor (cSCF, 25 μ g/kg body weight subcutaneously, once daily) for five consecutive days. Leukapheresis was performed with the COBE Spectra apheresis system (CaridianBCT, Lakewood, CO). The machine was primed with autologous blood. A dual-lumen venous catheter was inserted and connected to the COBE machine. During the procedure, the dogs were constantly monitored for level of sedation or signs of distress and a slow infusion of 10% calcium gluconate was given to prevent cramping.

As preparation for transplantation, all animals received a single myeloablative dose of 920 cGy total body irradiation (TBI) administered from a linear accelerator at 7 cGy/min. The animals received broad-spectrum antibiotics and recombinant cG-CSF after transplantation, until the absolute neutrophil count was $>1000/\mu$ l. The animals also received cyclosporine to inhibit immune responses to the enhanced green/yellow fluorescent protein (EG/YFP) transgenes from the day before transplant to 106 days after transplantation, with tapered doses from day 70 to day 106 (dog G380), or to day 92 after transplantation with tapered doses from day 19 to day 92 (dog G480).

Foamy and lentiviral vector production

The foamy vector Δ PF contains an EGFP transgene expressed from a murine phosphoglycerate kinase (PGK) promoter and has been described (Kiem *et al.*, 2007). The HIV vector pRRLsincpPTgkEYFPpre was kindly provided by L. Naldini (San Raffaele Telethon Institute for Gene Therapy, Milan, Italy) and contains an SIN RRL (Dull *et al.*, 1998) vector backbone with EYFP expressed from a human PGK promoter and a woodchuck posttranscriptional regulatory element. Foamy viral vector preparations were produced by calcium phosphate transfection with 12 μ g of p Δ PF, 12 μ g of pCiGSAPsi, 1.6 μ g of pCiPS, and 0.75 μ g of pCiES in a total volume of 800 μ l for each 10-cm tissue culture dish. Vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentiviral vectors were prepared by calcium phosphate-mediated three-plasmid transfection of 293T cells. Twenty-seven micrograms of transfer vector construct, 17.5 μ g of second-generation gag-pol packaging construct pCMV Δ R8.74, and 9.5 μ g of VSV-G expression construct pMD.G were used for transfection of 12×10^6 293T cells overnight in 25 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum. The cells were treated with 10 mM sodium butyrate during the first of three 12-hr vector supernatant

collections. Supernatants were filtered through 0.22- μ m pore size filters and concentrated 100-fold by ultracentrifugation before freezing and storing at -70°C . All vector stocks were titered by transducing HT1080 cells with the use of limiting dilutions of the stock and analysis for EG/YFP expression by flow cytometry.

CD34 enrichment, transduction, and analysis of transgene expression

The CD34 enrichment method has been described previously (Goerner *et al.*, 2001; Neff *et al.*, 2002; Kiem *et al.*, 2007). CD34-enriched cells from peripheral blood (PB) were exposed to foamy or lentiviral vectors at an MOI of 5 for 18 hr in 75-cm² canted-neck flasks (Corning, Corning, NY) coated with CH-296 (RetroNectin; Takara Shuzo, Otsu, Japan) at a concentration of 2 μ g/cm² in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS (GIBCO-BRL/Invitrogen, Gaithersburg, MD) and with sodium pyruvate, L-glutamine, penicillin, and streptomycin (GIBCO-BRL/Invitrogen) in the presence of Fms-like tyrosine kinase-3 ligand (Flt3-L), cSCF, and cG-CSF at a concentration of 50 ng/ml each. After overnight transduction, nonadherent and adherent cells were pooled, counted, and infused intravenously into the animal. EGFP- and EYFP-expressing cells were quantitated by flow cytometric analysis or by real-time polymerase chain reaction (PCR) as previously described (Kurre *et al.*, 2002; Kiem *et al.*, 2007).

Results and Discussion

We transplanted two dogs conditioned with 920 cGy TBI with mobilized autologous peripheral blood CD34⁺ cells

FIG. 1. Engraftment in dogs transplanted with autologous foamy and lentiviral vector-transduced peripheral blood stem cells. The absolute neutrophil counts (A) and platelet counts (B) observed after transplantation are displayed for both transplanted dogs. Solid lines mark the interpolated time course of average cell numbers.

TABLE 1. TRANSDUCTION AND ENGRAFTMENT OF CANINE CD34⁺ CELLS

Dog	Vector	No. of CD34-enriched cells ($\times 10^6$) before culture	Purity of CD34-enriched cells	MOI	In vitro marking ^a	No. of infused cells ($\times 10^6$)	Days to ANC >500/ μ l	Days to platelets >20,000/ μ l
G380	Foamy virus	20	86.5%	5	11.6%	9.0	10	23
	Lentivirus	20	86.5%	5	4.4%	20.0	10	23
G480	Foamy virus	18	86%	5	14%	9.6	14	11
	Lentivirus	18	86%	5	9.5%	15.6	14	11

Abbreviations: ANC, absolute neutrophil count; MOI, multiplicity of infection.

^aPercentage of EGFP (foamy)- or EYFP (lenti)-positive cells in transduced CD34-enriched cultures 10 days after vector exposure.

transduced with an EGFP-expressing foamy viral vector and also with an EYFP-expressing lentiviral vector. CD34⁺ cells were divided into two arms and exposed separately to either foamy or lentiviral vectors at an MOI of 5 (Table 1). Before infusion, transduced cells were washed so that in our approach, repopulating cells would be transduced with either a foamy or lentiviral vector but not both. The vectors were prepared with the viral envelope that would likely be used for clinical applications; the foamy viral vector was prepared with the foamy envelope because foamy vectors do not pseudotype efficiently (Pietschmann *et al.*, 1999), and the lentiviral vector was prepared with a VSV-G pseudotype. After vector exposure higher numbers of viable cells were present in the lentiviral vector preparation for both dogs, so more cells were infused from the lentiviral vector arm than from the foamy viral vector arm of the experiment. We cannot be sure what this toxicity was due to in the foamy viral arm of these experiments, but one difference is that the frozen foamy preparations contained dimethyl sulfoxide (DMSO), which may have contributed. We have since modified our protocol for handling foamy viral vectors after thawing to remove DMSO by dialysis. With this new vector preparation method we do not see any significant difference in toxicity to dog

CD34⁺ cells between concentrated foamy and lentiviral vector preparations. For dog G480, pretransplant marking in CFUs was also assessed by fluorescence microscopy on day 14. Foamy viral vector marking was detected in 14% of CFUs, and lentiviral marking occurred in 23% of CFUs. Both animals engrafted rapidly, reaching a stable absolute neutrophil count (ANC) >500/ μ l within 10 or 14 days. Figure 1A and B display ANC and platelet counts after transplantation for both animals. Engraftment was similar to our previous results using either lentiviral vectors or foamy viral vectors (Kurre *et al.*, 2002; Horn *et al.*, 2004; Kiem *et al.*, 2007) and faster than historic controls that received hematopoietic stem cells transduced with gammaretroviral vectors in a 3-day transduction protocol (Goerner *et al.*, 1999, 2001). Engraftment was stable in both dogs long term, and complete blood counts were within normal values (11,100/ μ l for G380, and 12,590/ μ l for G480) over 1 year posttransplantation.

The transduction frequency of hematopoietic repopulating cells was measured by flow cytometric detection of EGFP and EYFP in PB granulocytes and lymphocytes after transplantation (Fig. 2A and B). We observed stable long-term (>700 days) expression from both the foamy and lentiviral vectors in both animals. For the foamy viral vector, marking in lymphocytes

FIG. 2. Transgene expression in dogs transplanted with autologous foamy and lentiviral vector-transduced peripheral blood stem cells. The percentage of EGFP expressing leukocytes detected by flow cytometry are shown for dogs G380 (A) and G480 (B) at various times after transplantation.

was as high as 4.5%, and as high as 4.7% in granulocytes. For the lentiviral vector, marking was approximately 2-fold higher than for foamy vectors at early time points for animal G380, with the percentage of EYFP-expressing lymphocytes as high as 9.5%, and as high as 9.3% in granulocytes. However, in dog G380 the percentage of cells expressing the lentiviral vector transgene declined to closely match the percentage of foamy viral vector transgene-expressing cells by day 939 post-transplantation. In dog G480 the percentage of transgene-expressing cells were remarkably similar and stable long term for both vector types in both lymphocytes and granulocytes, at approximately 2% for each arm. Real-time PCR analysis of marking showed that the average proviral copy number per cell was similar for both vector systems. For lentiviral vectors, marking was 2.3-fold above transgene expression (G380 and G480), and for foamy viral vectors marking was 2.7-fold (G380) or 4.4-fold (G480) above transgene expression levels. In this experimental setting there was no obvious difference between the relative ability of the two vector types to mediate transgene expression in myeloid and lymphoid cells.

We previously described high-level long-term marking in canines for both foamy and lentiviral vectors (approximately 4–10% for lentiviral and 15–20% for foamy viral). In these previous studies we also found that both vector systems were capable of transducing long-term repopulating hematopoietic cells capable of producing myeloid cells and lymphoid cells and observed no obvious differences in marking between B and T lymphocytes (Horn *et al.*, 2004; Kiem *et al.*, 2007). When the gene transfer levels based on transgene expression from both arms (foamy and lentiviral) of the current study are combined, approximately 10–14 and 4% of cells express the transgene in dogs G380 and G480, respectively. This is slightly lower than we observed in previous experiments (Kiem *et al.*, 2007). For the lentiviral vector we used a much lower MOI in this study (5) than was used previously (100) (Horn *et al.*, 2004). For the foamy viral vectors, the lower marking levels we observed here might be explained in part by the fact that frozen foamy vector stocks were used rather than freshly prepared foamy vector stocks, which we had used previously (Kiem *et al.*, 2007). Improved methods for preparing foamy viral vectors have been developed since these animals were transplanted, so this should not be a problem for clinical trials. The short transduction protocol should be effective for maintaining the engraftment potential of *ex vivo*-cultured stem cells. We used relatively low MOIs, which should reduce the risk of vector-mediated leukemia, yet marking levels were attained that should be sufficient to cure several hematopoietic diseases including X-linked SCID, ADA deficiency, and CGD. To date there has been no evidence of malignancy, and the two animals remain healthy. Both vector types can be prepared at high titers as SIN vectors with expression from internal promoters, and may be safer than the long terminal repeat (LTR)-driven gammaretroviral vectors used in the French and British SCID-X1 studies. Our data support the use of foamy viral vectors as an effective alternative to lentiviral vectors in clinical trials.

Acknowledgments

This work was supported in part by grants HL36444, DK47754, HL074162, HL92554, DK56465, AI063959, DK77806, HL85107, and HL53750 from the National Institutes

of Health (Bethesda, MD). H.P.K. and D.W.R. are Markey Molecular Medicine Investigators. The authors thank Michele Spector, DVM, the technicians in the canine facilities of the Fred Hutchinson Cancer Research Center, and the investigators of the Program in Transplantation Biology who participated in the weekend treatments. The authors thank Amgen for providing canine-specific growth factors, and the technicians of the hematology and pathology laboratories of the Fred Hutchinson Cancer Research Center. The authors thank Erik Olson for help with vector stock production, and also acknowledge the assistance of Bonnie Larson and Helen Crawford in preparing the manuscript.

Author Disclosure Statement

No competing financial interests exist.

References

- Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M.G., and Bordignon, C. (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 296, 2410–2413.
- Bauer, T.R., Jr., Allen, J.M., Hai, M., Tusshong, L.M., Khan, I.F., Olson, E.M., Adler, R.L., Burkholder, T.H., Gu, Y.C., Russell, D.W., and Hickstein, D.D. (2008). Successful treatment of canine leukocyte adhesion deficiency by foamy virus vectors. *Nat. Med.* 14, 93–97.
- Beard, B.C., Keyser, K.A., Trobridge, G.D., Peterson, L.J., Miller, D.G., Jacobs, M., Kaul, R., and Kiem, H.-P. (2007). Unique integration profiles in a canine model of long-term repopulating cells transduced with gammaretrovirus, lentivirus, and foamy virus. *Hum. Gene Ther.* 18, 423–434.
- Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* 72, 8463–8471.
- Falcone, V., Schweizer, M., and Neumann-Haefelin, D. (2003). Replication of primate foamy viruses in natural and experimental hosts (Review). *Curr. Top. Microbiol. Immunol.* 277, 161–180.
- Gaspar, H.B., Parsley, K.L., Howe, S., King, D., Gilmour, K.C., Sinclair, J., Brouns, G., Schmidt, M., Von Kalle, C., Barington, T., Jakobsen, M.A., Christensen, H.O., Al Ghonaium, A., White, H.N., Smith, J.L., Levinsky, R.J., Ali, R.R., Kinnon, C., and Thrasher, A.J. (2004). Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* 364, 2181–2187.
- Gaspar, H.B., Bjorkegren, E., Parsley, K., Gilmour, K.C., King, D., Sinclair, J., Zhang, F., Giannakopoulos, A., Adams, S., Fairbanks, L.D., Gaspar, J., Henderson, L., Xu-Bayford, J.H., Davies, E.G., Veys, P.A., Kinnon, C., and Thrasher, A.J. (2006). Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol. Ther.* 14, 505–513.
- Goerner, M., Bruno, B., McSweeney, P.A., Buron, G., Storb, R., and Kiem, H.-P. (1999). The use of granulocyte colony-stimulating factor during retroviral transduction on fibronectin fragment CH-296 enhances gene transfer into hematopoietic repopulating cells in dogs. *Blood* 94, 2287–2292.
- Goerner, M., Horn, P.A., Peterson, L., Kurre, P., Storb, R., Rasko, J.E.J., and Kiem, H.-P. (2001). Sustained multilineage gene

- persistence and expression in dogs transplanted with CD34⁺ marrow cells transduced by RD114-pseudotype oncoretrovirus vectors. *Blood* 98, 2065–2070.
- Hacein-Bey-Abina, S., Le Deist, F., Carlier, F., Bouneaud, C., Hue, C., De Villartay, J.P., Thrasher, A.J., Wulffraat, N., Sorensen, R., Dupuis-Girod, S., Fischer, A., Davies, E.G., Kuis, W., Leiva, L., and Cavazzana-Calvo, M. (2002). Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N. Engl. J. Med.* 346, 1185–1193.
- Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M., and Fischer, A. (2003). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* 348, 255–256.
- Hendrie, P.C., Huo, Y., Stolitenko, R.B., and Russell, D.W. (2008). A rapid and quantitative assay for measuring neighboring gene activation by vector proviruses. *Mol. Ther.* 16, 534–540.
- Hooks, J.J., and Gibbs, C.J., Jr. (1975). The foamy viruses [review]. *Bacteriol. Rev.* 39, 169–185.
- Horn, P.A., Keyser, K.A., Peterson, L.J., Neff, T., Thomasson, B.M., Thompson, J., and Kiem, H.-P. (2004). Efficient lentiviral gene transfer to canine repopulating cells using an overnight transduction protocol. *Blood* 103, 3710–3716.
- Kiem, H.-P., Allen, J., Trobridge, G., Olson, E., Keyser, K., Peterson, L., and Russell, D.W. (2007). Foamy virus-mediated gene transfer to canine repopulating cells. *Blood* 109, 65–70.
- Kurre, P., Morris, J., Andrews, R.G., Kohn, D.B., and Kiem, H.-P. (2002). Kinetics of fluorescence expression in nonhuman primates transplanted with GFP retrovirus-modified CD34 cells. *Mol. Ther.* 6, 83–90.
- Neff, T., Horn, P.A., Valli, V.E., Gown, A.M., Wardwell, S., Wood, B.L., Von Kalle, C., Schmidt, M., Peterson, L.J., Morris, J.C., Richard, R.E., Clackson, T., Kiem, H.-P., and Blau, C.A. (2002). Pharmacologically regulated *in vivo* selection in a large animal. *Blood* 100, 2026–2031.
- Ott, M.G., Schmidt, M., Schwarzwaelder, K., Stein, S., Siler, U., Koehl, U., Glimm, H., Kuhlcke, K., Schilz, A., Kunkel, H., Naundorf, S., Brinkmann, A., Deichmann, A., Fischer, M., Ball, C., PILZ, I., Dunbar, C., Du, Y., Jenkins, N.A., Copeland, N.G., Luthi, U., Hassan, M., Thrasher, A.J., Hoelzer, D., Von Kalle, C., Seger, R., and Grez, M. (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat. Med.* 12, 401–409.
- Pietschmann, T., Heinkelein, M., Heldmann, M., Zentgraf, H., Rethwilm, A., and Lindemann, D. (1999). Foamy virus capsids require the cognate envelope protein for particle export. *J. Virol.* 73, 2613–2621.
- Trobridge, G., Josephson, N., Vassilopoulos, G., Mac, J., and Russell, D.W. (2002). Improved foamy virus vectors with minimal viral sequences. *Mol. Ther.* 6, 321–328.
- Trobridge, G.D., Miller, D.G., Jacobs, M.A., Allen, J.M., Kiem, H.-P., Kaul, R., and Russell, D.W. (2006). Foamy virus vector integration sites in normal human cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1498–1503.

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Received for publication October 21, 2008;
accepted after revision January 22, 2009.

Published online: April 1, 2009.