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Human CNS cultures exposed to HIV-1 gp120 reproduce dendritic injuries of HIV-1-associated dementia

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Abstract

HIV-1-associated dementia remains a common subacute to chronic central nervous system degeneration in adult and pediatric HIV-1 infected populations. A number of viral and host factors have been implicated including the HIV-1 120 kDa envelope glycoprotein (gp120). In human post-mortem studies using confocal scanning laser microscopy for microtubule-associated protein 2 and synaptophysin, neuronal dendritic pathology correlated with dementia. In the present study, primary human CNS cultures exposed to HIV-1 gp120 at 4 weeks *in vitro* suffered gliosis and dendritic damage analogous to that described in association with HIV-1-associated dementia.

Introduction

HIV-1-associated dementia (HAD) is a late, subacute to chronic dementia characterized by a progressive and severe decline in cognitive and motor function. HAD remains a major debilitating consequence of HIV-1 infection. It is an independent risk factor for death from AIDS and the most common form of dementia in young adults worldwide [1-5]. Evidence of a reduction in the incidence of HAD [6,7] and reports of cognitive improvement in cases of mild dementia with highly active antiretroviral therapy (HAART) have been presented [8]. Other studies have failed to identify a lower incidence of HAD post-HAART and a number of experts note the potential for a changing tempo of HAD from a precipitous dementia to one with a more protracted course and greater incidence in patients with relatively preserved CD4 counts [3,4,8]. It is premature to accurately predict how HAART will affect the incidence of HAD in the long term. HAART clearly does not afford complete protection and the potential for an increase in the prevalence of HAD has been raised by many [2,3,6,8-11].

HIV-1 associated neuronal damage has been characterized with evidence for both cytotoxic and subcytotoxic injuries. Evidence of loss of large neurons in the orbitofrontal, temporal and parietal regions [12] has been demonstrated in association with HAD. Other investigations have failed to demonstrate a correlation between neuronal loss and HAD [13].

Studies of HIV-1 associated neuronal damage using synaptic and dendritic markers [14] have shed additional light on the nature of the neuronal injury in HAD. Cases with severe HAD suffered a 40% loss of dendritic area in frontal cortex and a 40-60% loss of dendritic spine density in comparison with non-demented controls [12]. It was suggested that disruption of post-synaptic elements, characterized by sinuous, shortened, and vacuolated dendrites may be the primary lesion leading to the reduction in synaptic density and the development of dementia [14]. These and subsequent studies suggested that decreases in microtubule associated protein (MAP2) and synaptophysin (SYN) immunoreactivity may be more

sensitive markers of neuronal injury [15] perhaps identifying a more subtle primary injury.

The HIV-1 envelope glycoprotein gp120 has been linked to the pathogenesis of HAD from several lines of evidence. Both whole virus and gp120 alone have been shown to be toxic to murine, avian and human CNS cultures [16,17]. Individual studies provided evidence that gp120 acts synergistically with NMDA receptor agonists [18]. HIV-1 neurotoxicity was blocked in vitro by anti-gp120 antibodies but not by anti-CD4 antibodies [17] indicating that its toxicity was not dependent on CD4 receptor binding. Several groups have demonstrated that neurotoxicity associated with gp120 exposure may involve chemokine receptor activation [19-23] and may be further influenced by Apolipoprotein-E genotype [24]. Hippocampal neurons in mixed murine cultures are protected from gp120 by estrogenic steroids [25] while corticosterone exacerbates the gp120 inhibition of glutamate uptake [26].

Most evidence supports the theory that gp120 neuronal toxicity is largely mediated through its interactions with non-neuronal cells (microglia/monocytes and astrocytes) as reviewed by Kaul *et al.* and Scorziello *et al.* [3,27]. Activated microglia release compounds such as nitric oxide, proinflammatory cytokines and glutamatergic excitotoxins, which can lead to neuronal membrane destabilization and [28-32]. In point of fact, conditioned media from gp120-treated microglia was shown to be neurotoxic to murine hippocampal cultures [33]. The ability of gp120 to cause the upregulation of inducible nitric oxide synthase has been suggested in several studies [34,35] including our own (Walsh *et al.*: Anioxidant protection from HIV-1 gp120-induced neuroglial toxicity. *J Neuroinflammation* 2004, 1:8). Furthermore, studies have shown that gp120 increases free radical generation, impairs antioxidant defences and increases lipid peroxidation in cultures [36]. The alteration of cell cycle protein expression has recently been shown to be associated with neuronal damage caused by HIV [37].

The mechanism(s) of neuronal damage in this setting remains controversial and there are few human models available in which to study this human-specific disease. Gliosis and neuronal dendritic injury have been well characterized in association with HAD in post-mortem studies and the present studies were undertaken to derive a human culture system in which to study the pathogenesis of these alterations. We report the findings of gliosis and neuronal dendritic injury in primary mixed human CNS cultures exposed to recombinant gp120. This provides an additional tool for the study of HAD pathogenesis.

Materials and methods

Human primary CNS cultures

Human CNS tissue cultures were initiated from post-mortem 16 to 18 week gestational age forebrain samples submitted to the Department of Pathology, London Health Sciences Centre following institutional guidelines and Research Ethics Board approval. The tissue was dissected in fresh Dulbecco's Modified Eagle Medium, centrifuged and resuspended in a serum-free and pyruvate-free medium as previously described [38,39]. Suspension cultures were initiated at a density of 5×10^6 cells/cm³ in T-75 flasks (resulting in free-floating neuroglial aggregates). Monolayer cultures (for confocal microscope analysis) were plated at a concentration of 1×10^6 cells/cm³ onto poly-ornithine (Sigma, Mississauga, ON, Canada) and laminin (Gibco, Burlington, ON, Canada) coated glass coverslips in 12 well plates. By preserving cells for imaging in an intact state, monolayer preparations were optimal for confocal immunofluorescent quantitative analysis of changes in expression of structural proteins MAP2 and glial fibrillary acidic protein (GFAP). All cultures were incubated, humidified, at 37°C in 10% CO₂ and fed biweekly by half media exchange. All experiments for quantitative analyses by confocal microscopy were run in duplicate from three separate primary cultures.

Gp120 exposure

At four weeks in vitro, cultures were exposed to 1 nM purified recombinant gp120_{SF2} (Austral Biologicals, San Ramon, CA) via half media exchange as previously described [38]. Cultures were incubated with gp120 for 72 hours (or less, as in the case of the time series study of apoptosis, necrosis and proliferation). This dose of gp120 was selected from a dose response experiment that revealed no visible injury at levels below 1 nM and considerable cellular injury and nuclear debris at levels above. Hence 1 nM was used as the lowest dose with a measurable effect at 72 hours (figure 1).

Immunofluorescence and confocal imaging

Immunofluorescence and confocal imaging followed previously published protocols [38,40]. Briefly, seventy-two hours post-gp120 exposure, cultures were rinsed twice with phosphate buffered saline (PBS) and fixed for 30 minutes with 4% paraformaldehyde. After two PBS rinses, cultures were blocked with 5% horse serum with 0.1% Triton X100 for 1 hour and incubated with monoclonal mouse anti-human MAP2 (Sigma, Mississauga, ON, Canada, 1:500 dilution) and polyclonal rabbit anti-human GFAP (Sigma, Mississauga, ON, Canada, 1:1000 dilution) antibodies simultaneously for two hours at room temperature. Paired monolayers were incubated with mouse anti-human Class III beta tubulin (C3βT) (Sigma, Mississauga, ON, Canada, 1:1000 dilution, recognizes neuron specific microtubule protein) and polyclonal rabbit anti-

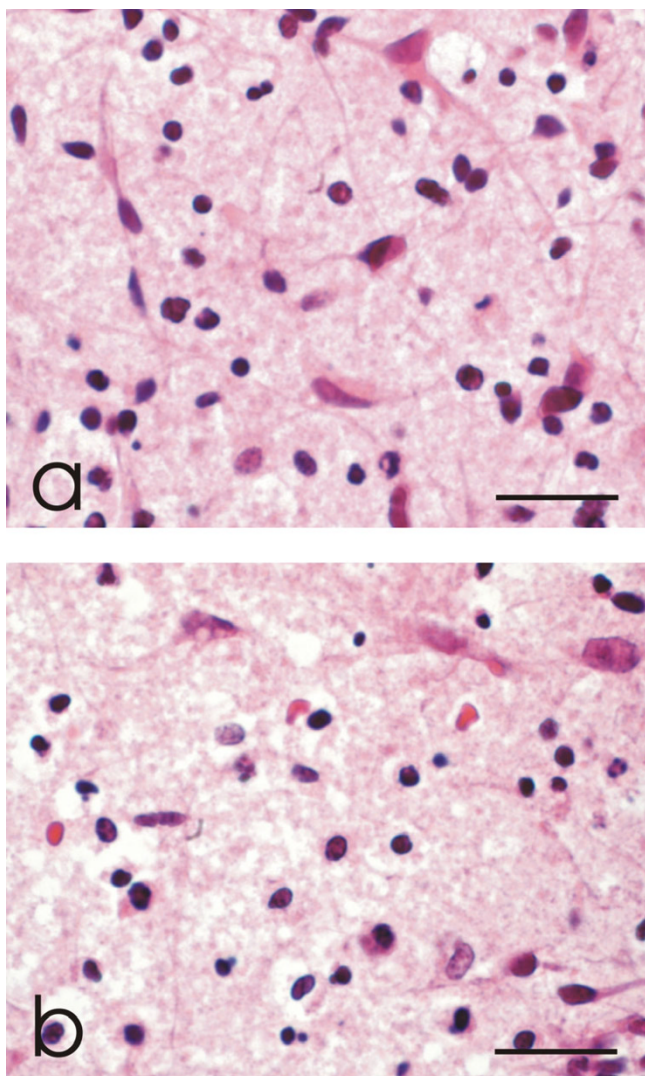


Figure 1
Seventy-two hour exposure to 1 nM gp120 causes observable cellular injury. Representative photomicrographs of control (a) and gp120 exposed (b) cultures. There is nuclear pyknosis, neuropil vacuolation and fewer visible cell processes in cultures exposed to gp120 for 72 hours. H&E, all bars = 25 μ m.

human GFAP antibodies for two hours at room temperature. The cells were rinsed with PBS and incubated in the dark with Texas Red conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA, 1:200 dilution) and fluorescein isothiocyanate (FITC) conjugated goat anti-mouse (Sigma, Mississauga, ON, Canada, 1:500 dilution) for one hour at room temperature. The cells were rinsed with PBS and incubated for 5 minutes with Hoechst nuclear stain in PBS (Sigma, Mississauga, ON, Canada,

1:100 dilution). Following a final PBS wash, the monolayers were mounted directly onto glass slides with Gelvatol fade resistant aqueous mounting media. Negative controls were prepared in the absence of primary antibody.

All cultures were imaged in a blinded fashion on a Zeiss LSM 410 confocal microscope equipped with Krypton/Argon and Helium/Neon lasers as previously described [38]. Texas Red, FITC and Hoechst signals from twelve random fields per coverslip were collected with a 63 \times objective lens under oil immersion. Five serial vertical z-planes were imaged within each field of view with a plane thickness of 0.9 μ m. Positive and negative controls were run with all experimental sets and all related culture sets were imaged in single sessions. Thresholds were set to eliminate background fluorescence if present.

Cell counts were performed manually. Identification of neurons and astrocytes was conservatively defined by circumnuclear expression of neuronal or astrocytic antigens (MAP2 or GFAP) leading to a slight but consistent underestimate of both populations.

The intensity of immunofluorescent staining in each sample was measured, as determined by the average pixel intensities for each fluorophore. Texas Red and FITC signals were normalized to the Hoechst signal.

Apoptosis, necrosis and cellular proliferation

Paired free floating neuroglial aggregate cultures were fixed at 0, 2, 6, 12, 24 and 72 hours post-gp120 exposure. The cultures were rinsed with PBS and fixed in 4% paraformaldehyde for 30 minutes. The cells were then suspended in 5% agar and embedded in paraffin blocks. 4 μ m sections were cut from each sample and analysed for apoptosis by terminal dUTP nick end labelling (TUNEL) (Intergen, Purchase, NY). Positive nuclei were identified by dark brown staining of shrunken or clumped nuclei. Ten random fields from each section were viewed under a 40 \times objective and the percentage of apoptotic nuclei in relation to total (methyl green counterstained) nuclei was determined. Ki-67 (Vector, Burlington, ON, Canada) positive nuclei were enumerated relative to total nuclei in 10 random fields. Immediately prior to fixation the media was sampled and assayed for lactate dehydrogenase (LDH) according to manufacturer's directions (Sigma, Mississauga, ON, Canada). Positive and negative controls were run with all sets.

Statistical analysis

For quantification of MAP2 and GFAP staining in the CSLM images, data were analyzed by Student's *t*-test. Data obtained from assays of apoptosis, necrosis and cellular proliferation were analyzed by one-way ANOVA followed

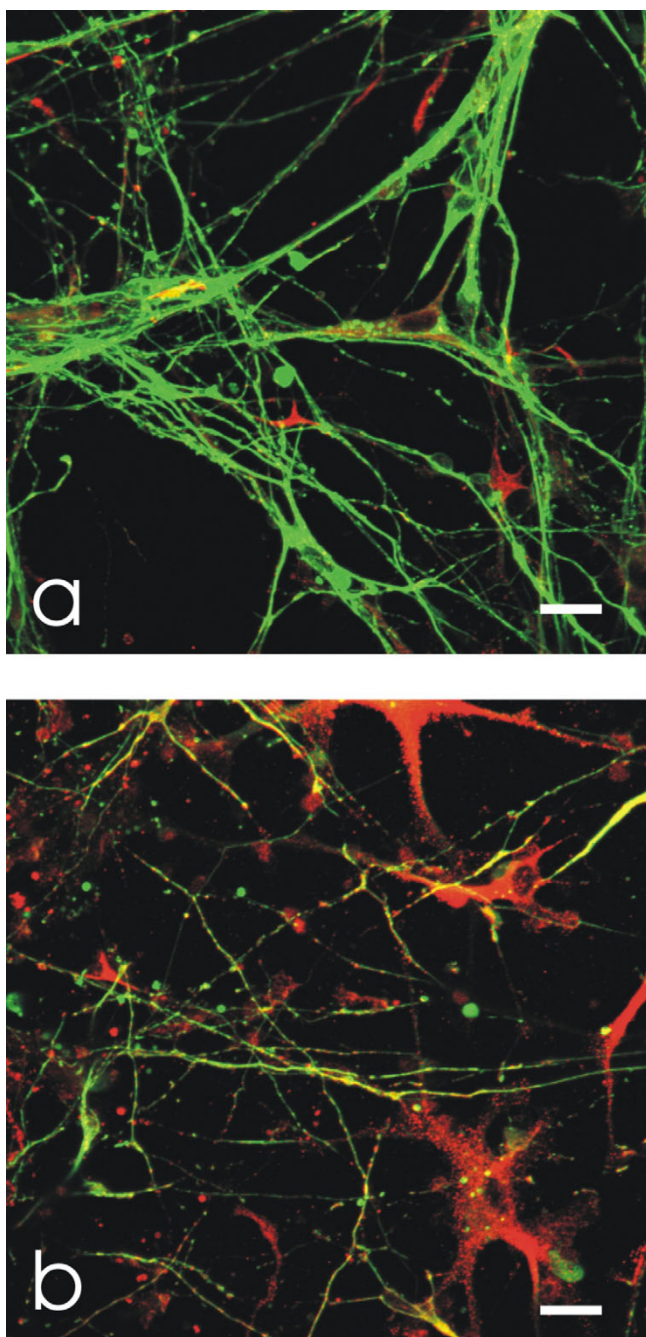


Figure 2
Gp120 exposure results in astrocytic hypertrophy and a reduction in dendritic complexity. Representative immunofluorescent images from 4-week monolayer control cultures (a) and cultures exposed to gp120 for 72 hours (b) stained for C3 β T (green) and GFAP (red). Neuronal processes in the gp120 exposed condition appear reduced, sinuous, varicosed and vacuolated in comparison to controls. All bars = 20 μ m.

by a Tukey's multiple comparison post-hoc test. In both cases, probabilities of $p < 0.05$ were considered significant.

Results

Routine light and confocal immunofluorescent microscopy of more than 20 separate primary cultures revealed several consistent qualitative morphological changes in neurons and astrocytes associated with a gp120 exposure including nuclear pyknosis and a reduction in fine cellular processes (figure 1). Neuronal processes in gp120-exposed cultures were fewer, more sinuous, varicosed and vacuolated compared to controls (figure 2a). Astrocytes exposed to gp120 became more prominent in number and size (figure 2b).

Quantitative analysis of confocal images revealed a 37% decrease in MAP2 immunoreactivity ($p < 0.05$, Student's *t*-test) and 43% increase in GFAP immunoreactivity ($p < 0.02$, Student's *t*-test) following gp120 exposure (figure 3). No significant differences were found in counts of total or MAP2-associated nuclei between experimental and control conditions. An 84% increase in the number of GFAP-associated nuclei was observed after gp120 exposure ($p < 0.01$, Student's *t*-test). No increase in total nuclei and no significant proliferation (see below) suggested that the increase in GFAP-associated nuclei was the result of astrocytic hypertrophy and recruitment of immature glia. There was no evidence of colocalization of MAP2 and GFAP.

Apoptosis was not significantly increased in gp120-exposed cultures at any time point compared with controls within 24 hours of exposure (Tukey's). At 72 hours post exposure there was a small increase in the incidence of TUNEL-positive nuclei compared with all other time points except for 2 hours. Proliferation as estimated by Ki-67 immunohistochemistry (percentage of Ki-67 positive nuclei) showed no significant difference between conditions. Similarly, cellular necrosis, as assayed by LDH release, was not significantly increased with gp120-exposure (figure 4).

Discussion

HAD and the Minor Cognitive Motor Disorder (MCMD) remain common, debilitating and costly complications of HIV-1 infection and independent risk factors for death in AIDS [1]. Recent post-mortem investigations of HAD identified neuronal dendritic pathology as a correlate of dementia [15,41]. Recent clinical evidence has suggested that some cases of HAD show a degree of improvement on HAART [6-8,42] and although apoptosis may occur in the setting of HAD, the correlation between apoptosis and dementia is poor [43]. Taken together, these findings and the present report support the theory that the primary

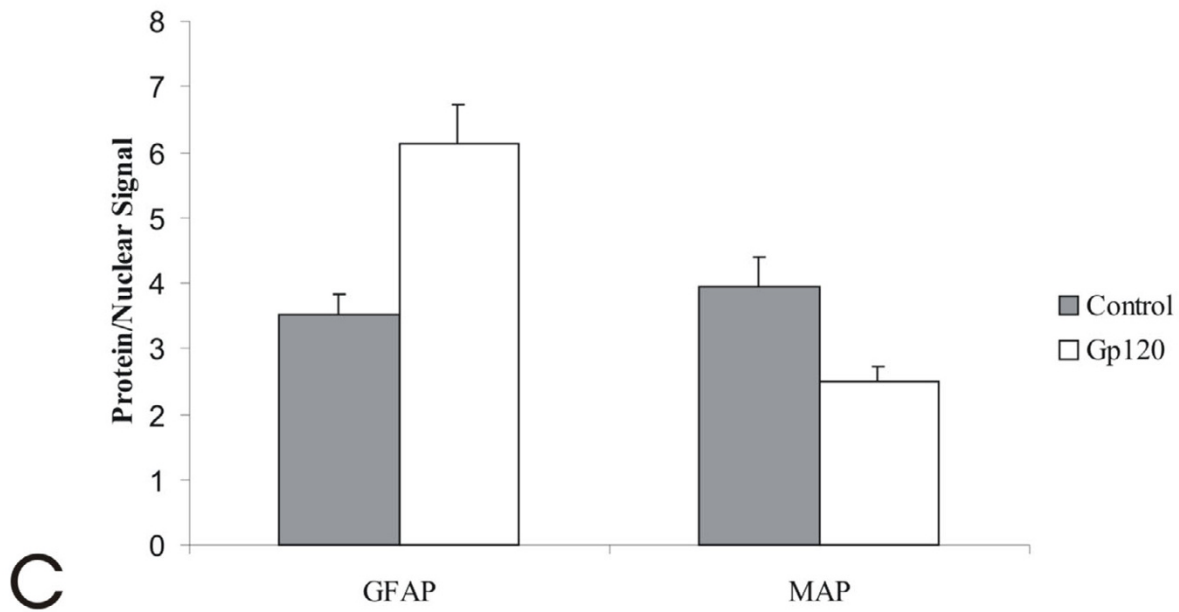
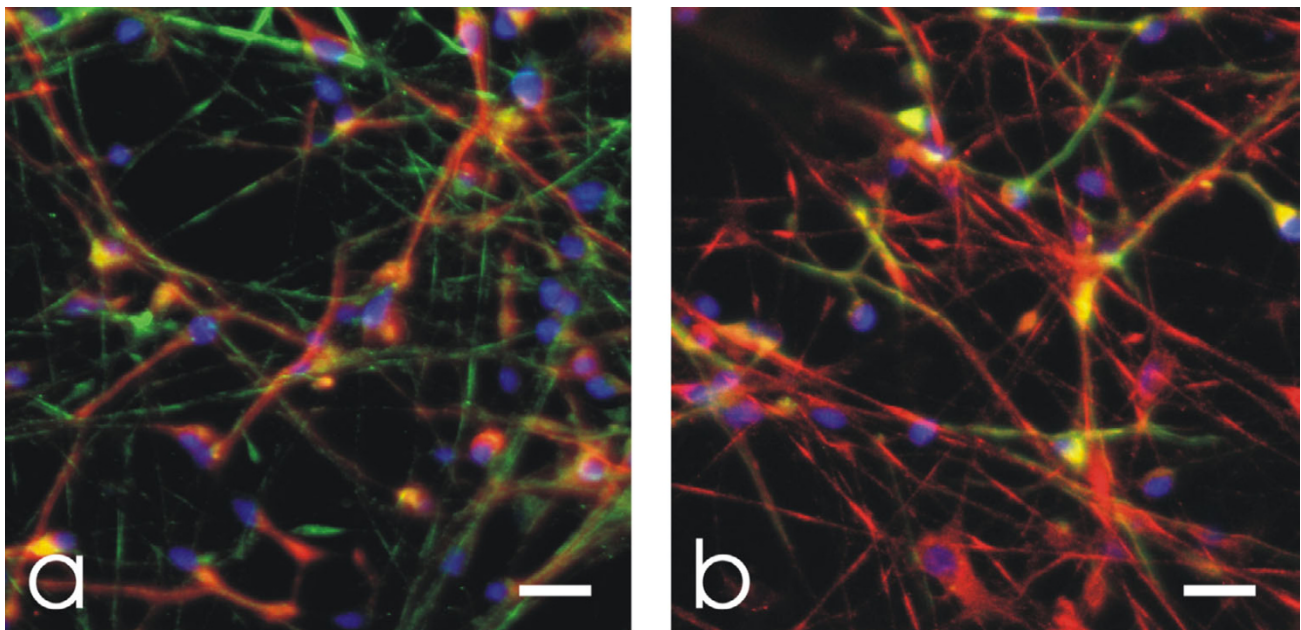


Figure 3
Quantitative analysis confirmed gp120 induced astrocytic hypertrophy and reduced dendritic complexity. Representative immunofluorescent images from 4-week monolayer control cultures (a) and cultures exposed to gp120 for 72 hours (b) stained for MAP2 (green) and GFAP (red). All bars = 20 μ m. Quantitative immunofluorescent analysis of the effect of 72 hour gp120 exposure on MAP2 and GFAP expression (normalized to nuclear staining) using confocal scanning laser microscopic images is shown in (c). Bars represent normalized mean pixel intensities from 12 random fields +/- SEM. Gp120 treated cultures demonstrate a 37% decrease in MAP2 immunoreactivity ($p < 0.02$) and a 43% increase in GFAP immunoreactivity ($p < 0.01$) in comparison with controls. Error bars: +/- 1 standard error.

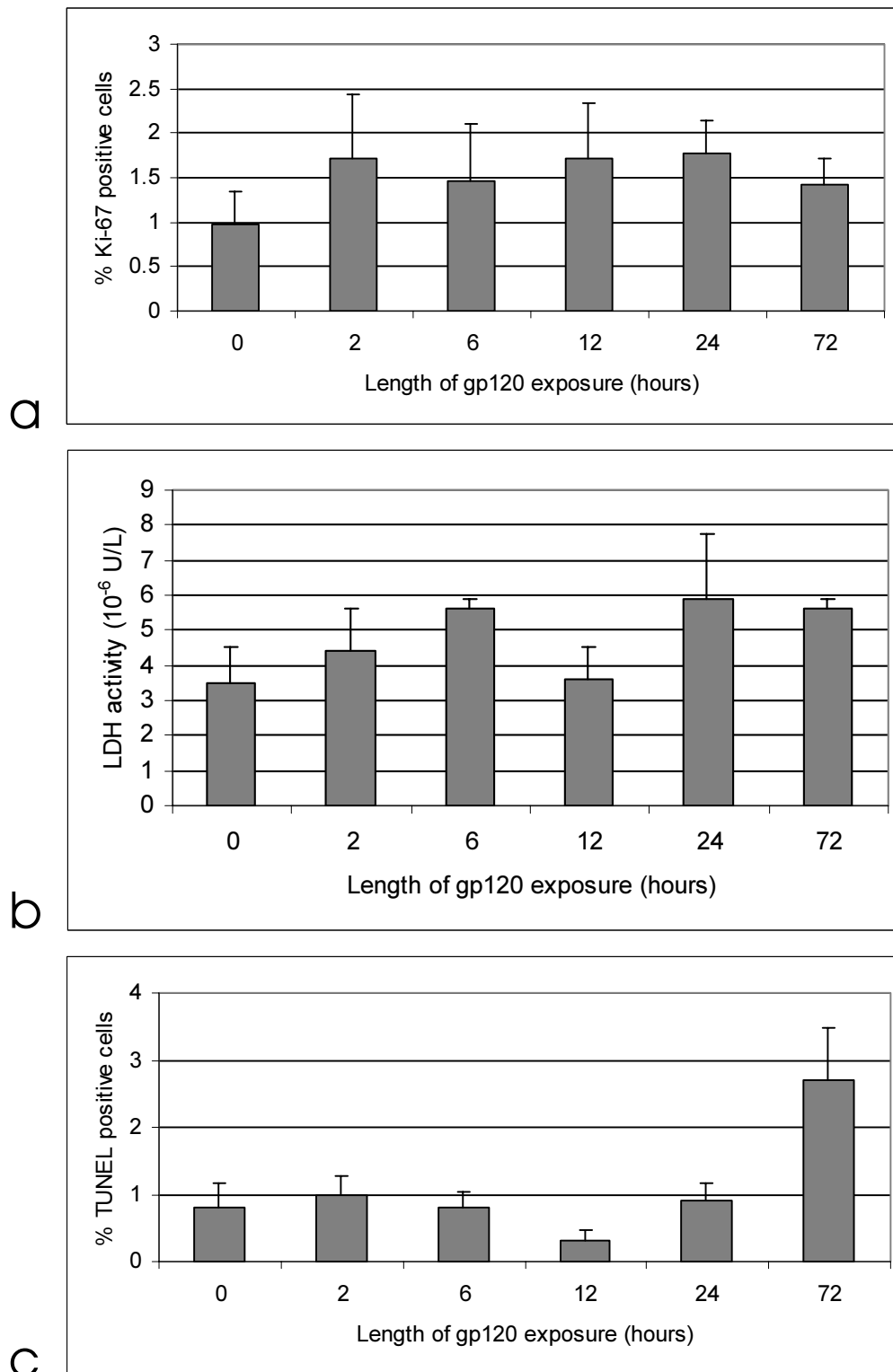


Figure 4
Gp120 exposure did not induce cell proliferation, necrosis or TUNEL within 24 hours of exposure. Gp120 exposure time series data for Ki-67 (a), LDH (b) showing no significant differences at timepoints between 0 and 72 hours for proliferation or necrosis. TUNEL (c) data suggest a small increase in apoptosis over baseline at 72 hours after gp120 exposure in comparison to all timepoints except 2 hours. Error bars: \pm 1 standard error.

insult and reversible component of dementia, may be one of neuronal dysfunction and subtle dendritic injury with cumulative injuries leading to more extensive dendritic damage, cell death and an irreversible component of dementia. Further studies are needed to examine the association between dendritic injury, neuronal loss and the reversibility of dementia. Apart from post-mortem studies of human brain, there is a limited opportunity to study the pathogenesis of HAD in human cells. The system described herein represents such a tool.

In the present study we have identified a qualitative and quantitative injury to the dendritic arbour of gp120 exposed neurons. The density of processes was reduced and remaining processes showed pathological structural alterations (fragmentation, varicosities, etc.). Furthermore, the changes in dendritic architecture were accompanied by a significant decrease in the volume and intensity of MAP2 immunoreactivity. Gp120 exposed cultures also demonstrated astrocytic hypertrophy and an increase in total GFAP immunoreactivity. These findings are reminiscent of those described in HAD *in vivo* [15,41] and provide further evidence that gp120 is a contributing factor in human neuronal injury.

TUNEL data suggest a small increase in apoptosis over baseline at 72 hours after gp120 exposure in comparison to all timepoints except 2 hours. Subsequent studies (Walsh *et al.* Antioxidant protection from HIV-1 gp 120-induced neurological toxicity. *J. Neuroinflam* 2004, 1:8) suggest that this cytotoxic injury is preceded by morphologic alteration to astrocytes and neurons. Many other authors have also shown an apoptotic component of gp120 toxicity in a variety of experimental systems [12,16,18,44]. The apparent sequence of cytotoxic, and presumably reversible, injury (GFAP and MAP2 alteration) followed by cytotoxic, and presumably irreversible, injury (TUNEL) invites a comparison to HAD whereby HAART has been shown to provide some cognitive improvement (reversible component) but with some residual symptomatology (irreversible component) [8].

Ki-67 data suggest no significant change in DNA replication in response to gp120 exposure. Likewise, LDH analyses show no evidence of increased necrosis. In the absence of significant nuclear turnover, an increase in the number of astrocytes in gp120 exposed cultures suggests the possible recruitment of existing precursors to form new astrocytes as a component of the observed increase in GFAP-positive cells.

Conclusions

This culture system [38] offers certain advantages to the study of neurotoxicity associated with HIV-1 being derived from human tissue (of relevance in studying the

effects of a human-specific virus) grown under conditions that promote the maturation of neurons in the absence of astrocytic overgrowth. It has been adapted to studies of engraftment [40] and oxidative injury [45] and the present report documents its ability to reproduce neuropathological correlates of HAD, providing an additional tool for the study of dendritic injury in this form of dementia. The present study characterizes cytotoxic and cytotoxic injuries associated with gp120 exposure in human primary mixed CNS cultures.

Abbreviations used

C3 β T; Class III beta tubulin

CSLM; confocal scanning laser microscopy

FITC; fluorescein isothiocyanate

GFAP; glial fibrillary acidic protein

gp120; HIV-1 120 kDa envelope glycoprotein

HAART; highly active antiretroviral therapy

HAD; HIV-1 Associated Dementia (HAD)

HIV-1; Human Immunodeficiency Virus I

LDH; Lactate dehydrogenase

MAP2; microtubule-associated protein 2

MCMD; Minor Cognitive Motor Disorder

PBS; phosphate buffered saline

SYN; synaptophysin

TUNEL; terminal dUTP nick end labelling

Competing interests

None declared.

Authors' contributions

RH conceived of the study. RH, SI and KW designed and carried out the experiments and collected and analyzed the data in the laboratory of RH. RH, SI and KW co-wrote the manuscript. All authors read and approved the final manuscript.

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