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Modulation of the long terminal repeat promoter activity of small ruminant lentiviruses by steroids

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ABSTRACT

Production and excretion of small ruminant lentiviruses (SRLVs) varies with the stage of the host reproductive cycle, suggesting hormonal involvement in this variation. Stress may also affect viral expression. To determine if hormones affect SRLV transcriptional activity, the expression of green fluorescent protein (GFP) driven by the promoters in the U3-cap region of the long terminal repeats (LTRs) of different strains of SRLV was assessed in cell culture. High concentrations of steroids (progesterone, cortisol and dehydroepiandrosterone) inhibited expression of GFP driven by SRLV promoters. This effect decreased in a dose-dependent manner with decreasing concentrations of steroids. In some strains, physiological concentrations of cortisol or dehydroepiandrosterone (DHEA) induced the expression of GFP above the baseline. There was strain variation in sensitivity to hormones, but this differed for different hormones. The presence of deletions and a 43 base repeat in the U3 region upstream of the TATA box of the LTR made strain EV1 less sensitive to DHEA. However, no clear tendencies or patterns were observed when comparing strains of different genotypes and/or subtypes, or those triggering different forms of disease.

Introduction

The viruses that produce visna-maedi (VMV-like genotype A viruses) and caprine arthritis-encephalitis (CAEV genotype B viruses) are known collectively as small ruminant lentiviruses (SRLVs). Although infection with SRLVs is life-long, viral expression or excretion is not constant and is affected by stress and reproductive events, increasing at parturition and during lactation (Lerondelle and Ouzrout, 1990; Morin et al., 2003). VMV infections in sheep are reactivated by inducing lactation through the administration of oestradiol and progesterone (Morin et al., 2003). This variable viral load at different reproductive stages in the life of ewes and goats could be responsible for the inconsistent and weak correlations between results obtained by ELISA and PCR (Barquero et al., 2013).

The increased proviral load of SRLV at parturition and lactation could be related to variations in hormone levels, specifically progesterone, oestrogens, prolactin and glucocorticoids (Niermann and Buehring, 1997; Tejerizo et al., 2012), since these fluctuate through gestation and parturition. Oestrogen concentrations reach maximal values just before oestrus, but also increase towards the end of gestation until parturition (Challis et al., 1977). Concentrations of progesterone are high through gestation and decrease towards parturition. The maximum concentrations of glucocorticoids coincide with the beginning of parturition. Lactation is triggered when concentrations of prolactin and glucocorticoids increase.

In human immunodeficiency virus type 1 (HIV-1) infections, women have a lower plasma viral load (Farzadegan et al., 1998; Rezza et al., 2000) and higher $CD4^+$ T cell counts than men, suggesting that sex differences affect HIV-1 viral replication. Furthermore, women may have a higher risk of developing acquired immunodeficiency syndrome (AIDS) than men (Henderson et al., 1992; Farzadegan et al., 1998). In HIV-1 infected women, progesterone and oestrogen levels influence HIV-1 virion concentrations in blood, as estimated by RT-PCR (Asin et al., 2008). Sex differences have also been observed in mice infected with FIS-2 murine leukaemia virus (Bruland et al., 2003). Similar to HIV infection in human beings, cats infected with feline immunodeficiency virus (FIV) or feline leukaemia virus (FeLV) had increased levels of 17β-oestradiol, testosterone and cortisol, similar levels of progesterone and decreased levels of dehydroepiandrosterone (DHEA) compared to uninfected cats. These results suggest that FIV and FeLV infections are associated with deregulation of steroids, which might have consequences for disease progression (Tejerizo et al., 2012).

Steroids cross cell membranes by simple diffusion due to their lipophilic nature and bind to receptors, which are complexed to chaperones, such as heat shock protein 90. The hormone–receptor complexes act as intracellular transcription factors that bind with high affinity to hormone response elements (HREs) (Beato, 1991).

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One subfamily of steroid receptors includes the receptors for glucocorticoids (GR), progesterone (PR), androgens (AR) and mineralocorticoids (MR) (Cato et al., 1988). The other subfamily of steroid receptors includes the receptors for oestrogens (ER), vitamin D3, thyroid hormone and retinoic acid. The consensus amino acid sequence TGTYCT is the target/ligand for the GR/PR/AR/MR subfamily, whereas TGACC is the target/ligand for the ER subfamily (Beato, 1991).

The effects of hormones have been correlated with the expression of a range of retroviruses, including mouse mammary tumour virus (MMTV), Moloney murine sarcoma virus (MoMSV), endogenous murine and porcine retroviruses, bovine leukaemia virus (BLV), simian immunodeficiency virus (SIV), HIV and FeLV, and HREs have been identified in the long terminal repeat (LTR) regions of several of these retroviruses (Gomez-Lucia et al., 2007). However, to date, no studies have demonstrated the existence of these regulatory sequences in SRLV. The aim of the present study was to determine whether the activities of LTRs of different strains of SRLVs vary upon exposure to steroid hormones and thus may harbour functional HREs. The response of putative HREs to altered hormonal concentrations was assessed by inserting the LTR into an expression plasmid encoding green fluorescent protein (GFP).

Materials and methods

Viral strains and cell cultures

Six VMV strains from various clinical conditions and geographical origins were used as sources of LTR sequences: (1) EV1 (arthritic and pulmonary; Scotland, UK; GenBank Z31610; Sargan et al., 1991); (2) its laboratory variant EV1w, with a repeated sequence of 43 base pairs (bp) in the U3 region (Scotland, UK; GenBank Z31619.1; Sargan et al., 1991); (3) 258 (pulmonary; Zaragoza, Spain; GenBank KJ415282; this study); (4) 697 (outbreak of neurological disease; Castilla-León, Spain; GenBank HQ848062; Glaria et al., 2012); (5) KV1772 (outbreak of neurological disease; Iceland; GenBank S55323; clone described by Andrésson et al., 1993); and (6) 496 (outbreak of arthritis; Zaragoza, Spain; GenBank FJ195346; Glaria et al., 2009). Strains EV1, EV1w, 258, 697 and KV1772 were SRLV genotype A strains, whereas 496 was SRLV genotype B subtype B2.

Viruses were propagated in primary cultures of ovine skin fibroblasts (passage 12–14) from inguinal skin explants of seronegative ewes. Cells were grown at 37 °C in RPMI-1640 with 10% fetal bovine serum. At 70–80% confluency, cells were infected with 0.5 50% tissue culture infectious doses of each SRLV. The culture medium was changed after 3 days; on day 7, when the cells were markedly altered, the culture medium was discarded, the cells were washed with 10 mL phosphate buffered saline (PBS) and trypsinised. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen). PCR (Barquero et al., 2011) confirmed that uninfected skin fibroblasts did not contain SRLV provirus.

Hormones

Stock solutions of hormones (progesterone, cortisol and DHEA) were serially diluted in RPMI-1640 (PAA) from 1×10^{-2} M to final concentrations of 1×10^{-3} to 1×10^{-13} M. Progesterone (P-7556, Sigma-Aldrich) and cortisol (H-0396, Sigma-Aldrich) were water soluble, while DHEA (D-063, Sigma-Aldrich) required methanol for dilution. Since DHEA-induced apoptosis is inversely associated with glucose concentration (Yang et al., 2000), DHEA dilutions were supplemented with 3 mg glucose/mL.

Plasmid constructions and analysis

The LTR U3-cap regions of strains 496, 697 and KV1772 were amplified by PCR using oligonucleotide primers as described by Glaria et al. (2012), while the LTR U3-cap regions of strains EV1w, EV1 and 258 were amplified by PCR using primers U3-Fw (5′-CGCTCGAGACTGTCAGGACAGAGAACAAATG-3′; *Xho*I site underlined) and R-CAP-Rv (5′-GGCCTCTCCTGCCTGCCTGAACCATGGCG-3′; *Nco*I site underlined). PCR amplicons were first cloned into the pGEM-T-easy vector (Promega) and then subcloned into pAcGFP1-1 (Clontech) using *Xho*I and *Eco*RI restriction sites. The derived plasmids (pAcGFP-U3-cap) were confirmed by sequencing using BigDye Terminator v3.1 chemistry on a 3730 DNA Analyzer (Applied-Biosystems). Sequences were aligned using ClustalW2.[1]

[1] http://www.ebi.ac.uk/Tools/msa/clustalw2/.

Myb, AP1, nuclear factor κ light chain enhancer of activated B cells (NF-κB), nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2 (NFATC2), SP1, nuclear factor I (NFI) and signal transducer and activator of transcription (STAT) 1 transcription binding sites (TBS), with a putative role in hormonal response (Cato et al., 1988; Brüggemeier et al., 1990; Beato, 1991; Ghosh, 1992; Russo et al., 1999; Kino et al., 2000; Deroo and Archer, 2002; Bruland, 2003) were located using the Jaspar algorithm (Bryne, 2008). GR/PR (TGTYCT) and ER (TGACC) (Beato, 1991) sites were searched manually.

Transfection

Skin fibroblasts (1×10^5 cells per well in 24-well plates) were transfected with 1 μg each pAcGFP-U3-cap construct using 2 μL Lipofectamine 2000 (Invitrogen). After 6 h, the medium was changed and hormones were added at final concentrations of 1×10^{-3}, 1×10^{-5}, 1×10^{-7}, 1×10^{-9}, 1×10^{-11} and 1×10^{-13} M. Cells were cultured for 48 h, trypsinised and fixed with 0.5% paraformaldehyde in PBS. All experiments were repeated at least three times. Controls were non-hormone treated transfected cells and cells transfected with the plasmid containing the cytomegalovirus (CMV) promoter (pAcGFP-CMV). The CMV promoter was amplified from eucaryotic expression plasmid pCNCR-HA (Jauregui et al., 2012) using primers Fw: 5′-TCG CTCGAGTTCCGCGTTACATAACTTACG-3′ and Rv: 5′-TCGGAATTCGGTTCACTAA ACGAGCTCTG-3′ (*Xho*I/*Eco*RI restriction sites underlined). Once the sequence was verified, the amplicon was digested with the appropriate restriction enzymes and subcloned into pAcGFP. GFP expression was measured by flow cytometry (FACScalibur, BD Biosciences) using an excitation wavelength of 488 nm, with detection filters of 515 nm and 585 nm. Only cultures where the percentage of live cells expressing GFP was ≥8% were analysed. The mean fluorescence intensity (MFI) of GFP in the gated cells was normalised by subtracting the intensity value observed in non-hormone treated cells from that obtained at each hormone concentration. At least 1×10^4 cells of each population were analysed.

Statistical analysis

The Mann–Whitney *U* non-parametric test in SPPS.18, with a 95% confidence interval, was applied for mean MFI comparisons. Results were compared using the Bonferroni correction with a critical *P* value (α) of 0.05.

Results

At the concentrations and conditions used in this study, neither the steroids, nor Lipofectamine, affected cell survival, since cell viability was ≥85% in all cases. DHEA inhibited cell viability when glucose was not added to the dilution medium (cell mortality without glucose was 55.0% at 1×10^{-3} M and 16.6% at 1×10^{-11} M). When 3 mg/mL glucose was added to the dilution medium, the cell viability was comparable to exposure to the other hormones. The percentage of transfection was 8.1–31.3%. Assessment of the effect of progesterone, cortisol and DHEA on the expression of GFP driven by the LTR of VMV strains (Fig. 1) demonstrated that the three hormones were mostly inhibitory at high concentrations ($\geq 1 \times 10^{-5}$ M), with the exception of the effect of DHEA on strain 258. The inhibitory effect decreased upon dilution. In most cases, 1×10^{-3} M and 1×10^{-5} M were significantly inhibitory ($P < 0.05$) (Fig. 1). Expression of GFP driven by a CMV promoter (pCMV) in transfected cells was always high, both in non-hormone-treated cultures and in the presence of either progesterone or cortisol, even at 1×10^{-5} M, the highest concentration tested (data not shown).

Each hormone exerted some effect on the different strains, although no two strains had the same results with the three hormones studied (Fig. 1). When compared to EV1, the inhibitory effect of DHEA was decreased in the laboratory variant EV1w, which contains a 43 bp repeat in the U3 region upstream of the TATA box, suggesting the involvement of this U3-cap region in sensitivity to this hormone (Fig. 1). At physiological levels (0.6×10^{-7} to 3×10^{-11} M for progesterone, 1.4×10^{-7} to 2.8×10^{-11} M for cortisol and 1.6×10^{-7} to 5.2×10^{-11} M for DHEA), the effect of the hormones was not as inhibitory as that observed at higher concentrations and, in some cases (e.g. cortisol on strain 258 and DHEA on strain 258), expression of GFP was lower upon exposure to hormones than in infected cells not exposed to hormones (Fig. 1). Thus, the inhibitory pattern of progesterone across the strains was not seen with the other hormones for all the strains. Strains 496 (genotype B2)