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Lentiviral latency in peripheral CD4+ T cells isolated from feline immunodeficiency virus-infected cats during the asymptomatic phase is not associated with hypermethylation of the proviral promoter

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ABSTRACT

Lentiviral latency remains a principal obstacle to curative AIDS therapy. Transcriptional repression and latency permits lentiviruses to evade host immune responses and antiretroviral drugs. We have established a model of peripheral CD4+ T cell lentiviral latency in cats experimentally infected with feline immunodeficiency virus (FIV). Multiple mechanisms of lentiviral transcriptional repression have been proposed including epigenetic mechanisms resulting in promoter hypermethylation and/or chromatin condensation. Methylation of promoter-associated cytosines in the cytosine-guanine dinucleotide (CpG) has been associated with transcriptional repression in both eukaryotic promoters and integrated retroviral genomes. Using methylcytosine mapping, we examined the CpG methylation patterns in both the 5′ and 3′ long terminal repeats (LTR) of the FIV provirus in peripheral blood mononuclear cells, monocytes and CD4+ T cells isolated during the acute and asymptomatic phases of infection. Here we report no evidence that proviral promoter hypermethylation is associated with lentiviral latency in peripheral CD4+ T cells and monocytes obtained from experimentally FIV-infected cats.

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1. Introduction

Feline immunodeficiency virus (FIV) infection of cats is an important animal model of human immunodeficiency virus-1 (HIV-1) pathogenesis (Burkhard and Dean, 2003; Elder et al., 2010; Sparger, 2005). These two viruses are phylogenetically related (Savarino et al., 2007) and both infect naïve and activated CD4+ T cell subsets as well as monocytes in the susceptible host (Bendinelli et al., 1995; Burkhard and Dean, 2003; Joshi et al., 2004). FIV-infected cats develop an acute infection syndrome followed by a prolonged asymptomatic period and terminal immunodeficiency syndrome (Barlough et al., 1991; Ikeda et al., 1996; Joshi et al., 2004). Our laboratory has established a model of lentiviral cellular latency in experimentally FIV-infected cats during the asymptomatic phase of infection (Murphy et al., 2012). Cats infected with a biological isolate of FIV clade C for approximately 3 years demonstrated undetectable plasma viral loads by sensitive real-time PCR assays from approximately 10 months post-infection onward. Viral DNA was repeatedly detected in peripheral CD4+ T cells isolated from infected cats whereas viral RNA was not detected at multiple time points during the early chronic phase of infection. In addition, circle junction viral DNA products, thought to be indicators of ongoing viral replication (Teo et al., 1997), were not detectable from uncultured, magnetic column-isolated CD4+ T cells. These findings are consistent with cellular latency in peripheral CD4+ T cells. The ability to isolate latently FIV-infected CD4+ T lymphocytes from FIV-infected cats provides a platform for the study of *in vivo* mechanisms of lentiviral latency.

Lentiviral latency remains a principal obstacle to curative AIDS therapy (Mok and Lever, 2007). Transcriptional repression and latency permit lentiviruses to evade host immune responses and antiretroviral drugs. Methylation of promoter-associated cytosines in the 5′ cytosine-guanine dinucleotide (CpG) has been associated with transcriptional repression in mammalian cells (Lorincz et al., 2000) and integrated retroviral genomes (Harbers et al., 1981; Hu et al., 1984; Koiwa et al., 2002). A clear relationship has been demonstrated between methylation density and transcriptional repression (Lorincz et al., 2000). Viral promoter methylation has been proposed as an important mechanism of lentiviral latency (Chavez et al., 2011; Kauder et al., 2009; Mok and Lever, 2007) and methylation of CpG dinucleotides in the HIV-1 promoter has been shown to be associated with latency (Bednarik et al., 1990; Blazkova et al., 2009; Chavez et al., 2011; Duverger et al., 2009). Cytosine methylation is thought to induce transcriptional repression *via* two general mechanisms – the direct inhibition of transcription

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factor binding (Watt and Molloy, 1988) and/or the recognition and recruitment of a class of proteins that induces a restrictive chromatin state (Boyes and Bird, 1991; Hendrich and Bird, 1998). Studies have demonstrated that 5-azacytidine, a DNA methylation antagonist, activates the integrated FIV promoter in *in vitro* reporter assays, providing evidence that the FIV promoter may be inhibited by DNA methylation (Ikeda et al., 1996). In addition, recent studies in our laboratory indicate that the FIV promoter isolated from latently infected *in vivo*-derived CD4+ T cells is physically associated with chromatin in a restrictive state (McDonnel et al., 2012).

The patterns of proviral promoter methylation in FIV-infected and *in vivo*-derived peripheral blood mononuclear cells (PBMC), CD4+ T cell subsets and monocytes have not been reported. The FIV 5′ long terminal repeat (LTR), comprised of the U3, R and U5 regions, functions as the viral promoter. The FIV-C LTR has 10 CpG dinucleotides available for cytosine methylation. The HIV-1 LTR contains 10 CpG dinucleotides within the U3, 0 in R, 1 in U5 and 3 in the gag leader regions (Blazkova et al., 2009; Duverger et al., 2009). We formulated the hypothesis that a pattern of hypermethylation of the integrated feline immunodeficiency proviral promoter region is associated with lentiviral latency in peripheral CD4+ T cells isolated from FIV-infected cats during the asymptomatic phase of infection. In order to address this hypothesis, we utilized an experimental method, sodium bisulfite-mediated methylcytosine mapping (Chavez et al., 2011), coupled with nested PCR amplification, amplicon cloning and sequencing to assess the specific methylation patterns of CpG dinucleotides within both the 5′ and 3′ proviral LTRs. Here we report no evidence that proviral promoter hypermethylation is associated with latency in peripheral CD4+ T cells and monocytes obtained from experimentally FIV-infected cats.

2. Materials and methods

2.1. Animals and virus

Six FIV specific pathogen free (SPF) kittens were purchased from the breeding colony of the Feline Nutrition Laboratory, University of California at Davis (UC Davis). At time of purchase, the kittens ranged in age from 4 to 5 months and were housed in the Feline Research Laboratory of the Center for Companion Animal Health, UC Davis. The kittens were intramuscularly inoculated with FIV-C-Pgmr viral inoculums (kittens 165, 184, 187 and 186). Two control kittens (183 and 185) were mock-inoculated with 1 ml of sterile culture media. The FIV-C-Pgmr isolate was provided by Drs. E. Hoover and N. Pedersen. The kittens were monitored as described previously (Murphy et al., 2012). The study protocol was approved by the UC Davis Institutional Animal Care and Use Committee.

2.2. Hematologic assessment of cats

Whole blood samples were obtained from the infected and mock-infected cats every 2–4 weeks. Complete blood cell counts were performed on a Coulter counter (Coulter ACT diff, Beckman Coulter) and differentials were performed on cytologic smears of whole blood. The total number of white blood cells was further confirmed with the BD Unopette System (Beckton, Dickinson and Company). Total PBMC isolated by Ficoll-Hypaque gradient centrifugation were phenotyped for CD4+ and CD8+ lymphocyte frequency by flow cytometry using anti-feline CD4 (FE1.7B12) and anti-feline CD8α (FE1.10E9). Secondary antibody, fluorescein-conjugated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) was utilized for detection. Samples were analyzed on a FACScan instrument (Becton Dickinson, San Jose, CA) using FloJo v8.6.3 flow cytometry analysis software (Tree Star, Ashland, OR). Absolute CD4+ and CD8+ cell counts in blood were derived from absolute white blood cell counts and subset frequencies determined by flow cytometry. The CD4/CD8 ratio was determined by dividing the absolute number of CD4+ cells by the absolute number of CD8+ cells.

2.3. In vitro infection of SPF PBMC and ex vivo activation of FIV-infected PBMC

Blood was obtained from a SPF FIV-negative cat (Feline Nutrition Research Colony, UC Davis) *via* jugular venipuncture- and PBMC were isolated by density gradient centrifugation through Ficoll-Hypaque 1044 (Sigma–Aldrich). PBMC were cultured for 24 hours at 2×10^6 cells/ml in RPMI 1640 media containing 2.05 mM L-glutamine (Hyclone – ThermoFisher Scientific, Logan, UT), 10% (v/v) fetal bovine serum (Hyclone), 100 U penicillin/ml and 100 μg streptomycin/ml (Pen/Strep 100× solution, Hyclone), 100 U/ml recombinant human interleukin 2 (Hoffman – La Roche Inc.) and 0.5 mg/ml Concanavilin A (Con A, ThermoFisher Scientific). After 24 hours, the media was replaced with fresh media lacking Con A. On day 4, the PBMC were placed in fresh media and infected with FIV-C (7×10^6 copies RNA/ml). On day 11, PBMC were harvested for nucleic acid isolation (RNA/DNA Minikit, Qiagen) and bisulfite treatment. FIV infection was confirmed with real-time PCR for FIV gag (presence of viral DNA and cDNA), as described previously (Murphy et al., 2012).

2.4. Transcriptional reactivation of latently infected feline PBMC

Isolated PBMC from FIV-infected cats (184, 186, 187, 165) and an uninfected cat (183) at 50 weeks post infection were cultivated *in vitro* using previously described protocols (Murphy et al., 2012). Preculture DNA samples (day 0) were isolated from the PBMC using a commercial kit (Qiagen DNA Mini kit, Qiagen). PBMC were harvested on day 7 or 13 and processed for genomic DNA and total RNA using a commercial kit (AllPrep RNA/DNA Minikit, Qiagen). The RNA was DNAse treated (Turbo DNAse, Ambion), and reverse transcribed (Origene First Strand cDNA Synthesis) according to the manufacturers' protocols. Nucleic acid was assayed for the presence of FIV gag, 2-LTR CJ- and feline GAPDH as previously described (Murphy et al., 2012). Transcriptional reactivation was defined as detectable 2 LTR CJ amplicons and concurrently detectable FIVgag cDNA.

2.5. CD4+ lymphocyte and monocyte purification

Feline whole blood was collected as described above. Isolated PBMC were resuspended and separated through a combination of magnetic-bead columns (Miltenyi Biotec Inc.) and flow cytometric cell sorting as described previously (Murphy et al., 2012). Briefly, cells were enumerated using an automated cell counter (Coulter A^{C}T diff, Beckman Coulter) and approximately 2.0×10^7 were used for primary antibody (Ab) binding. Leukocytes were initially depleted of monocytes, B cells and granulocytes *via* an LD column (MACS Separation Columns, Miltenyi Biotec) and a cocktail of mouse anti-feline antibodies: anti-CD11b (clone CA16.3E10-IgG1), anti-CD8α (clone FE1.10E9-IgG1), anti-CD21 (clone CA2.1D6-IgG1), (antibodies provided by Peter Moore, UC Davis) and goat-anti mouse IgG-microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The flow-through cell fraction was treated with mouse anti-feline CD25 (clone 9F23; gift of Koichi Ohno, University of Tokyo) and applied onto a MS column (MACS Separation Columns, Miltenyi Biotec) along with goat-anti mouse IgG-microbeads (Miltenyi Biotec). Column-sorted cells were treated with a blocking antibody (1 mg/ml, CA2.1D6,

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