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Mass spectrometry analysis reveals differences in the host cell protein species found in pseudotyped lentiviral vectors

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ABSTRACT

Lentiviral vectors (LVs) have been successfully used in clinical trials showing long term therapeutic benefits. Studying the role of cellular proteins in lentivirus HIV-1 life cycle can help understand virus assembly and budding, leading to improvement of LV production for gene therapy. Lentiviral vectors were purified using size exclusion chromatography (SEC). The cellular protein composition of LVs produced by two different methods was compared: the transient transfection system pseudotyped with the VSV-G envelope, currently used in clinical trials, and a stable producer cell system using a non-toxic envelope derived from cat endogenous retrovirus RD114, RDpro. Proteins of LVs purified by size exclusion chromatography were identified by tandem mass spectrometry (MS/MS). A smaller number of cellular protein species were detected in stably produced vectors compared to transiently produced vector samples. This may be due to the presence of co-purified VSV-G vesicles in transiently produced vectors. AHNAK (Desmoyokin) was unique to RDpro-Env vectors. The potential role in LV particle production of selected proteins identified by MS analysis including AHNAK was assessed using shRNA gene knockdown technique. Down-regulation of the selected host proteins AHNAK, ALIX, and TSG101 in vector producer cells did not result in a significant difference in vector production.

1. Introduction

Lentiviral vectors (LVs) have been applied in several clinical trials for the treatment of monogenic disorders such as beta-thalassaemia [1], Wiscott-Aldrich syndrome [2] and Metachromatic Leukodystrophy (MLD) [3]. Besides ex vivo therapy of hematopoietic stem cells (HSC), LVs can efficiently transduce dendritic cells [4–6] given them the potential to be used as vaccines in larger groups of patients. LVs are also widely used as experimental tools for stable genetic modifications of cells in research laboratories, e.g. shRNA libraries [7] and gene editing [8].

Most commonly lentiviral vectors are produced by transient transfection. This method has several disadvantages as vectors can only be produced over a short time and production is labour-intensive as well as difficult to scale up. Furthermore the presence of DNA plasmids in produced vectors requires their purification, complicating downstream-processing. With the increasing need for large amounts of LVs a packaging cell line stably expressing LVs is desirable. Packaging cell lines such as tetracycline-dependent systems [9–11] have been developed, with variable vector production abilities. The producer cell line STAR stably expresses high levels of a codon-optimised HIV-1 *gag-pol* introduced by transduction with murine leukaemia virus (MLV) derived vectors as well as *rev* and *tat* [12]. STAR cell produced vectors are pseudotyped with a gammaretroviral envelope RDpro as it can efficiently transduce HSC as shown for vectors produced by STAR-RDpro cells [13] and shown for the next generation of stable RDpro-pseudotyped producer cells (WinPac) in comparison to transiently produced VSV-G pseudotyped vectors [14] and is not cytotoxic [15], unlike VSV-G which has been used in transiently produced LVs in current clinical trials.

Studies of wild-type HIV-1 virus showed that proteins such as the transcription factor 1-alpha (EEF1A1), programmed cell death 6-interacting protein (ALIX or AIP1), annexin A2 or 5 and alpha-enolase [16–18] were associated with virus particles. These proteins were also identified in studies on crude or purified lentiviral vectors [19,20]. Several groups [19–21] have used mass spectrometry (MS) to identify vector associated host cell proteins. Further analysis of vector-associated host proteins can point the way towards fundamental virus-host interaction in viral vector assembly, in particular, the protein-protein and protein-RNA interactions during viral particle assembly and

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formation. Ultimately this knowledge can be applied to improve vector production.

In this study we purified lentiviral vectors using size exclusion chromatography (SEC) a purification method that was used during processing of a HIV-1 based VSV-G pseudotyped lentiviral vector in a phase I clinical trial [22] and then used LC-MS/MS to analyse the protein composition in the purified vectors generated by transient production pseudotyped with VSV-G and by stable producer cell line, STAR, pseudotyped with the RDpro envelope protein.

We further analysed the effect of these proteins on vector production using small hairpin RNA (shRNAs) -mediated gene regulation. Our results showed insignificant effects of knock-down expression on vector production levels.

2. Materials and methods

2.1. Lentiviral vectors

Lentiviral vectors were either produced by transient transfection of HEK 293T cells [23] or by a stable producer cell line [12]. Six different vector samples were prepared. Transiently produced vectors were 1) VSV-G-GFP: transfection of DNA plasmids coding for VSV-G Env, structural protein HIV-1 Gag/Pol, Rev and a SIN-vector genome with GFP marker gene driven by a human phosphoglycerate kinase 1 (PGK) promoter; 2) VSV-G-Empty: transfection of DNA plasmids coding for VSV-G Env, HIV-1 Gag-Pol and Rev, 3) Gag/Pol-GFP: transfection of DNA plasmids coding for Gag/Pol, a vector genome and Rev. 4) VSV-G only: a sample produced by transfection of only the VSV-G envelope and the SIN-LV genome plasmid. Stably produced vectors were packaged by STAR-RDpro-HV or STAR-RDpro cells [12]. STAR-RDpro-HV, derived from HEK 293T cells, are continuously expressing RDpro envelope protein, Gag/Pol, a non-SIN vector genome with marker protein GFP, driven by a spleen focus-forming virus (SFFV) promoter and Rev, producing vector sample 'RDpro-GFP' (sample 5). STAR-RDpro express RDpro, Gag/Pol and Rev but no vector genome and produced vector sample 'RDpro-Empty' (sample 6). For vector sample preparation, cells were seeded on 15 cm dishes. For vector samples 1) to 4) cells were transiently transfected 24 h later. Following a medium change 24 h later, vector containing supernatant was collected 24 h and 48 h post transfection. Supernatants of each plate were concentrated by ultracentrifugation to a final volume of 900 µl. Transiently produced vector samples were concentrated 40 fold and stably produced vectors 240 fold.

2.2. Lentiviral vector purification

For vector purification, a Gilson liquid chromatography system was used, consisting of a pump (model 306), an autosampler (model 231XL) as well as a fraction collector (model FC203B) connected to a temperature controlled rack. Sample injection was controlled by the software Trilution LC 2.1 and sample elution and detection were controlled by Unipoint 5.11 (all parts and software by Gilson, Middleton, WI, USA). Samples were separated by a cooling jacked XK16/70 column packed with Sephacryl-500–HR medium (both by GE Healthcare, Little Chalfont, UK). Sephacryl-500–HR medium consists of cross-linked copolymer beads of allyl dextran and N,N′-methylene bisacrylamide with an average particle size of 47 µm, allowing the separation of macromolecules in the range of 4×10^4 to 2×10^7 relative molecular mass (M_r). TEN buffer containing 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.4 was used as sample running buffer (Sigma-Aldrich, UK). For size exclusion chromatography 900 µl of crude vector sample were purified per column at a flow rate of 0.8 ml/min. Void peak fractions from a total of 8 runs of 900 µl crude vector samples were pooled and dialysed using slide-A-Lyzer dialysis cassettes (3–12 ml capacity, molecular cut-off 10 000 Da, Thermo Fisher Scientific, UK) overnight in 3 L of 10 mM ammonium bicarbonate (ABC), pH 8.0 (Sigma-Aldrich). Dialysed samples were freeze-dried using an Edwards E2M2 High Vacuum Pump and resuspended in distilled water. Total protein amounts were quantified by Bradford Protein Assay (BioRad, Hercules, CA, USA) using BSA protein standard (Thermo Fisher Scientific).

2.3. Purified lentiviral vector characterisation using LC-MS/MS

Purified and concentrated lentiviral vector samples were digested with trypsin in the presence of 0.05% Rapigest (an enzyme-compatible detergent to ensure protein denaturing; Waters, Milford, MA, USA) and 50 mM ammonium bicarbonate, pH 8.5 for 3 h at 37 °C. HCl was added to terminate digestion and ensure breakdown of Rapigest.

LC-MS/MS was carried out using a MS system (Thermo Fisher Scientific, Hemel Hampstead, Herts, UK) equipped with a nano-electrospray ion source and two mass detectors i.e. linear trap (LTQ) and orbitrap, coupled with an Ultimate 3000 nano-LC system, comprising a solvent degasser, a loading pump, a nano-pump, and a thermostated autosampler. After an automated injection, the extracted peptides from each digestion were desalted in a trapping cartridge (PepMap reversed phase C18, 5 µm 100 Å, 300µ id x 5 mm length; Thermo Fisher Scientific), eluted on to a C18 reversed phase nano-column (3 µm, 100 Å, 5 cm length; Thermo Fisher Scientific) followed by a 60 min separation under a column flow rate of 0.3 µl/min using linear gradient of 5–70% acetonitrile and 0.1% formic acid (both by Sigma-Aldrich). Separated and eluted peptides were ionised by electrospray ionisation followed by a MS survey scan (mass-to charge-ratio, m/z 400–2000) in the LTQ, sequentially selecting the five most abundant ions of peptides eluting from the LC at that time, before being passed on to the Orbitrap. The total cycle time for each MS/MS was approximately 30 milliseconds. The Orbi-trap took accurate mass measurement with the resolution of 30 000 parts per million (ppm) and ions were then fragmented in the linear ion trap by collision induced dissociation at collision induced energy of 35%. Subsequently, fractionated ions were separated according to their m/z ratio. Data was collected in data dependent MS/MS mode with dynamic exclusion set to 2 counts. Data analysis including mass spectra processing and database searching was carried out using Thermo Proteome Discoverer 1.2. with built-in Sequest (Thermo Fisher Scientific). Initial mass tolerances for protein identification by MS were set to 10 ppm. Up to two missed tryptic cleavages were considered and methionine oxidation was set as dynamic modification. Peptide sequences by MS/MS were only included when Xcorrelation scores were greater than 1.5, 2 or 2.2 for charge states 1, 2 and 3, respectively. An unambiguous identification was considered when at least two peptides matched to the protein. The protein FASTA databases were downloaded from www.uniprot.org, release 2012-03 including the complete entries from *homo sapiens* (taxon identifier 9606), *bos taurus* (9913), human immunodeficiency virus type 1 group M subtype B (isolate HXB2) (HIV-1) (11706), vesicular stomatitis indiana virus (strain San Juan) (VSIV) (11285), RD114 virus (11834), RDpro Env (Ikeda et al., 2003) AA sequence: (Bell et al., 2010, Ikeda et al., 2003), and GFP (P42212).

2.4. Knock-down of protein expression in vector producer cells using shRNA

Selected host proteins were knocked-down using lentiviral vector particles carrying the pGIPZ vector genome (LVs-GIPZ). PGIPZ target sequences were: TSG101- TGCAATAACTTATTCTGGG, ALIX- TAATCT GCAGCCTGATTAG, AHNAK- TAGATCAGGAGCTCCTACG) (Thermo Fisher Scientific). Transduced 293T cells were selected in DMEM with puromycin (10 µg/ml) twenty-four hours after transduction. After a minimum of 10 days in selection medium vector samples and producer cells were analysed for host protein knock-down levels and vector levels by western blotting.