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A nuclear localization signal in the matrix of spleen necrosis virus (SNV) does not allow efficient gene transfer into quiescent cells with SNV-derived vectors

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Abstract

A major limitation in gene therapy for vectors derived from Moloney murine leukemia virus (MLV) is that they only deliver genes into dividing cells. In this study, a careful comparison of spleen necrosis virus (SNV)-derived vectors with MLV and human immunodeficiency virus (HIV)-1 retroviral vectors indicated that SNV vectors can deliver genes 4-fold more efficiently than MLV vectors into aphidicolin-arrested cells, although at a 25-fold lower efficiency than HIV-1-derived vectors. Furthermore, the addition of a NLS in the SNV matrix (MA) that mimics the one located in HIV-1 MA did not increase the ability of SNV vectors to transfer genes into arrested cells. Also, we found that the RD114 envelope was able to pseudotype SNV viral particles in a very efficient manner.
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Keywords: Retrovirus; Spleen necrosis virus; Quiescent cells; Vector; Gene therapy; RD114

Introduction

Gene transfer into hematopoietic stem cells has been proposed for the treatment of inherited diseases that affect blood cells (Klein and Baum, 2004). It is usually performed in vitro using Moloney murine leukemia virus (MLV)-based vectors, but it is necessary to stimulate cell division to allow the transgene integration (Klein and Baum, 2004). Indeed, in order for the preintegration complex (PIC) of the retrovirus to enter the cell nucleus, the nuclear membrane must be dissolved (Goff, 2001; Lewis and Emerman, 1994; Miller et al., 1990; Roe et al., 1993). Then, the therapeutic gene will integrate into the DNA of the cell, and it will be transmitted to daughter cells (Goff, 2001). It has been shown that the lentiviral vector is clearly superior to the MLV vector for transduction of quiescent, primitive human hematopoietic progenitor cells (Case et al., 1999; Miyoshi et al., 1999; Sutton et al., 1998) and it seems that this property could be attributed to the active nuclear import of the human immunodeficiency virus (HIV) PIC. The pathogenic nature of HIV could limit its clinical use, and the production of the lentiviral vector usually performed in transient transfection renders the scale-up of the vector cumbersome (Reiser, 2000).

A vector derived from a retrovirus that is naturally nonpathogenic to humans and that could efficiently transport its PIC through the nuclear membrane would be ideal for gene transfer into hematopoietic stem cells. Few years ago, a vector derived from the gammaretrovirus spleen necrosis virus (SNV) has been genetically engineered to allow gene delivery into quiescent cells (Parveen et al., 2000). A nuclear localization signal (NLS) was artificially created in the matrix (MA) since it was believed that the NLS located in the N-terminal region of HIV-1 MA was critical for the entry of the virus PIC into the nucleus of quiescent cells (Bukrinsky et al., 1993; Bukrinsky et al., 1992; von Schwedler et al., 1994). SNV retroviral vectors are not infectious to human cells (Gautier et al., 2000), and therefore the pseudotyping with other envelopes (Env) is a

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necessary requirement for human applications. RD114 Env could be a good candidate because the RD114 receptor is highly expressed on human hematopoietic stem cells (Tailor et al., 1999), and above all, it has been reported that MLV and HIV vectors pseudotyped with RD114 Env can transduce human CD34$^+$ cells very efficiently (Kelly et al., 2000; Sandrin et al., 2002).

Results and discussion

First, the capacity of RD114 Env to pseudotype SNV viral particles was investigated. SNV and RD114 Env show approximately 50% identity at the amino acid level (Overbaugh et al., 2001), and they belong to the same receptor interference group (Koo et al., 1992). MLV and SNV recombinant retroviruses containing the MLV RNA vector GFP3 (Qiao et al., 2002) and displaying a vesicular stomatitis virus G protein (VSV-G) or a RD114 Env at their surface were produced in transient transfection. It has been previously reported that SNV gag polyprotein packages efficiently MLV RNA (Certo et al., 1999; Embretson and Temin, 1987) and that VSV-G Env is able to pseudotype SNV viral particles (Dornburg, 2003). The pseudotyping efficacy of SNV particles by RD114 Env was quantified by measuring viral titers by GFP analysis. The titer measured by FACS of SNV particles pseudotyped by RD114 Env was 1.9×10^4 GFP transducing unit (TU)/ml, and it was 2.5 times lower than the one found with VSV-G Env pseudotyped SNV viruses. This difference in titer was similar to the one found with MLV viral particles: 4.6×10^5 and 1.2×10^6 GFP TU/ml (Fig. 1). Therefore, we could conclude that SNV viral particles can be pseudotyped in an efficient manner by RD114 Env.

The next step was to create a NLS in SNV MA as previously described by Parveen et al. (2000) in order to produce viral particles that could transfer genes into quiescent cells. The NLS in SNV MA was generated by PCR-mediated site-directed mutagenesis; the alanine in position 28 and the glycine in position 29 were, respectively, changed for a tyrosine and a lysine. This mutant shares 4 amino acids with the NLS of HIV-1 MA, and it was shown to be the most efficient for generating viral particles that could deliver genes into quiescent cells (Parveen et al., 2000). Aphidicolin-arrested HT-1080 cells were infected with SNV, SNV* (SNV with the NLS in MA), HIV-1 or MLV retroviral particles pseudotyped with VSV-G Env. Two days later, GFP fluorescent cells were counted under a microscope to evaluate the viral titers. The percentage of the number of infected aphidicolin-treated HT-1080 cells (A) divided by the number of infected dividing cells (D) was used to compare the vectors. A/D of 0.6% for MLV particles and 50.8% for HIV-1 particles were found, and the values obtained were in the range of those that have been reported by others (Hatziioannou and Goff, 2001; Naldini et al., 1996; Trobridge and Russell, 2004). The A/D were 1.9% and 2.9% for SNV and SNV* vectors, respectively (Fig. 2). Although the A/D values for SNV vectors were higher than the one found for MLV, these vectors were 25-fold less efficient than HIV-1-derived vector to infect aphidicolin-arrested cells. Moreover, the addition of a NLS in SNV MA did not improve the ability of SNV vector to deliver genes into quiescent cells as previously reported (Parveen et al., 2000). We obtained similar results with the D17 cell line that was used by Parveen et al. to demonstrate that SNV* recombinant viruses could infect efficiently quiescent cells (data not shown).

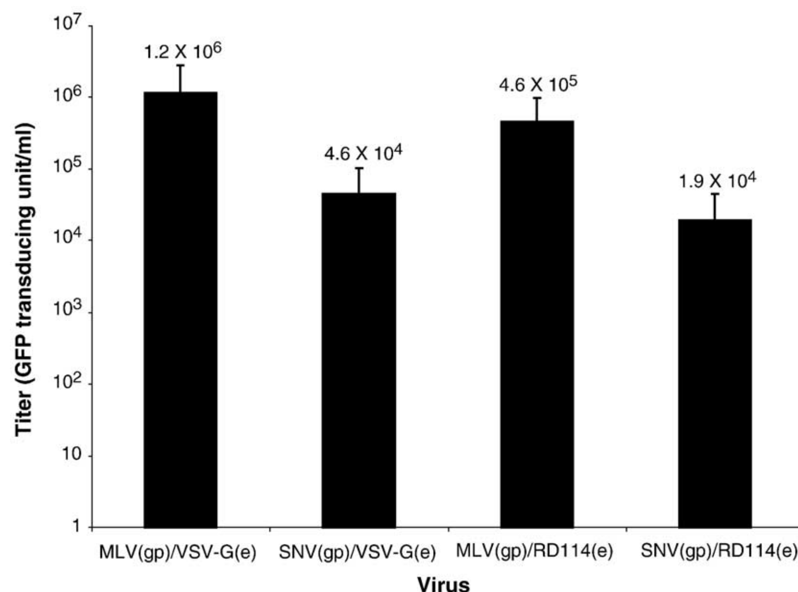

Fig. 1. RD114 Env pseudotypes SNV viral particles. Titers are represented in GFP transducing unit per ml, and they are the average ± SD of 3 independent transfection and infection experiments. The abbreviations gp and e stand for gag-pol and Env, respectively.

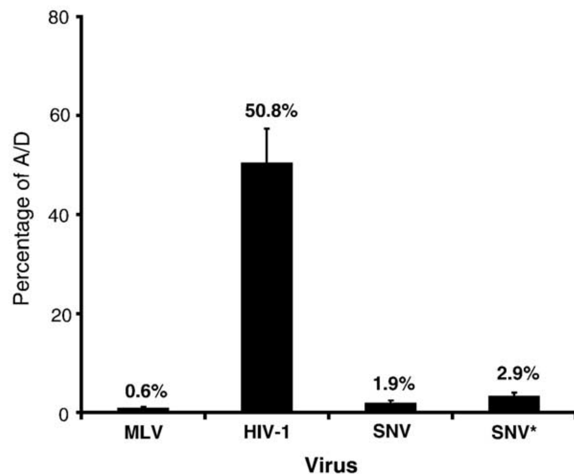

Fig. 2. A nuclear localization signal inserted into SNV MA does not allow the efficient retroviral infection of quiescent cells. A/D is the number of infected aphidicolin-treated HT-1080 cells divided by the number of infected dividing cells. The A/D ratios were calculated using the following titers for quiescent and dividing cells, respectively: (a) MLV: 1.5×10^4 and 2.7×10^6 GFP TU/ml, (b) HIV-1: 6.6×10^6 and 1.3×10^7 GFP TU/ml, (c) SNV: 1.1×10^3 and 5.9×10^4 GFP TU/ml and (d) SNV*: 1.5×10^3 and 5.1×10^4 GFP TU/ml. Viral titers were measured by counting GFP fluorescent cells. The values are represented by the average ± SD of an experiment done 3 times.

More recently, Dornburg's group used SNV* viral particles to transduce neuronal cells in vivo, but they could not be certain that the neurons were quiescent at the time of infection since the injections were made in newborn mice (Parveen et al., 2003). The brain of these mice was still not fully differentiated, and brain cells could still undergo some cell division.

Many studies have strongly challenged the need of MA NLS for the infection of nondividing cells by HIV-1 (Fouchier et al., 1997; Freed et al., 1995), and it has also been shown that the entire globular domain of MA (which contains the NLS) was dispensable for the infection of nondividing cells (Reil et al., 1998). It has also been demonstrated that the HIV-1 MA can efficiently replace the MLV MA without affecting the virus infectivity. Nevertheless, the incorporation of the HIV-1 MA in MLV did not allow the virus to replicate in growth-arrested cells (Deminie and Emerman, 1994).

A NLS located in the HIV-1 integrase has also been implicated in the nuclear entry of the PIC using the importin/karyopherin pathway (Gallay et al., 1997), and a NLS present in the integrase of Rous sarcoma virus is suspected to be responsible for the property of this virus to infect nondividing cells (Hatziioannou and Goff, 2001; Katz et al., 2002). Different NLSs fused to the MLV integrase of a replication-competent virus were also tested for their ability to render MLV infection cell cycle-independent. Although the viruses could replicate, they could not infect efficiently quiescent cells (Seamon et al., 2002).

In conclusion, our data suggest that SNV viral particles are more efficient than MLV vectors to infect aphidicolin-arrested cells, although they are 25-fold less efficient than HIV-1-derived vectors. Also, our results indicate that the addition of a NLS in SNV MA does not change the capacity of SNV vectors to transduce quiescent cells as it was reported by Parveen et al. (2000). Finally, we show in this study that RD114 Env can pseudotype SNV particles efficiently.

Materials and methods

Plasmid construction

The SNV gag-pol plasmids were constructed as follows: gag-pol was excised in *Sal*I/*Pme*I from the SNV provirus pPB101 (Bandyopadhyay and Temin, 1984) (ATCC, Manassas, VA) and was cloned under the control of the cytomegalovirus immediate early enhancer/promoter region (CMVi.e.) in the pCI plasmid (Promega, Madison, WI). A version in which SNV gag-pol was under the control of the elongation factor-1α promoter linked to the 5′RU5 untranslated region of HTLV-1 (EF1α-RU5/HTLV) was also constructed. The EF1α-RU5/HTLV sequence was isolated from the plasmid pOrf-hCx43 (InvivoGen, San Diego, CA) and was cloned instead of the CMVi.e. in the pCI plasmid. The NLS in SNV MA was generated by PCR-mediated site-directed mutagenesis (the protocol will be available upon request), and expression vectors with gag-pol SNV* were also constructed similarly to the wild-type versions. pVPack-Gag-Pol contained MLV gag-pol under the control of a CMVi.e promoter, and it was commercially obtained (Stratagene, La Jolla, CA). The RD114 Env expression vector was also derived from pCI, and it was constructed as follows: the 2.2-kb RD114 envelope gene from the proviral clone Sc3c (a gift from Dr S. J. O'Brien) was digested by *Apa*I/*Hinc*II followed by klenow treatment, and was cloned in pCI digested by *Sma*I to give pCI-RD114Env. The plasmid pMD2.G that encodes the VSV-G envelope, and the lentiviral vector pHIV-GFP and the MLV vector GFP3 that express EGFP have been described elsewhere (Dull et al., 1998; He et al., 1997; Qiao et al., 2002).

Cell lines, transfection and infection

293T (human embryonic kidney), HT-1080 (human fibrosarcoma, ATCC CCL-121) and D17 cells (dog osteosarcoma, ATCC CRL-6248) were cultured with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Bio Cell, Drumondville, Canada) and antibiotics. For the production of recombinant viruses, subconfluent 293T cells were transfected by the calcium phosphate procedure with 9.5 μg of gag-pol plasmid (SNV or MLV derived), 12.5 μg of GFP3 vector and with 3 μg of pMD2.G or pCI-RD114Env plasmid in a 10-cm tissue culture dish. To produce the lentiviral vector, 16.7 μg of pHIV-GFP and 8.3 μg of

pMD2.G were cotransfected into cells. After 48 h, 10 ml of supernatant containing virus was harvested for titration. The titer was determined as follows: HT-1080 cells were seeded at 2×10^5 cells/well in a 6-well plate the day prior infection, and the cells were infected with 1 ml of SNV supernatant or 100 μl of MLV supernatant in the presence of 8 μg/ml Polybrene (PB). The fluorescence of the cells was analyzed with a flow cytometer (Beckam Coulter, Brea, CA, USA) 48 hours later, and the titer in GFP TU/ml was calculated using the following formula (Sastry et al., 2002): titer = $(F \times Co/V) \times D$. F is the frequency of GFP-positive cells determined by flow cytometry; Co is the total number of target cells infected; V is the volume of the inoculum; D is the virus dilution factor.

Aphidicolin-arrested HT-1080 cells were used to assess the capacity of SNV, SNV*, MLV and HIV-1 recombinant viruses pseudotyped with VSV-G Env to infect quiescent cells. Cells plated at 1.3×10^5 in 35-mm dish were synchronized in serum-free medium overnight, and the next morning, they were treated with aphidicolin (1 μg/ml) at the time of infection. Cells stained with propidium iodide at different time points during the next 48 h showed a strong block in G1/S as expected (data not shown). Arrested cells were infected with 500 μl of SNV or SNV* virus, 50 μl of MLV virus or 0.5 μl of HIV virus. Control untreated cells that were dividing were infected with 50 μl of SNV or SNV* virus, 5 μl of MLV virus or 0.5 μl of HIV virus. In this experiment, the viral supernatant volumes were chosen based on titers and the susceptibility of each virus to infect quiescent cells. Forty-eight hours after infection, titers were measured by counting the number of GFP-positive cells under a fluorescent microscope in 10 independent fields.

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