Didanosine causes sensory neuropathy in an HIV/AIDS animal model: impaired mitochondrial and neurotrophic factor gene expression

Yu Zhu, Joseph M. Antony, Jose A. Martinez, D. Moira Glerum, Valentine Brussee, Ahmet Hoke, Douglas Zochodne and Christopher Power

Departments of Medicine and Medical Genetics, University of Alberta, Edmonton AB, Canada T6G 2S2, Department of Clinical Neuroscience, University of Calgary, Calgary AB, Canada T2N 4N1 and Departments of Neurology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

Correspondence to: Dr C. Power, Department of Medicine, 611 Heritage Medical Research Centre, University of Alberta, Edmonton AB, Canada T6G 2S2
E-mail: chris.power@ualberta.ca

Antiretroviral toxic neuropathy (ATN) has become a common peripheral neuropathy among HIV/AIDS patients, for which the underlying pathogenesis is uncertain. Indeed, no models exist for ATN that assess the interaction between retroviral infection and antiretroviral therapy. Herein, we developed ex vivo and in vivo models of ATN induced by didanosine (ddl) following infection by the lentivirus, feline immunodeficiency virus (FIV), permitting us to address the working hypothesis that ddl mediates ATN through mitochondrial injury in neurons. We investigated neuronal morphology, neurobehavioural testing, viral load, mitochondrial and neurotrophic factor gene expression after ddl treatment of FIV-infected and uninfected animals or dorsal root ganglia (DRG) cultures. Ddl caused concentration-dependent neuronal injury in cultured feline DRGs (P < 0.05), together with reduced viral replication and diminished expression of mitochondrial cytochrome C oxidase subunit I gene (mtCOX I) and the neurotrophin, brain-derived neurotrophic factor (BDNF). Indeed, BDNF treatment reversed neuronal injury caused by FIV infection in the presence or absence of ddl exposure (P < 0.05). In vivo FIV infection revealed delays in withdrawal latency to a noxious stimulus, which were exacerbated by ddl treatment. Epidermal density of nerve endings was reduced after FIV infection (P < 0.05), especially with ddl treatment. Although viral replication in blood was suppressed in ddl-treated animals (P < 0.05), ddl had a limited effect on viral abundance in DRGs of the same animals. Ddl decreased mtCOX I expression in DRG neurons of FIV-infected animals (P < 0.05). BDNF expression was downregulated by ddl in DRG Schwann cells following FIV infection. Thus, ddl treatment during FIV infection resulted in additive pathogenic effects contributing to the development of ATN, which was associated with mitochondrial injury on neurons and reduced BDNF production by Schwann cells in DRGs, highlighting the convergent pathogenic effects that antiretroviral drugs might have in patients with HIV infection.

Keywords: HIV; FIV; didanosine; BDNF; mitochondria; neuropathy

Abbreviations: ATN = antiretroviral toxic neuropathy; ddl = didanosine; FIV = feline immunodeficiency virus; DRG = dorsal root ganglion; NGS = normal goat serum


Introduction

Painful sensory neuropathy has become the principal neurological complication of human immunodeficiency virus type 1 (HIV-1) infection in the industrialized world (Verma et al., 2005). The prevalence of peripheral neuropathy has increased among HIV/AIDS patients with the greater use of nucleoside reverse transcriptase inhibitors (NRTIs), particularly zacitabine (ddC), stavudine (d4T) and didanosine (ddl), leading to the recognition of the disorder, termed antiretroviral toxic neuropathy (ATN). Distal sensory polyneuropathy (DSP), which is directly caused by HIV-1 infection of the peripheral nervous system, and ATN affect over 50% of HIV-infected patients in North American clinics (Moyle and Sadler, 1998; Morgello et al., 2004), and share similar clinical features (Simpson and Tagliati, 1995; Schifitto et al., 2001). These features include

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symptoms of numbness, burning pain and paresthesias, typically with a symmetrical onset in the lower extremities accompanied by reduced or absent deep tendon reflexes although in ATN, implementation of the neurotoxic NRTIs within the preceding 6 months is evident. The corresponding pathological features include distal degeneration of sensory axons, described as ‘dying back’, reduced density of intraepidermal nerve fibres, of small and large myelinated fibres in nerves, and in particular, of unmyelinated fibres (Pardo et al., 2001). In fact, dorsal root ganglion (DRG) neuronal loss has also been demonstrated in association with DSP (Bradley et al., 1998). The occurrence of ATN and DSP not only affects the quality of life of patients with HIV-1 infection (Pandya et al., 2005), but also limits antiviral therapeutic options. Ideally, a pathogenesis-based treatment for ATN would allow patients to continue NRTI therapy that includes potentially neurotoxic antiretroviral drugs. However, the pathogenesis of ATN remains unclear although NRTI-related mitochondrial injury has been implicated (Lee et al., 2003).

Highly active antiretroviral therapy (HAART) reduces the rate of HIV-1 replication, thus leading to improved immunity and clinical well being (Brinkman et al., 1998a). The NRTIs serve as the backbone of combination HAART regimens, exerting antiretroviral activity via drug phosphorylation, incorporation into viral DNA, DNA chain termination, which inhibits the viral reverse transcription enzyme (Brinkman et al., 1998a; Carr et al., 2000; Carr and Cooper, 2000). Several adverse effects of HAART including ATN have been reported, which are dose-dependent (Dagan et al., 2002; Lee et al., 2003). While earlier studies from our group have suggested the mitochondrial injury (Keswani et al., 2003a) might underlie the development of ATN, definitive in vivo and ex vivo evidence for mitochondrial injury in defined neural cell types is lacking. Of note, the NRTIs are also competitive inhibitors of human mitochondrial DNA polymerase-gamma, which is a nuclear-encoded protein required for mitochondrial DNA replication (Kakuda, 2000).

In the present study, using an animal model of HIV-1 infection, feline immunodeficiency virus, FIV, we investigated the mechanism(s) underlying development of ATN. FIV is a naturally occurring lentivirus, like HIV-1, causing AIDS in domestic cats (Hartmann, 1998), and is one of the established animal models for HIV-1 infection (Burkhard and Dean, 2003; Kennedy et al., 2004). It shares many similarities with HIV-1 in terms of viral properties and pathological aspects (Patrick et al., 2002). FIV-infected animals exhibit immunosuppression together with rapid onset of DSP (Kennedy et al., 2004), and moreover, FIV infection causes neuronal injury in DRG cultures and previous studies from our group have shown that selective FIV infection of macrophages ex vivo cultured and necropsied-derived feline DRGs. In fact, FIV infection of DRGs results in neuronal death, neurite retraction and neuronal soma atrophy (Zhu et al., 2005). Herein, we hypothesized that the prototypic neurotoxic NRTI, ddI, exacerbates peripheral neuropathy during lentivirus infection in additive manner through mitochondrial injury in DRG neurons. The experimental strategy involved comparisons of ddI’s effects using both in vivo and ex vivo models that permitted comparison of the ddI’s actions with and without concurrent lentivirus infection and its consequences (immunosuppression and innate immune activation). Our present studies revealed that ddI treatment induced ATN together with DRG neuronal injury. Indeed, ddI caused mitochondrial injury with reduced mitochondrial cytochrome C oxidase subunit I (mtCOX I) abundance in neurons together with diminished brain-derived neurotrophin factor (BDNF) expression in proximate Schwann cells.

### Methods and Materials

#### Virus preparation

The FIV strain used in this study was an infectious neurovirulent recombinant molecular clone, V1-Ch, derived by transfection of CrFK cells and amplification in feline peripheral blood mononuclear cells (PBMCs), as described previously (Johnston et al., 2000). Culture supernatants from FIV-infected feline PBMC, which served as sources of infectious virus for these experiments, were cleared of cellular debris by centrifugation and titered by limiting dilution, as described previously (Power et al., 1998).

#### Experimental animals and tissue collection

Specific pathogen-free neonatal (day 1) kittens were infected with 0.2 ml of infectious (10⁴ TCID₅₀/ml) or heat-inactivated virus (control cats) in accordance with CCAC guidelines, as described previously (Power et al., 1998). FIV-infected and mock-infected animals were treated with ddI (33 mg/kg daily) by oral gavage starting at 6 weeks post-infection until 12 weeks post-infection. Kittens were weaned at 6 weeks and monitored until 12 weeks of age at which time, all animals were euthanized, as previously described (Power et al., 1998; Kennedy et al., 2004). Sera were collected and stored at −80°C immediately. L5 DRGs were collected and fixed in 4% PBS-buffered paraformaldehyde for 12 h at 4°C and submerged in PBS containing 20% sucrose overnight at 4°C following wash in PBS. The fixed DRGs were embedded in O.T.C and fast frozen in isopentane with dry ice, and stored at −80°C until sectioned. L6 DRGs were immediately frozen on dry ice and kept at −80°C until used. The footpads were collected, fixed in PLP, transferred to cryoprotectant (20% glycerol) and kept at −20°C. The sural nerves were fixed in 2.5% glutaraldehyde in 0.025M cacodylate buffer.

#### Neurobehavioural testing

Timed latency to hind limb withdrawal response (three trials, 1 min apart) to brief radiant heat aimed at the ventral aspect of the paw from a heat lamp applied through a plexiglass plate was assessed among uninfected control (n = 9), FIV-infected (n = 9), FIV-infected with ddI treatment (n = 6) and FIV-uninfected animals with ddI treatment (n = 5), as previously reported (Kennedy et al., 2004).
Flow cytometry analysis
Peripheral blood monocytes (PBMCs) were isolated from blood of FIV-infected and uninfected animals with or without ddI treatment as previously reported (Zhu et al., 2005). PBMCs were labelled with anti-feline CD4 or anti-feline CD8 monoclonal antibodies. FITC-conjugated goat anti-mouse IgG1 antibody was applied after primary antibodies’ labelling. Omitting the primary antibodies served as controls. Analysis was performed using the FACScan (Becton Dickinson) flow cytometer. Cells (1 × 10⁶) were analysed for each sample (Power et al., 1998).

Nerve morphometry
Sural nerves were processed for epon embedding (Zochodne et al., 1997). Briefly, samples were fixed in 2.5% glutaraldehyde in 0.025 M cacodylate buffer overnight, serially washed in 0.15 M cacodylate buffer, post-fixed in 2% osmium tetroxide in 0.12 M cacodylate, dehydrated using a series of graded alcohols and propylene oxide and embedded in epon. Transverse sections (1.0 μm thick) 15-mm distal to the sciatic trifurcation were cut with an ultramicrotome (Reichert, Austria) utilizing glass knives and stained with toluidine blue. Morphological examination of specimens was performed using Scion Image (Scion, Frederick, MD). Video images were obtained with a light microscope (Axioskope, Zeiss, Toronto, Ontario, Canada) and attached digital camera (Axiocam, Zeiss, Toronto, Ontario, Canada) interfaced with a computer. The computer-assisted image analysis allowed for the determination of the number, caliber and size frequency of intact myelinated fibres. All counting was performed with the microscopist blinded to the identity of the animal group.

Electrophysiology
Electrophysiological recordings were made under anesthesia (Viking I; Nicolet, Madison, WI), as reported elsewhere in rats (Zochodne and Ho, 1992). Sensory conduction velocity in cat was recorded by stimulating distally and recording proximally at fixed distances. Temperature near nerves was kept constant at 37 ± 1°C using a subdermal thermistor and heating lamp.

Epidermal nerve density
The footpads fixed by PLP were sectioned at 50 μM thickness. Immunohistochemistry was performed using an anti-protein gene product 9.5 (PGP9.5) antibody (Chemicon, Temecula, CA, USA). An examiner, blinded to the identity of the animal group, counted the number of intraepidermal nerve fibres that stained with the anti-PGP9.5 antibody, which was expressed as the number of fibres/mm² of skin (Kennedy et al., 2004).

Feline dorsal root ganglion (DRG) cultures
Culture plates and chamber slides (Nunc, Naperville, IL) were coated with a 1:2 dilution (in media v/v) of matrigel (BD Biosciences, Quebec, Canada). DRGs from adult FIV seronegative healthy cats and FIV-infected cats were removed under a dissecting microscope. Cleanly dissected DRGs were incubated at 37°C for 100 min in digestion media containing 0.5 mg/ml trypsin (Gibco BRL, Burlington, Canada), 1 mg/ml collagenase type IA (Sigma-Aldrich, Oakville, Canada) and 0.1 mg/ml DNAase type I (Roche Diagnostics Corporation) in DMEM (Sigma-Aldrich, Oakville, Canada). Digestion medium was removed by centrifugation at 1500 rpm for 5 min and cells were washed twice with culture medium (DMEM containing 10% heat-inactivated fetal bovine serum, 5% horse serum (Gibco BRL, Burlington, Canada), 2 mM l-glutamine and 1% N-2 supplement (Gibco BRL, Burlington, Canada), 0.1 mg/ml penicillin/streptomycin (Gibco BRL, Burlington, Canada) and 5% L929 cell-conditioned medium. The tissue solution was triturated using a sterile glass pipette until a homogeneous cell suspension was obtained and adjusted to a concentration of 0.1 × 10⁶ cells/ml. Cells including neurons, Schwann cells and macrophages (Zhu et al., 2005) were seeded into 8 well chamber slides (250 μl/well) or 24 well plates (1 ml/well) and incubated at 37°C, 5% CO₂. On the next day, medium was changed, and every third day thereafter. DRG cultures were composed of macrophages, neurons and Schwann cells, as previously reported (Zhu et al., 2005).

FIV infection with ddI, or BDNF treatment of DRG cultures
Following 7 days of ex vivo differentiation, DRG cultures from healthy animals and FIV-infected animals were treated with different concentrations of ddI (1, 10, 100 μM) for 4 days, or DRG cultures from healthy animals were infected with FIV V1-Ch at different input titres (TCID₅₀ 10⁴/ml; 10⁶/ml/well for 8 well chamber slides and 100 μl/well for 24 well plates), incubated at 37°C with 5% CO₂ for 6 h, following which the cultures were washed to remove input virus and cultured under humid conditions at 37°C with 5% CO₂ for 2 days, then treated with 10 μM of ddI and/or BDNF (60 ng/ml) for another 4 days. The DRG cells were fixed with 4% PBS-buffered paraformaldehyde or collected in Trizol for RNA and DNA extraction.

Immunofluorescence detection and confocal microscopy analysis
Two percent PBS-buffered paraformaldehyde fixed cultured DRG cells on chamber slides were incubated with PBS containing 30% normal goat serum (NGS) overnight at 4°C to block non-specific staining. The sections and slides were exposed either to mouse anti-MAP-2 (clone HM-2, 1:100 dilution, Sigma), or mouse anti-human mitochondrial COX I (1:100 dilution, Invitrogen, USA), or mouse anti-human GFAP (1:100 dilution, Pharmingen, USA), and rabbit anti-human BDNF (1:100 dilution, Santa Cruz, USA), overnight at 4°C followed by washing in PBS, then incubated with either Cy3 conjugated goat anti-mouse (1:1000 dilution; Molecular Probes, Eugene, OR) for 2 h at room temperature in dark followed by repeated washing in PBS. The slides were mounted with Gelvatol. The specificity of staining was confirmed by omitting the primary antibody. Images were captured on a LSM510 META (Carl Zeiss Microimaging, Inc.) confocal laser-scanning microscope and analysed using LSM 5 Image Browser software (Carl Zeiss Microimaging, Inc.). A Zeiss Axioskop 2 upright microscope (Oberkochen, Germany) and Spot system (Diagnostic Instruments, Sterling Heights, MI) provided digital images for quantitative analyses of neurite length and soma size of neurons using the public domain program Scion Image (Scion, Frederick, MD).

Quantitation of neuronal injury and loss
Following completion of the immuno-labelling protocol, slides were also imaged for subsequent measurements of neuronal soma...
area, maximal neurite length per neuron, using a minimum of 25–50 neurons per individual treatment from three separate wells by an examiner unaware of the slide identity. Using ScionImage image analysis software (Scion Corporation, Frederick, MD, USA), each parameter was assessed, as previously reported (Hannila and Kawaja, 2003). In addition, cell survival was measured in terms of the number of MAP-2 immunopositive neurons per unit area in triplicate (Zhu et al., 2005). All experiments were repeated at least twice and performed in triplicate.

Feline macrophage cultures and FIV infection

Feline macrophages were isolated from the pelvic and femoral bone marrow of healthy specific pathogen-free (SPF) cats, as described previously (Riches and Underwood, 1991). The cells were cultured in DMEM containing 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FBS and 10% L929 cell-derived-conditioned medium as a source of macrophage colony-stimulating factor-1 in 10% CO₂ in plastic dishes permitting cellular differentiation, resulting in monolayer cultures that were >95% pure macrophages. These cells were infected with FIV (TCID₅₀ 10⁴/ml; 100 μl/well for 2 days. The supernatant was collected and stored at –80°C for further use.

Feline Schwann cell cultures

Schwann cells were prepared from 12-week-old healthy cats and purified by a modified Brockes’ method (Brockes et al., 1979). The purified Schwann cells were replaced in 24 well plates at a density of 10,000 cells/well as previously reported (Keswani et al., 2003b) to allow for treatment. Following 4 days of treatment with supernatant of FIV-infected macrophage culture in presence or absence of ddI (10 μM), the Schwann cells were collected in Trizol (Life Technologies, Gaithersburg, MD) and stored at –80°C for RNA and DNA extraction.

RNA and DNA extraction

DRGs from FIV-infected and uninfected animals with or without ddI treatment animals, DRG culture cells and Schwann cells were homogenized and lysed in 1 ml Trizol (Life Technologies, Gaithersburg, MD) and 1 ml of plasma from FIV-infected and uninfected with or without ddI treatment, extracted in Trizol, were cleared by centrifugation, and protein levels were quantified using a Bradford assay (BIO-RAD, Mississauga, ON). Equal amounts of protein (20 μg/sample), determined by Coomassie blue staining and subsequent detection of housekeeping proteins, were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blocked with 10% milk in TBST (25 mM Tris-buffered saline and 0.1% Tween 20). A monoclonal antibody recognizing mtCOX I (Invitrogen, U.S.A) was diluted 1:500 in TBST containing 5% milk, and then incubated with membrane at 4°C overnight. Membrane was washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Lab Inc., Westgrove, PA) diluted 1:2000 in 5% milk-TBST.

Real-time PCR

First-strand cDNA was synthesized by using aliquots of 1 μg of total RNA, reverse transcriptase and random primers (Zhu et al., 2005). A real-time PCR protocol using primers that detect the FIV pol gene was used to determine the number of copies of viral RNA/ml in serum of FIV-infected and uninfected animals with or without ddI treatment, as previously reported (Kennedy et al., 2004). Specific genes were quantified by real-time PCR using i-Cycler IQ system (Bio-Rad, Mississauga, ON). cDNA prepared from total RNA of cultured DRG cells and DRG tissues was diluted 1:1 with sterile water and 5 μl were used per PCR reaction. One nanogram of genomic DNA from cultured DRG cells and DRG tissues was applied per PCR reaction. The primers used in the real-time PCR were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Forward primer, 5’-AGC CTT CTC CAT GGT GGT GAA-3’; reverse primer, 5’-CGG AGT CAA CGG ATT TGG TCG-3’; feline mtCOX I: forward primer, 5’-TGC CAC GAC GTT ATT CTG A-3’; reverse primer, 5’-GTG GTA CGG AGG AGG ACA-3’ (annealing temperature 50°C). BDNF: forward primer, 5’-GAA AGT CCC GGT ATCCAA AG-3’; reverse primer, 5’-CCA GCC AAT TCT CTT TT-3’ (annealing temperature 50°C). TrkB: forward primer, 5’-TGG CAT CAC CAA CAG TCA GCT-3’; reverse primer, 5’-TCC TTG CGT CCA TTG TCA CT-3’ (annealing temperature 54°C). Insulin-like Growth Factor-I (IGF-I): forward primer, 5’-GCT CTT CAG TCC TTC GTG GGA-3’; reverse primer, 5’-AGA TCA CAG CTC CGG AAG CA-3’ (annealing temperature 58°C); GFAP: forward primer, 5’-GAG ATC GCC ACC TAC AG-3’; reverse primer, 5’-CAC ATC CTT GRG CTC CG-3’ (annealing temperature 52°C). Semi-quantitative analysis was performed by monitoring real-time increase in fluorescence of SYBR-Green dye. Real-time fluorescence measurements were performed, and a threshold cycle value for each gene of interest was determined, as reported previously (Power et al., 2003). All data were normalized to the GAPDH mRNA threshold cycles level and expressed as mRNA relative fold change (RFC).

Western blot

DRG tissues from FIV-infected and uninfected animals with or without ddI treatment, extracted in Trizol, were cleared by centrifugation, and protein levels were quantified using a Bradford assay (BIO-RAD, Mississauga, ON). Equal amounts of protein (20 μg/sample), determined by Coomassie blue staining and subsequent detection of housekeeping proteins, were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blocked with 10% milk in TBST (25 mM Tris-buffered saline and 0.1% Tween 20). A monoclonal antibody recognizing mtCOX I (Invitrogen, U.S.A) was diluted 1:500 in TBST containing 5% milk, and then incubated with membrane at 4°C overnight. Membrane was washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Lab Inc., Westgrove, PA) diluted 1:2000 in 5% milk-TBST. Immunoreactive proteins were detected by chemiluminescence (Roche Diagnostics, Laval, QC). The membrane was stripped and blocked with 10% milk-TBST 1 h at room temperature, then incubated with horseradish peroxidase-conjugated β-actin (1:2000 dilution, Santa Cruz Biotechnology, Inc, California) 1 h at room temperature. Following the wash with TBST, protein was detected by chemiluminescence (Roche Diagnostics, Laval, QC).

Statistical analysis

Statistical analyses were performed using GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA), using non-parametric ANOVA, together with post hoc tests for histopathological changes and unpaired Student’s t-test for mRNA alteration, viral load and lymphocyte counts. P-values <0.05 were considered significant. Unless otherwise stated, all post hoc significant comparisons indicate differences between the control and individual treatment groups, i.e. FIV-infection, FIV-infection with ddI treatment or ddI alone.
Results
ddi is a widely used antiretroviral drug associated with ATN (Simpson and Tagliati, 1995) and thus, we investigated its effects during FIV infection. We initially assessed the actions of ddi on cultured feline DRG neurons with or without concurrent FIV infection. In healthy DRG cultures, neurons exhibited neuritic processes (neurites) with large cell bodies (soma) (Fig. 1A), but in DRG cultures treated with varying concentrations of ddi for 4 days, toxic effects of ddi on neurons were observed at 10 μM (Fig. 1B) and 100 μM in terms of soma atrophy and reduced neurite length was evident. Quantitative analyses revealed ddi caused (C) soma atrophy and (D) neurite retraction in a concentration-dependent manner although (E) neuronal survival was unaffected by ddi. Similar effects on (F) neuronal soma size, (G) neurite length and (H) neuronal viability were observed in DRG cultures derived from chronically FIV-infected animals (means ± SEM, *P < 0.05, **P < 0.01) (original magnification 400×).

However, ddi treatment did not result in neuronal loss (Fig. 1E), compared to untreated controls. Likewise, higher concentrations of ddi (10 and 100 μM) also induced neuronal injury in neurite length (Fig. 1G) and soma size (Fig. 1F) in DRG cultures from chronically (12 weeks) FIV-infected animals. Again, ddi did not cause neuronal loss in DRG cultures derived from FIV-infected animals (Fig. 1H). These data suggested that ddi was neurotoxic ex vivo but chronic FIV infection had minimal effect on ddi’s neurotoxic effects.

Since lentivirus infection of neural tissues involves ongoing infections of macrophages migrating from circulation (Patrick et al., 2002), we examined the effects of ddi during acute FIV infection of feline DRGs.

Fig. 1 ddi is neurotoxic in DRG cultures. (A) In healthy MAP-2 immunopositive DRG neurons, large soma and neurites were evident but (B) after 4 days of ddi treatment (10 μM), neuronal injury in terms of soma atrophy and reduced neurite length was evident. Quantitative analyses revealed ddi caused (C) soma atrophy and (D) neurite retraction in a concentration-dependent manner although (E) neuronal survival was unaffected by ddi. Similar effects on (F) neuronal soma size, (G) neurite length and (H) neuronal viability were observed in DRG cultures derived from chronically FIV-infected animals (means ± SEM, *P < 0.05, **P < 0.01) (original magnification 400×).
Of note, FIV (and HIV-1) infects macrophages in the DRG while Schwann cells and neurons do not exhibit viral antigens or genome (Zhu et al., 2005). Importantly, application of high titre FIV (10^4 TCID_{50}/ml) to cultured feline Schwann cells failed to show any evidence of FIV infection (data not shown), using an established nested PCR protocol (Johnston et al., 2000). DRG cultures were treated with ddI (10 μM) for 4 days after FIV-infection. High viral RNA concentrations were present in supernatants from FIV-infected DRG cultures (Fig. 2A) although ddI treatment non-significantly reduced the viral abundance in FIV-infected DRG cultures compared to untreated FIV-infected DRG cultures. ddI treatment aggravated FIV-induced DRG neuronal injury in terms of exacerbated soma atrophy (Fig. 2C), neurite retraction (Fig. 2B), but it did not affect neuronal survival (Fig. 2D). In fact, while acute FIV infection caused neuronal loss (Fig. 2D), concurrent ddI treatment appeared to limit neuronal death in acutely FIV-infected cultures, perhaps due to the partial suppression of FIV replication. Nonetheless, these observations of acute FIV infection causing neuronal loss resembled previous reports (Zhu et al., 2005) although also indicating that NRTIs induced ex vivo DRG neuronal injury (Keswani et al., 2003a).

The mechanisms underlying the development of lentivirus-related neuropathy during antiretroviral drug treatment remain unclear. To investigate this issue, we examined host gene expression in DRGs during FIV infection in the presence of ddI treatment. NRTIs have been suggested to disrupt mitochondrial DNA synthesis resulting in peripheral neuropathy and other disorders during HIV infection (Hulgan et al., 2005). Thus, we measured the DNA and mRNA levels of feline mtCOX I-encoding sequences. The combination of FIV infection and ddI treatment (10 μM) decreased abundance of mtCOX I RNA (Fig. 3A) (P < 0.05) and DNA levels (Fig. 3B) in DRG cultures. As neurotrophic factors maintain neuronal survival and neurite outgrowth in the PNS, we investigated the expression of several neurotrophic factors in this system. ddI treatment downregulated BDNF transcript expression in FIV-infected and uninfected DRG cultures (Fig. 3C). Similarly, decreased expression of the BDNF receptor, TrkB was also observed in ddI-treated DRG cultures with or without FIV infection (Fig. 3D). ddI and FIV infection also suppressed IGF-I mRNA expression in DRG cultures (data not shown). We explored the question of whether the above gene expression changes in DRGs occurred in cultured feline Schwann cells. Given that lentivirus-infected and -activated macrophages secrete neurotoxic factors (Gonzalez-Scarano and Martin-Garcia, 2005) we examined the effects of supernatants from FIV-infected macrophages on feline Schwann cells (SC). There were no changes in DNA levels of mtCOX I in SC cultures treated with the supernatants of FIV-infected macrophage cultures in presence or absence of ddI (Fig. 3E). However, the supernatants of FIV-infected feline macrophage cultures with and without ddI treatment suppressed the expression of BDNF in SC cultures (Fig. 3F), indicating that the indirect effects of FIV infection affected the expression of BDNF in SC.
intriguing findings with regard to BDNF, DRG cultures were treated with BDNF (60 ng/ml) for 4 days. BDNF not only attenuated FIV-induced neuronal soma atrophy ($P < 0.05$), but also substantially improved FIV-induced neurite retraction ($P < 0.01$) in the presence or absence of ddI, indicating BDNF prevented DRG neuronal injury caused by FIV and with ddI treatment.

FIV infection shares many similarities with HIV-1 infection in terms of pathogenic mechanisms underlying peripheral and central nervous systems’ damage. Hence, we used an in vivo model to further evaluate ddI’s effects on the development of peripheral neuropathy, by examining the effects of ddI on immunity and peripheral nerves in FIV-infected cats. Although lower CD4+ T cell levels

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**Fig. 3** ddI reduces mtCOX I and BDNF expression. ddI (10 μM) treatment diminished mtCOX I (A) transcript levels in FIV-infected and ddI-treated DRG cultures. (B) Conversely, mtCOX I DNA levels were not suppressed in acutely infected DRG cultures. ddI suppressed (C) BDNF expression RNA levels compared to controls and FIV-infection and also (D) TrkB expression compared to controls. Although ddI and FIV-infected macrophage supernatant did not alter the expression of mtCOX I DNA levels in Schwann cell (SC) cultures (E), supernatants from FIV-infected macrophages with or without concurrent ddI treatment caused diminished BDNF transcript production (F) ($n = 4$). In DRG cultures with FIV infection and ddI treatment, (G) BDNF significantly attenuated FIV-induced neuronal soma size atrophy. (H) FIV infection with and without ddI treatment shortened the neurite length compared to the mock group but BDNF substantially reversed neurite retraction in these groups (means ± SEM, *$P < 0.05$, **$P < 0.01$).
(Fig. 4A) and increased CD8+ T cell levels (Fig. 4B) were observed in the blood of FIV-infected animals compared to healthy controls (mock) by week 12 post-infection, ddI treatment did not influence CD4+ and CD8+ T cell levels in blood of FIV-infected and uninfected animals. ddI substantially reduced viral replication in blood in FIV-infected animals. Both FIV infection and ddI treatment caused a delay in withdrawal response latency to a noxious stimulus, which was exacerbated in FIV-infected animals receiving ddI (means ± SEM, n = 4, *P < 0.05).

ddI treatment also reduced the number of axons in the sural nerves from FIV-infected animals compared to untreated or uninfected animals, especially small diameter axons (Fig. 5A). Similar to the findings in patients with HIV-related DSP or ATN (Martin et al., 2003; Tagliati et al., 1999), nerve conduction studies showed no changes in velocity of sensory nerves among FIV-infected and uninfected animals with or without ddI treatment (Fig. 5B) and similarly, sensory nerve action potential amplitudes did not differ among groups (data not shown). Since small diameter sensory fibres are most affected in DSP and ATN (Pardo et al., 2001), we examined nerve fibre density in the epidermis of footpads from FIV-infected animals, or with ddI treatment and control animals (Fig. 5C). These studies disclosed that while FIV infection reduced the number of PGP9.5 immunopositive nerve endings in epidermis (Fig. 5D), concurrent ddI therapy worsened axonal loss in the FIV-infected animals (Fig. 5E). By counting the PGP9.5 immunostained fibres, we measured the extent of innervation of epidermis, indicating that fewer epidermal nerve fibres were observed in FIV-infected animals compared to uninfected control animals while ddI treatment synergistically diminished the number of nerve fibres in FIV-infected (Fig. 5F). These neurobehavioural and neuro-pathological findings suggested that in vivo ddI treatment exerted neuropathogenic effects in conjunction with FIV infection.

Given the above neurotoxic findings caused by ddI, we next examined the effects of ddI on both viral and host gene expression in the present in vivo system. Although ddI significantly diminished viral RNA abundance in DRGs of FIV-infected cats (Fig. 6A), relatively high viral levels remained detectable in DRGs from FIV-infected cats receiving ddI treatment. Similar to the findings in cultured DRGs, FIV infection and ddI treatment significantly decreased expression of mtCOX I DNA (Fig. 6B) and mRNA levels (Fig. 6C) in DRGs from animals. Immunodetection of mtCOX I in DRGs revealed that it was principally detected in neurons although present in other cell types (Fig. 6D). Western blotting with the same monoclonal antibody disclosed that both FIV infection and ddI treatment suppressed mtCOX I immunoreactivity in DRGs (Fig. 6E), which was confirmed by comparisons of the relative ratios of mtCOX I to beta-actin.
immunoreactivity in DRGs from each group of animals (Fig. 6F). These observations indicated that mitochondrial injury was evident during FIV-infection but exacerbated by ddI treatment, potentially leading to the development of ATN.

Since our ex vivo studies disclosed evidence implying that neurotrophin expression levels were reduced during FIV infection and ddI treatment (Fig. 3), we also examined the relative levels of different neurotrophins in vivo. Recapitulating the ex vivo observations, BDNF transcript levels were suppressed in the DRGs of FIV-infected animals, especially after ddI treatment compared to uninfected controls (Fig. 7A); however, expression levels of the BDNF receptor, TrkB, did not differ between ddI treated and untreated groups regardless of the FIV infection status (Fig. 7B). We did not observe changes in NGF or IGF-1 transcript levels in DRGs from the different experimental groups (data not shown). Similarly, TrkC levels did not differ among groups, while NT3 transcripts were not detected in the present DRGs (data not shown). To pursue the identity of the BDNF-secreting cells, immunostaining of DRGs with BDNF- (Fig. 7G, red) and GFAP- (Fig. 7F, green) specific antibodies was performed, revealing that BDNF was chiefly co-localized with GFAP immunopositive cells (Fig. 7H, yellow), indicating that Schwann cells (GFAP immunopositive) were the principal source of BDNF in DRGs. Co-localization of BDNF with CD18 was not observed although some BDNF immunoreactivity was present in NF200 immunopositive cells (data not shown). Indeed, more BDNF immunoreactive cells were evident in DRGs from healthy animals (Fig. 7C) compared to those derived from FIV-infected animals (Fig. 7D), and minimally detected in FIV-infected animals receiving ddI treatment (Fig. 7E), which was similar to the findings in Schwann cell cultures (Fig. 3). These findings suggested that while mitochondrial injury was a component of ddI-induced ATN, decreased BDNF production by proximate Schwann cells might also contribute to the development of ATN.

Discussion

In the present studies, we provide the first in vivo report of the development of ATN in an established model of...
HIV/AIDS caused by ddl treatment at clinically utilized concentrations, which resulted in axonal injury with associated neurobehavioural changes. Moreover, ddl treatment caused damage to mitochondria and a selective reduction in expression of an important neurotrophic factor, BDNF, within DRGs. The pathogenic effects of ddl on mitochondria were limited to neurons while reduced BDNF expression was chiefly observed in Schwann cells. These observations underscore the complex perturbations among different cell types within the DRG, which give rise to ATN, especially the antiretroviral drug-mediated ‘off target’ effects.

The NRTI drugs consisting of zalcitabine (ddC), zidovudine (AZT), lamivudine (3TC), stavudine (d4T), abacavir (ABC) and didanosine (ddl) are essential components of the antiretroviral therapy for HIV-1 infection in clinical practice, which effectively inhibit viral replication and improve immunity although each drug also can lead to adverse effects (Dagan et al., 2002). Dose-dependent peripheral neuropathy is commonly seen following treatment with ddC, d4T and ddl and is the major treatment-limiting adverse effect of nucleoside analogues (Lee et al., 2003). To date, there is no effective clinical therapy for antiretroviral toxic neuropathy, since the pathogenesis of ATN is not understood. Several reverse transcriptase inhibitors effective against HIV-1 are also active against FIV. Hence, an animal model such as FIV infection represents a useful strategy to investigate the mechanisms underlying ATN. Keswani et al. (2003a) reported that NTRIs caused neurotoxicity on primary rat DRG sensory neurons ex vivo; interestingly, ddC had the greatest adverse effects on neuronal viability, followed by ddl and d4T.
Oral administration of ddI led to distal degeneration of unmyelinated sensory axons in HIV gp120 transgenic mice (Keswani et al., 2006). In the current studies, ddI significantly inhibited FIV replication in DRG cultures; but coincidentally, higher concentrations of ddI treatment also increased neuronal injury in FIV-infected DRG cultures. These findings suggested that ddI was toxic ex vivo and that the extent of neuronal injury depended on the concentration of ddI used and FIV infection. The loss of small diameter fibres is the prototypic feature of HIV-1 DSP and ATN, which underlies the accompanying altered pain and temperature perception, reported by HIV/AIDS patients (Simpson et al., 1998). We observed that ddI treatment induced axonal loss, especially small diameter axons, in sural nerves and reduced dermal fibre density in skin from ddI-treated FIV-infected animals, similar to that observed in HIV-1-infected patients receiving NRTI therapy. We also assessed withdrawal responses to noxious stimuli, which revealed delays in withdrawal latency in FIV-infected animals, especially with ddI treatment. These observations revealed that FIV-infected animals with ddI treatment exhibited neuropathological and clinical features of DSP and ATN observed in HIV-infected patients.

The NRTIs are structural analogs of adenosine, guanosine, cytidine or thymidine, which serve as substrates to DNA polymerase and viral reverse transcriptases. Chemical modifications in the 3-OH of deoxyribose, which normally forms the 3’–5’ phosphodiester bond of the DNA molecule, prevents the addition of the next nucleotide leading to premature termination during replication (Kakuda, 2000). However, NRTIs also inhibit the function of DNA polymerase-γ, which is the sole enzyme responsible for the replication and repair of the mitochondrial genome (Moyle, 2000). Myopathies, lactic acidosis, hepatic steatosis, pancreatitis and neuropathy have all been associated with use of NRTIs (Dagan et al., 2002). The clinical and morphological manifestations of these pathologies are remarkably similar to those seen in various genetic mitochondrial disorders (Wallace, 1999). In an ex vivo model of ATN, mitochondrial membrane depolarization was observed in rat DRG neurons after treatment by ddC, ddI and d4T.
(Keswani et al., 2003a). Likewise, mitochondrial DNA and mRNA levels were also reduced in ddl-treated DRG cultures, especially in FIV-infected cultures although nonsignificantly. However, both FIV infection and ddl treatment significantly diminished mtCOX I DNA abundance in vivo, similar to previous studies showing that NRTIs reduced both mtDNA and mRNA levels (Arnaudo et al., 1991; de la Asuncion et al., 1998). These findings indicated that ATN might have resulted from disrupted mitochondrial oxidative metabolism secondary to reduction in neuronal mitochondrial DNA content (Chen et al., 1991; Brinkman et al., 1998b). There is also substantial evidence that HIV-1 triggers mitochondrial injury in various cell types including hippocampal neurons or astrocytes (Huang et al., 2000; Arnoult et al., 2004). There were several discrepancies in mtCOX I and in other host gene levels between cultured and necropsied-DRGs, which emphasizes the importance of using confirmatory in vivo methods. In the present studies, ddl did not efficiently diminish virus replication in the DRGs of FIV-infected animals in spite of significant inhibition of viral replication in blood of FIV-infected animals. Thus, these studies suggested that while ddl exerted mitochondrial toxic effects on neurons, it did not completely clear the peripheral nervous system of virus.

Although there are no curative therapies for DSP or ATN, a clinical trial showed that nerve growth factor (NGF) attenuated some of the clinical manifestations of DSP (Schifitto et al., 2001), suggesting the neurotrophic factors might be involved in the pathogenesis of DSP and ATN. Neurotrophins are one of the chief epigenetic factors that influence the development and survival of neurons. There are multiple neurotrophins influencing survival of sensory and motor neurons including NGF and BDNF, neurotrophin 3 as well as neurotrophin 4/5. Neurotrophins exert their effects by engaging their cognate receptors: TrkA, TrkB or TrkC. In our studies, ddl did not change the expression of NGF mRNA levels among DRGs and DRG cultures with or without FIV infection (data not shown). It is well known that BDNF is directly involved in neurite outgrowth, phenotypic maturation, morphological plasticity and synthesis of proteins required for neuronal and synaptic function (Huang and Reichardt, 2001). BDNF is anterogradely transported in both peripheral and central processes of sensory neurons (Zhou and Rush, 1996). Deprivation of endogenous BDNF can result in a dramatic reduction in the number of myelinated axons distal to a sciatic nerve lesion and a reduction in the elongation of regenerating axons (Zhang et al., 2000). Several studies showed that BDNF is necessary for the maintenance of the DRG neurons (Acheson et al., 1995; Stephens et al., 2005), and moreover, BDNF and its receptor TrkB protected neurons from HIV-1 gp120-induced neuronal degeneration (Nosheny et al., 2005). We observed that ddl downregulated both BDNF transcript and protein levels in DRGs from FIV-infected animals compared to controls, but there were no differences in expression of the BDNF receptor, TrkB, among these groups. However, ddl reduced both BDNF and TrkB transcript levels in DRG cultures with or without FIV infection compared to controls, implicating BDNF in the pathogenesis of ATN. Moreover, BDNF substantially improved FIV- and FIV with ddl-induced DRG neuronal injury ex vivo. BDNF has been shown to increase glucose utilization in response to energy demand associated with neuronal differentiation (Burkhalter et al., 2003). In fact, BDNF also improves rat brain mitochondrial respiratory coupling (Markham et al., 2004) and increases mitochondrial activity (El Idrissi and Trenkner, 1999). TrkB is localized on mitochondrial membranes (Wiedemann et al., 2006), indicating that BDNF could influence mitochondrial function. Thus, the interactions between BDNF and neuronal mitochondria require further elucidation and might provide new therapeutic strategies for ATN in the future.

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