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Lentiviral expression of HIV-1 Vpr induces apoptosis in human neurons

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Our recent studies have demonstrated that extracellular, recombinant human immunodeficiency virus type I (HIV-1) Vpr protein is highly neurotoxic in the microenvironment of differentiated mature human neurons and undifferentiated neuronal precursors. Although most of the direct neurotoxic effects of HIV-1 have been attributed previously to the envelope gene product, gp120, and the Tat regulatory protein, it was demonstrated that Vpr protein caused apoptosis comparable to that induced by gp120 protein in a dose-dependent manner in the neuronal system. Having observed the neurocytopathic effects of extracellular Vpr protein previously, the effects of virally expressed Vpr on nondividing, terminally differentiated human neurons were investigated. An HIV-1-based three-plasmid expression vector system was utilized to study the effects of intracellularly expressed Vpr. These virion preparations were then used to transduce neurons generated from the human neuronal precursor NT2 cell-line. Intracellularly expressed Vpr induced apoptosis within terminally differentiated neurons, as demonstrated by TUNEL assays. Additionally, virions lacking Vpr expression did not significantly induce apoptosis within these neurons. These results suggest that HIV-1 Vpr may also be leading directly to selective neurotoxicity through intracellular expression. Furthermore, human apoptosis gene microarray comparisons exhibited an up-regulation of Bcl-2-related mRNA, as well as other apoptosis genes involved in the mitochondrial apoptotic pathway, for the Vpr-transduced neuronal cells, when compared to Vpr-negative controls. Thus, Vpr delivered intracellularly, as well as extracellularly, is involved in the induction of significant neuronal apoptosis and may be one of the molecular mechanisms in HIV-1-induced encephalopathy. *Journal of NeuroVirology* (2002) **8,** 86–99.

Keywords: HIV-1; Vpr; neurons; apoptosis; encephalopathy; mitochondria

Introduction

Human immunodeficiency virus type I (HIV-1) infection in some individuals can lead to the acquired immunodeficiency syndrome (AIDS) dementia complex (ADC), characterized by diffuse motor, sensory, and cognitive dysfunctions. The cells in the human brain that are clearly the most commonly infected with HIV-1 are macrophages and microglia, however, variable lower levels of HIV-1 infection are demonstrated in neurons, microvascular endothelial cells (MVEC), and astrocytes within brain specimens of AIDS patients (Bagasra *et al*, 1996; Kohleisen *et al*, 2001; Canto-Nogues *et al*, 2001).

It is apparent from these findings that several cell-types in the central nervous system (CNS) are likely to become infected with HIV-1 *in vivo*, and the most probable cause of CNS injury in the brains of infected patients with neuropathological abnormalities appears to be neuronal dropout and neuronal apoptosis (Adie-Biasette *et al*, 1995; Gelbard *et al*, 1995; Petito and Roberts, 1995; Shi *et al*, 1996; Ohagen *et al*, 1999). Additionally, it has been previously shown

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that HIV-1 replicates in the mature differentiated NT2 neuronal cells (Hirka *et al*, 1991; Mukhtar *et al*, 2000; Mukhtar and Pomerantz, 2000). Although the underlying cause of HIV-1-induced dysfunctions of the CNS remains unknown, the HIV-1 regulatory protein, Vpr, has been implicated in the induction of apoptosis of human neuronal cells, primary rat cortical cells, and cultured hippocampal neurons (Piller *et al*, 1998; Huang *et al*, 2000; Patel *et al*, 2000). Recent observations have shown that Vpr induces apoptosis when added extracellularly to the medium of differentiated human neurons (Patel *et al*, 2000).

Vpr, an 96-amino acid regulatory gene product of HIV-1, has been described to have several unique biological properties. These include cytoplasmic to nuclear shuttling, cell-cycle G_2 arrest, positive effects on virion production and replication, incorporation into the virus particle, and an ability to oligomerize (Zhao *et al*, 1994; He *et al*, 1995; Jowett *et al*, 1995; Mahalingam *et al*, 1995; Yao *et al*, 1995; Gummuluru *et al*, 1999; Hrimech *et al*, 1999). Previously, Vpr was identified as nuclear import protein and more recently studies suggest Vpr functions as a nucleoplasmic shuttling protein with an export function dependent on the CRM1 nuclear export factor (DeNoronha and Sherman, 2001; Sherman *et al*, 2001; Vodicka *et al*, 2001). Perhaps the most intriguing property of Vpr is that apart from being expressed from the integrated proviral DNA of the HIV-1 genome, it has been reported to exist as a virion-encapsidated protein (Cohen *et al*, 1990; Yuan *et al*, 1990; Hrimech *et al*, 1999). It has been postulated that, as a virion-associated protein, Vpr acts early in viral replication and plays a role in guiding the HIV-1 pre-integration complex into the nucleus of nonproliferating cell-types (Heinzinger *et al*, 1994; Popov *et al*, 1998). As well, Vpr induces apoptosis in human fibroblasts, T-cell-lines and primary peripheral blood lymphocytes following cell-cycle G_2 arrest. In T-lymphocytes, Vpr was shown to induce apoptosis, following G_2 cell-cycle arrest, via the activation of caspases and independent of p53 stimulation (Shostak *et al*, 1999). Even though most of the evidence for the induction of apoptosis during HIV-1 infection implicates Tat and gp120 proteins, it has been suggested that Vpr encapsidated within virions is also involved in the direct cell killing effects of HIV-1 (Shi *et al*, 1996; Stewart *et al*, 1997; Yao *et al*, 1998; Mischiati *et al*, 1999; Ohagen *et al*, 1999). Stewart and coworkers found that Vpr expressed during a viral infection, in the absence of other viral components, was also capable of inducing apoptosis in T-cells. In the same study Vpr-induced apoptosis was decreased with the use of a synthetic caspase inhibitor, indicative of caspase activation (Stewart *et al*, 2000). Also, in a recent study transgenic mice expressing the Vpr gene were shown to have reduced numbers of T-lymphocytes in the thymus and periphery due to Vpr-mediated thymocyte apoptosis (Yasuda and Miyao, 2001).

We have recently demonstrated that extracellular Vpr protein induces apoptosis in human neurons via caspase-8 stimulation (Patel *et al*, 2000). However, the effects of Vpr delivered intracellularly, expressed from an integrated provirus and encapsidated within the virion itself, has not yet been studied in human CNS-based cells. Nondividing, terminally differentiated, mature neurons were cultured from the NT2 human teratocarcinoma precursor cell-line (Pleasure *et al*, 1992; Pleasure and Lee, 1993; Patel *et al*, 2000). To investigate the effects of Vpr expressed intracellularly, thereby mimicking viral infection, mature differentiated NT2 neurons were transduced with recombinant virus expressing cloned Vpr and mutant Vpr as proviral constructs, as well as the appropriate control viruses.

First, mature human neurons were analyzed for Vpr protein expression intracellularly through immunostaining. Then, the neurons transduced with recombinant virus containing Vpr were examined for apoptosis using the TUNEL assay. Finally, gene regulation in these neurons was studied utilizing apoptosis gene microarray technology.

Results

Recombinant HIV-1 vector virions

The HIV-1 p24 antigen values, obtained after transfection of 293T cells, for both major types of recombinant lentiviral vectors particles (i.e., with and without HIV-1 Vpr in the packaging construct as illustrated in Figure 1) were in the same concentration range of nanograms/ml. Specifically, the viral supernatants generated using the Vpr transgene along with Vpr in the packaging construct (Vpr++) were 100 to 250 ng/ml and the viral supernatants generated with Vpr as a transgene only (Vpr+−) were 130 to 225 ng/ml. The respective controls without Vpr in transfer vectors were also generated, and Vpr−+ as well Vpr−− were 310 and 160 ng/ml, respectively. The input of the viral supernatant on mature neurons was strictly normalized before addition (112 ng of p24 antigen equivalents), to achieve approximately 50% to 100% transduction efficiency.

HIV-1 Vpr vector virions express Vpr protein intracellularly in neurons

Mature neurons transduced with recombinant Vpr++ virus, containing Vpr as a transgene and in the packaging construct, were immunostained for intracellular expression of the Vpr protein. Figure 2A illustrates the expression of Vpr++ in the mature neurons, as well as in the nucleus of some basal cells in the cultures. Neuronal expression of wild-type Vpr 89.6 and Vpr mutants (A59P, H71C, HXB2) indicated that the Vpr transgenes were efficiently integrated into these cells. Similarly, Vpr expression of wild-type and mutants was observed in neurons transduced with Vpr+− virus containing Vpr only as

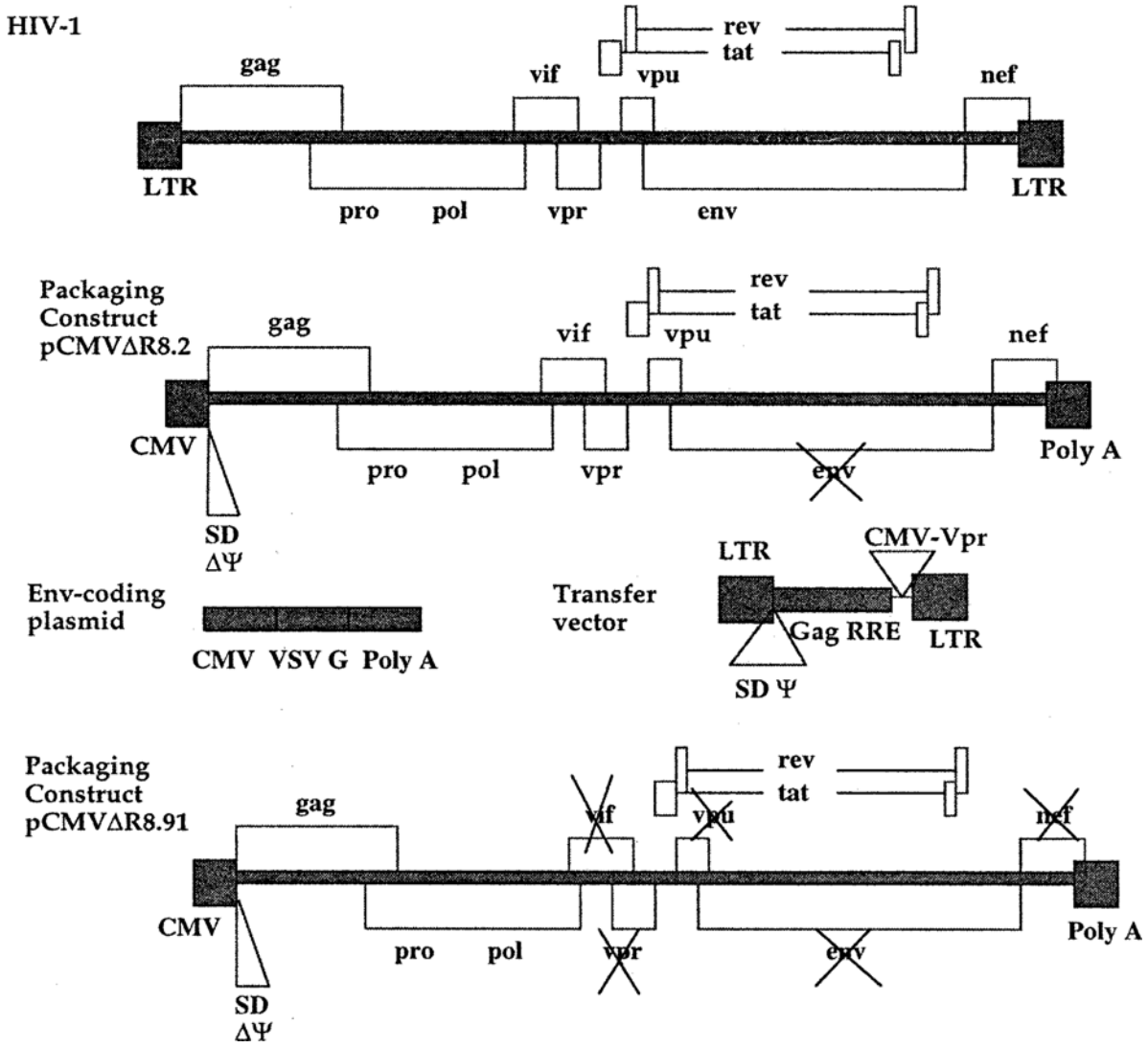
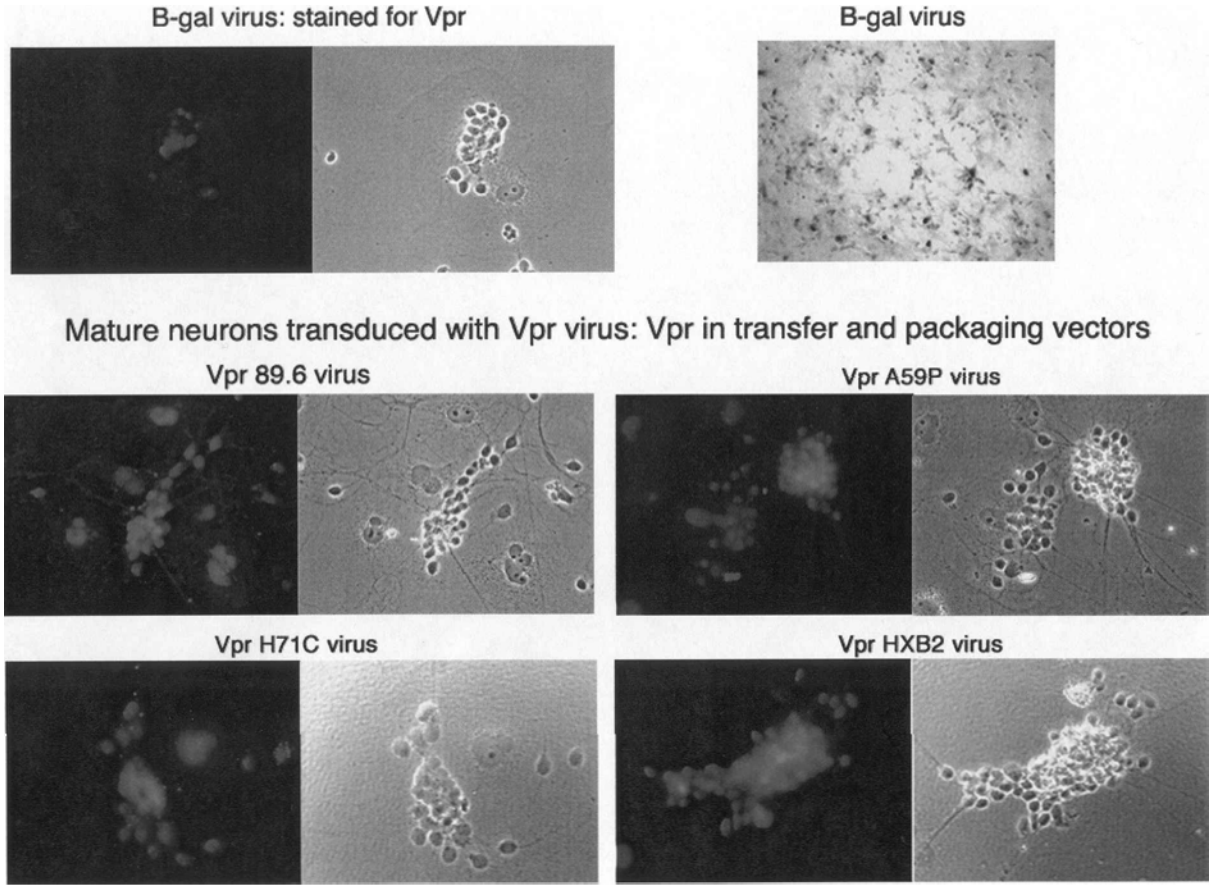


Figure 1 Schematic of the lentiviral triple vector plasmid system utilized for recombinant HIV-1 Vpr viruses. Replication-defective retroviral particles were generated by triple transfection of transfer vector pHR'CMVVpr or pHR'CMVlacZ, packaging construct pCMVΔR8.2 or pCMVΔR8.91, and envelope-encoding plasmid, pMD.G.

a transgene but only minimal amounts of Vpr stained when contained solely in the packaging vector (i.e., virion-encapsidated Vpr only) (Figure 2A). The corresponding viruses used as controls lacking the Vpr transgene were produced with and without Vpr in the packaging vector (i.e., containing and lacking virion-encapsidated Vpr), and were then used to transduce mature neurons. As these viruses contained the β-gal reporter gene, these cultures were analyzed for the expression of β-gal, as illustrated in the top panel of Figures 2A and 2B. The top panel of Figure 3A is a representative photomicrograph at low magnification of a field of cells transduced with Vpr+− virus with the Vpr 89.6 transgene, lacking Vpr in the packaging vector, and demonstrates a high transduction efficiency by Vpr-containing viruses. In summary, mature neurons transduced with the recombinant viruses displayed significant expression of the diverse transgenes.

Intracellular HIV-1 Vpr induces apoptosis of human neurons

Neuronal apoptosis was analyzed using the TUNEL assay. Neurons transduced with recombinant HIV-1 Vpr++ viruses, with Vpr in both the transfer and packaging vectors, exhibited prominent apoptosis in the wild-type Vpr 89.6-transduced cells (Figure 3B). Vpr++ viruses with mutant Vpr (i.e., A59P, H71C, HXB2) in the transfer vector and Vpr in the packaging vector revealed significantly less apoptotic induction compared to the wild-type Vpr transgene (Figure 3B). Apoptosis exhibited by the mutant Vpr transgenes A59P, H71C, and HXB2 was at the same



(A)

Figure 2 Immunostaining for expression of Vpr in mature neurons. (**A**) The two lower panels illustrate immunostaining with Vpr antibody for neuronal cells transduced with the Vpr++ virus containing the wild-type 89.6 or mutant Vpr (i.e., A59P, H71C, HXB2) in the transfer vector, with Vpr in the packaging vector (left side: fluorescent staining; right side: phase contrast photomicrograph of the same slide section). Top panels illustrate X-gal and Vpr immunostaining for the β-gal virus lacking the Vpr transgene but containing Vpr in the packaging construct. *(Continued)*

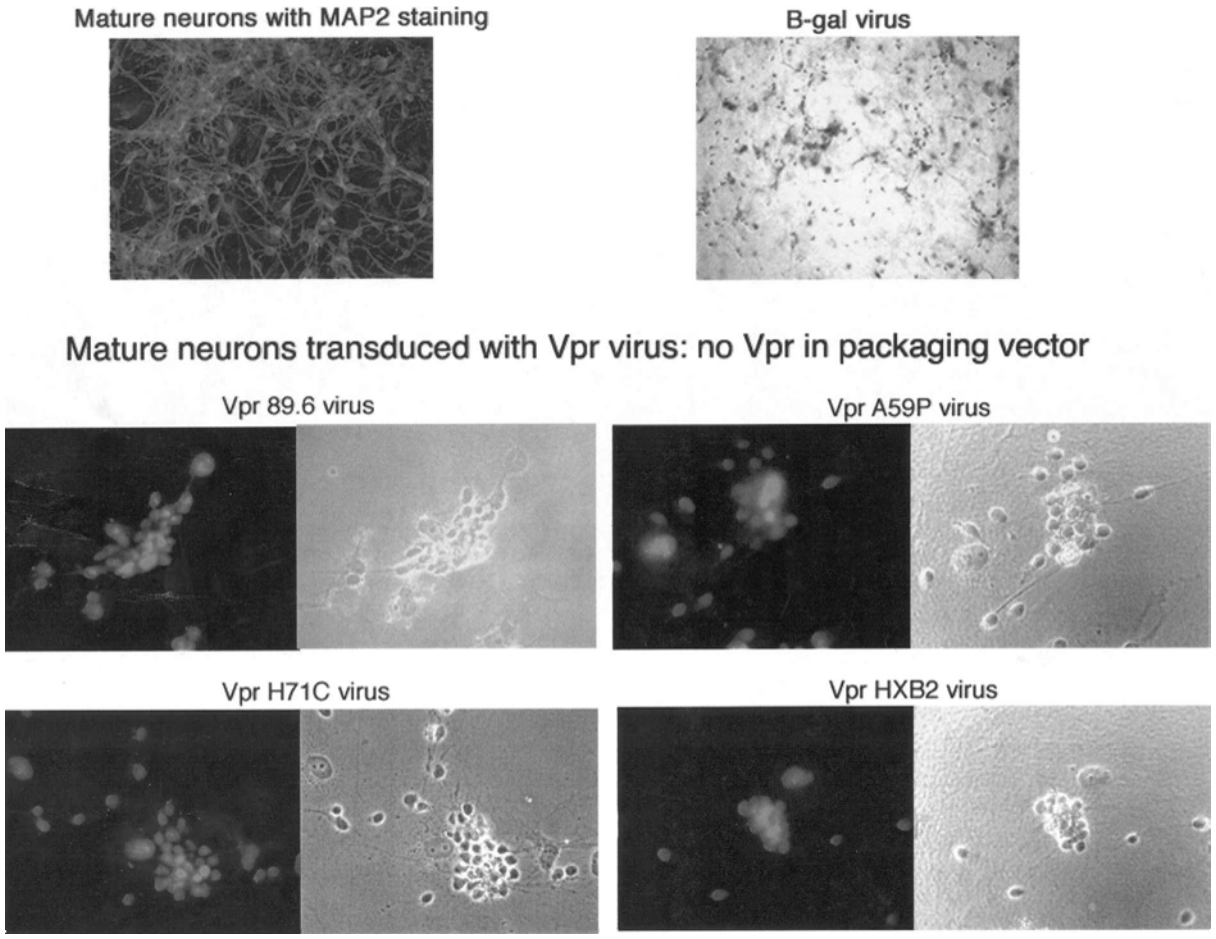
low levels as its respective control, the β-gal Vpr−+ virus, which lacked Vpr in the transduction vector (Figure 3B).

Similarly, the Vpr+− viruses containing Vpr as a transgene and lacking Vpr in the packaging vector exhibited significant apoptosis for wild-type Vpr 89.6 (Figure 3C). However, the mutant Vpr transgenes, A59P, H71C and HXB2, showed significantly decreased induction of apoptosis. The levels of apoptosis induced by mutant Vpr transgenes were approximately the same as the Vpr−− virus lacking completely the Vpr transgene.

Notably, there was no significant difference in the apoptosis levels induced by the β-gal viruses (lacking the Vpr transgene), with and without Vpr in the packaging construct (Figure 3D and lower power magnification in the middle and lower panels of Figure 3A). In contrast, the Vpr++ virus containing the Vpr 89.6 transgene, along with Vpr in the packaging construct, exhibited prominent induction of apoptosis. The Vpr+− virus containing Vpr 89.6 in the transduction vector but lacking Vpr in the packaging vector also showed a significant induction of apoptosis, suggesting that the presence of Vpr in the packaging vector alone (i.e., only virion-encapsidated Vpr) does not significantly influence the level of transgene induction of human neuronal apoptosis.

Analysis of neurons transduced with HIV-1 Vpr by human apoptosis gene microarray analysis

Vpr 89.6 transgene-containing virions, devoid of virion-encapsidated Vpr, and a Vpr−− virion vector bearing a β-gal *orf* in the transduction vector, but lacking Vpr in the packaging construct, were used to begin to dissect the intracellular molecular mechanisms of Vpr-mediated apoptosis in human neurons. Using gene microarray comparison of Vpr+− virus-treated neuronal cells to β-gal Vpr−− virus-treated neuronal cells, significant up-regulation was observed in the mRNA expression of TNF receptor associated factor 2 (TRAF2), interleukin-2 (IL-2) receptor α, IL-4 receptor α, nuclear factor-kappa B (NF-κB), and Bcl-2-associated death promoter (Bad) in the Vpr-transduced neurons (Figure 4 and Table 1).



(B)

Figure 2 (continued) (**B**) The two lower panels illustrate immunostaining with Vpr antibody for neuronal cells transduced with Vpr+− virus containing the wild-type 89.6 or mutant Vpr (i.e., A59P, H71C, HXB2) in the transfer vector only and lacking Vpr in the packaging construct (left side: fluorescent staining; right side: phase contrast photomicrograph of the same slide section). Top panel illustrates X-gal and MAP2 staining for the negative control, β-gal virus, lacking Vpr in both the transfer vector and the packaging construct.

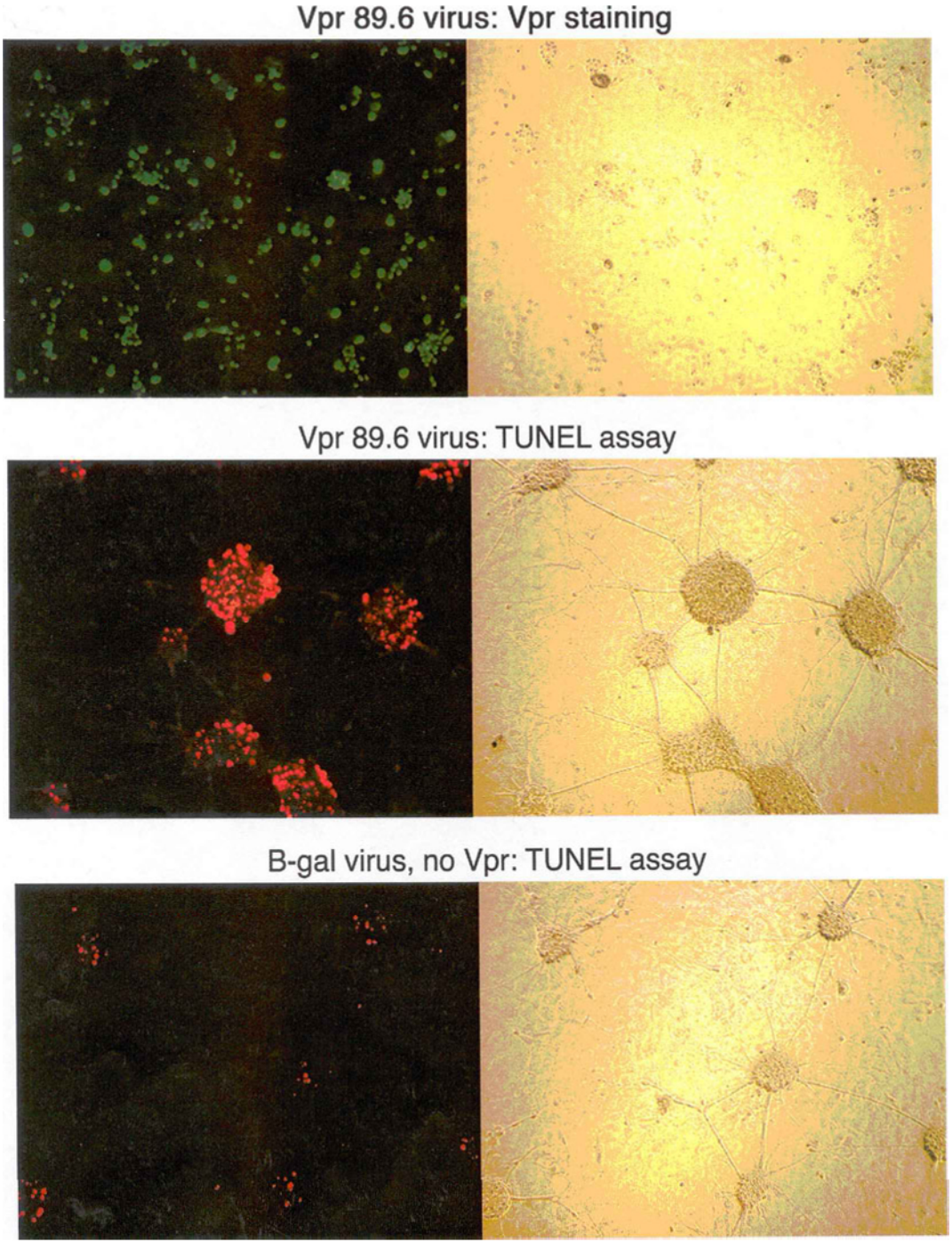
Table 1 is a compilation of the genes most strikingly up-regulated and the top five genes were the most up-regulated, by 2-fold or higher. TRAF2 was up-regulated by more than 5-fold, IL-2 receptor α was up-regulated by more than 4-fold, and IL-4 receptor α, NF-κB and Bad were up-regulated by more than 2-fold. TNFR1, Fas L, caspase-8, Bik, caspase-9, caspase-3 were all up-regulated by more than 1-fold, and up to 2-fold. These data were analyzed using genomic DNA to normalize the Vpr-treated and β-gal-treated cell samples, since some housekeeping genes were slightly up-regulated. The observation that Vpr 89.6 transgene-containing virions up-regulated the mRNA expression of Bad, TNFR1, TRAF2, NF-κB, Fas L, caspase-8, Bik, caspase-9, and caspase-3 indicates an induction of apoptosis through a mechanism most likely involving the mitochondria.

Table 1 Human apoptosis gene microarray analysis: Ratio of Vpr+− virus-transduced neurons to Vpr−− virus-transduced neurons

Genes up-regulated	*Fold increase*
1. TRAF2	>5 fold
2. IL-2 receptor α	>4 fold
3. Bad	>2–4 fold
4. IL-4 receptor α	>2–4 fold
5. NF-κB	>2–4 fold
6. TNF R1	>1–2 fold
7. Fas L	>1–2 fold
8. Caspase-8	>1–2 fold
9. Bik	>1–2 fold
10. Caspase-9	>1–2 fold
11. Caspase-3	>1–2 fold

Discussion

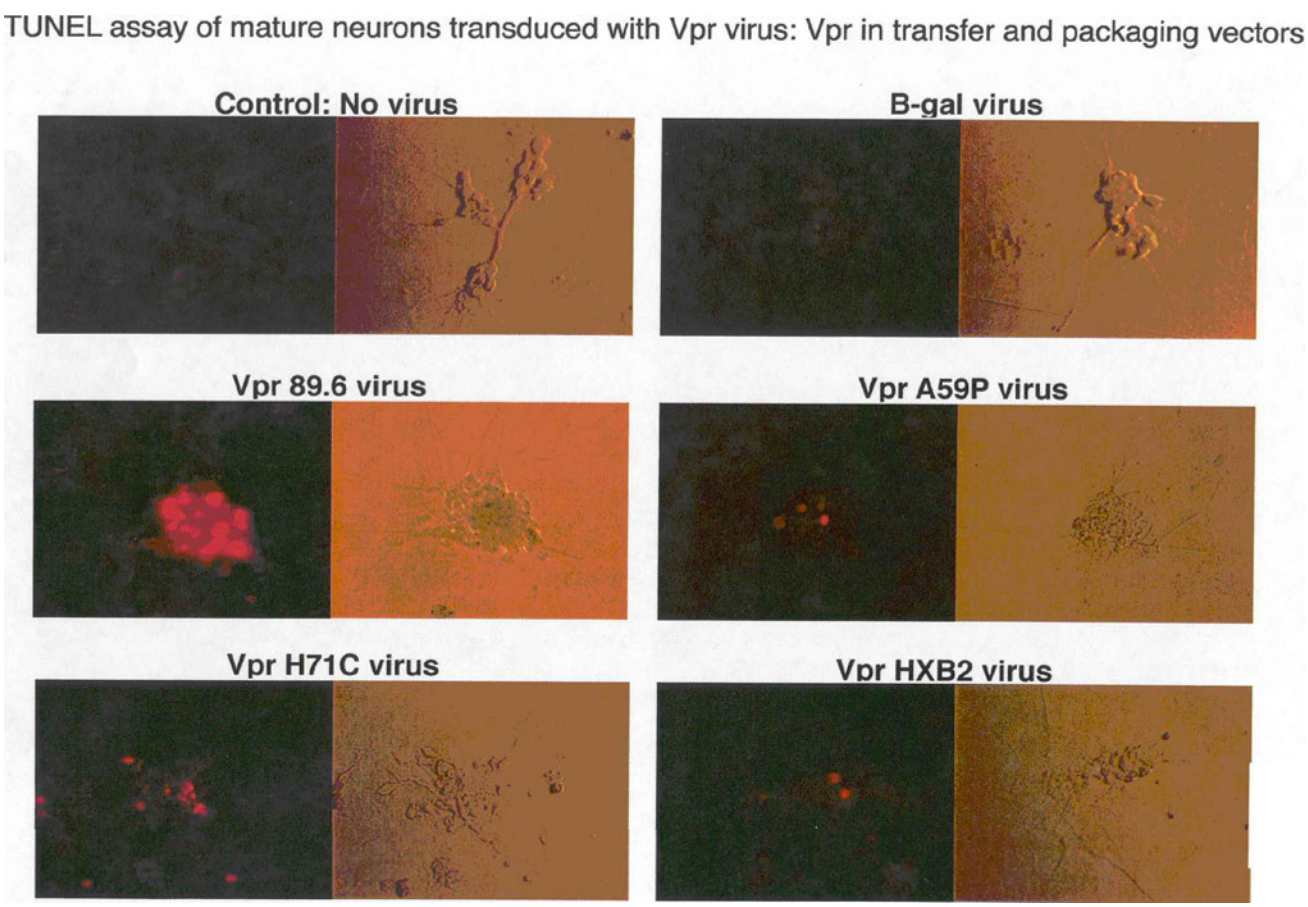
HIV-1 Vpr has been implicated in the induction of apoptosis of human T-cells during HIV-1 infections (Shostak *et al*, 1999). Recent studies have shown that extracellular Vpr protein induces apoptosis in certain cells of the human central nervous system (CNS), specifically terminally differentiated neurons



(A)

Figure 3 Apoptosis in neurons induced by intracellular HIV-1 Vpr. (**A**) TUNEL assay staining and immunostaining for expression of Vpr in neuronal cells transduced with Vpr+− virus containing the wild-type 89.6 transgene only, and TUNEL staining for neurons transduced with β-gal virus lacking Vpr in both the transfer vector and the packaging construct (left: fluorescent staining; right: phase contrast photomicrograph of the same slide section). *(Continued)*

and undifferentiated human neuronal precursor NT2 cells (Patel *et al*, 2000). Other studies have reported that Vpr induces apoptosis in human fibroblasts, T-cell-lines, and primary peripheral blood lymphocytes following cell-cycle G_2 arrest (Stewart *et al*, 1997). Although, the effects of Vpr as an inducer of apoptosis have more commonly been studied in cell-lines other than CNS-based cells, it has been reported that Vpr protein is found in a high concentration in the cerebrospinal fluid (CSF) of AIDS patients



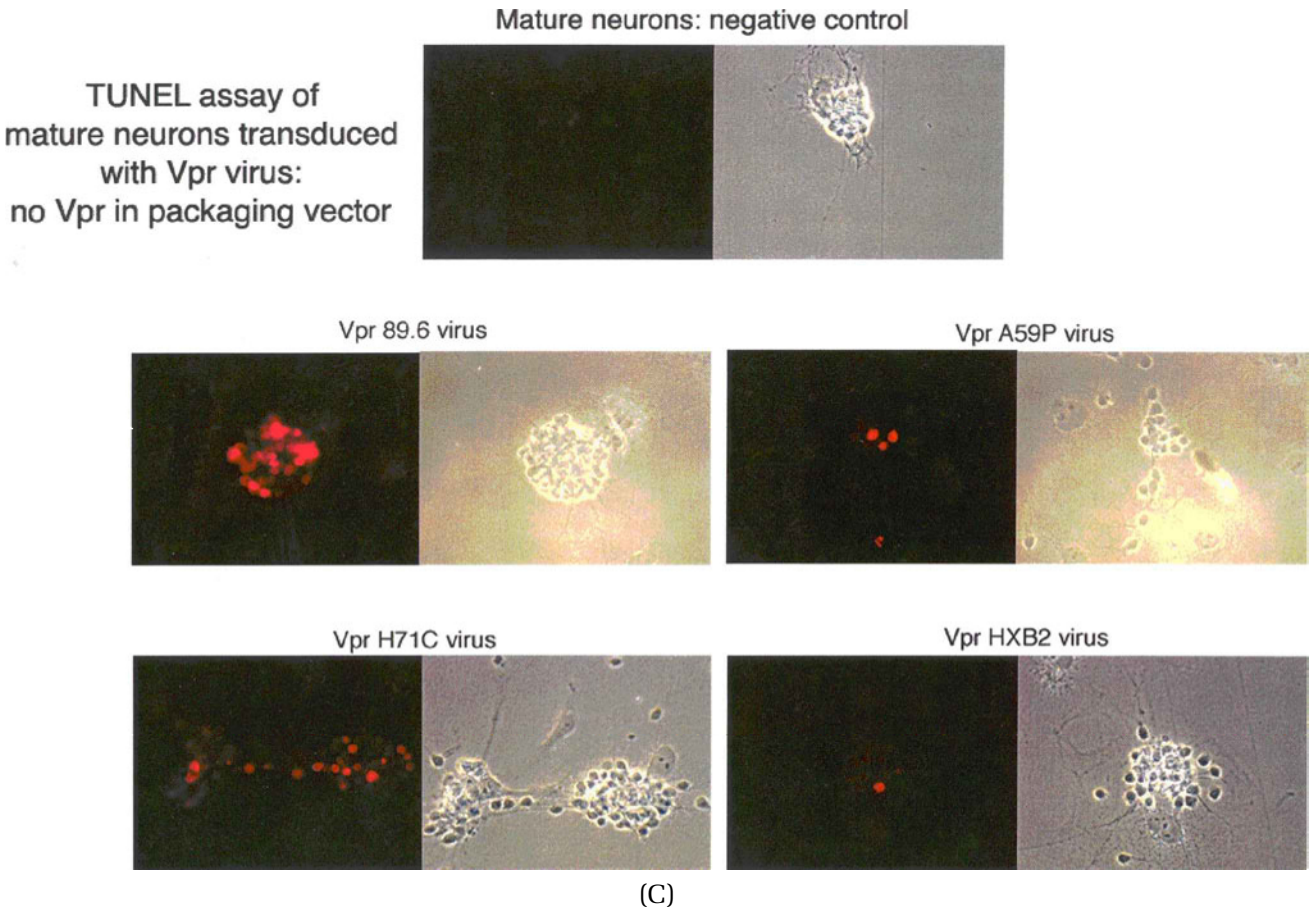
(B)

Figure 3 (Continued) (**B**) TUNEL assay staining for neurons transduced with Vpr++ virus containing the wild-type 89.6 or mutant Vpr (A59P, H71C, HXB2) in the transfer vector plus Vpr in the packaging vector (left side: fluorescent staining for the TUNEL assay; right side: phase contrast photomicrograph of the same slide section). *(Continued)*

(Levy *et al*, 1995). Therefore, we analyzed the effects of Vpr delivered intracellularly, expressed from an integrated proviral vector and encapsidated within the virions on human CNS-based cells.

The effects of Vpr were investigated in the context of the virus by using a lentiviral delivery system, where Vpr was expressed from the transfer vector, packaged in the virion vectors, and delivered into the cells for integration into the cell's genome. Furthermore, Vpr has been reported to exist within the virus particles as virion-encapsidated protein (Cohen *et al*, 1990; Yuan *et al*, 1990; Hrimech *et al*, 1999). Thus, we examined the intracellular delivery of Vpr in the presence and absence of virion-encapsidated Vpr protein (Vpr++ and Vpr+−). Concerns regarding efficient transduction in the absence of virion-encapsidated Vpr have been alleviated based on previous reports suggesting that neurons, as well as growth-arrested HeLa cells, can be efficiently transduced with virions devoid of virion-encapsidated Vpr (Blomer *et al*, 1997; Gasmi *et al*, 1999). In the present studies, the virus particles lacking the virion-associated Vpr efficiently transduced neuronal cells. The results indicate clear expression of Vpr protein within the neurons, with a high transduction efficiency, suggesting that the transfer vector DNA has stably integrated into the human neurons. Additionally, immunostaining for the expression of the mutant Vpr transgenes (A59P, H71C, and HXB2) indicated efficient expression of the various mutant Vpr proteins.

TUNEL assay staining demonstrated significant induction of apoptosis for the Vpr++ 89.6 virus. There was only a slight induction of apoptosis in the neurons expressing the Vpr mutants A59P, H71C, HXB2, as well as the neurons transduced with virus devoid of the Vpr transgene. Notably, decreased induction of apoptosis for Vpr mutants in our study compared well with previous findings, where mutant Vpr A59P was shown to impair all the functions of Vpr despite detectable expression (Mahalingam *et al*, 1997). Similarly, mutant H71C was previously reported to negate the cell-cycle arrest function of Vpr, as it did for the induction of apoptosis in our study (Mahalingam *et al*, 1997). Moreover, there was no quantitative difference in the level of apoptosis induced by the virus vectors lacking

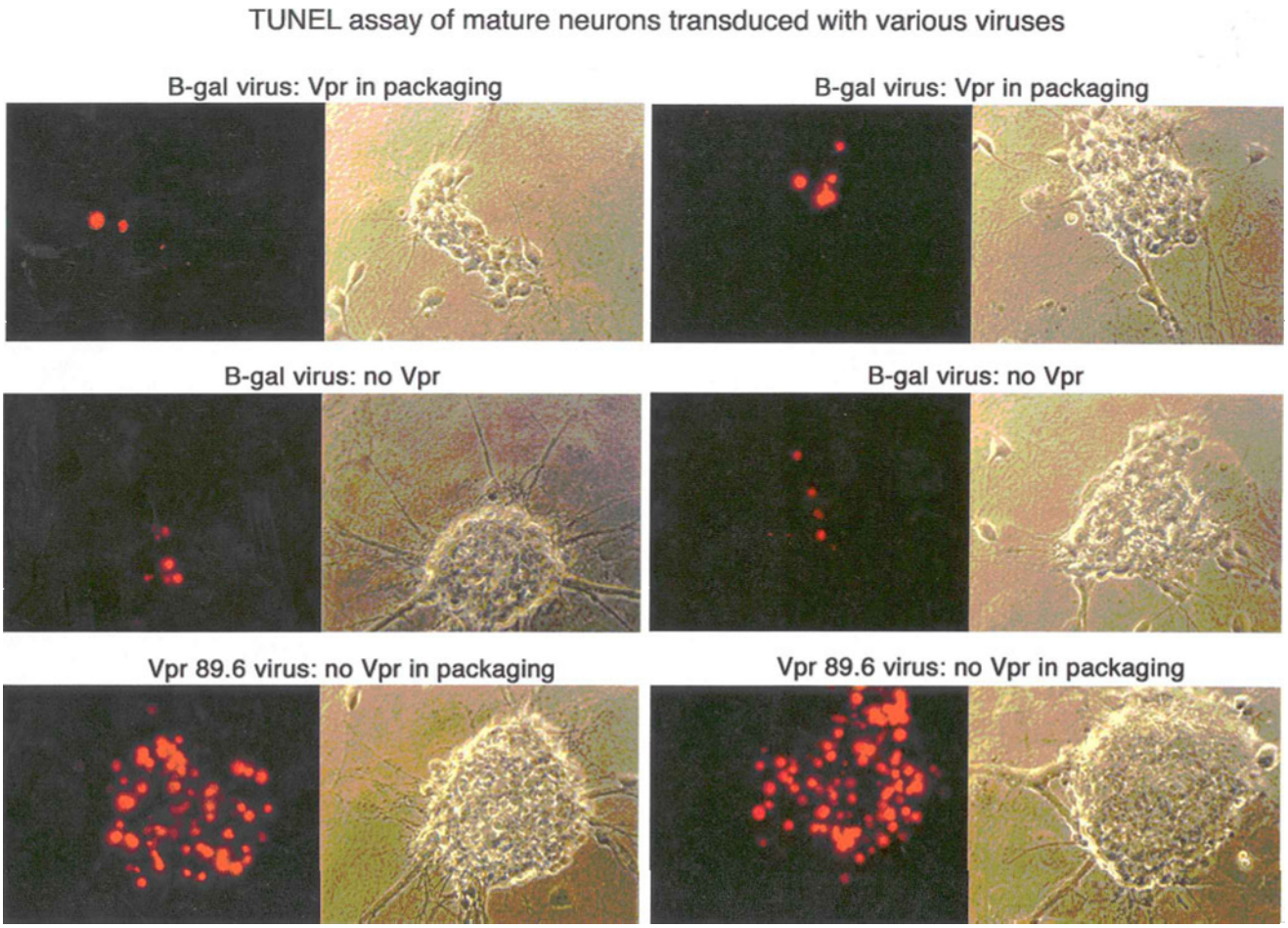


(C)

Figure 3 (Continued) (**C**) TUNEL assay staining for neurons transduced with Vpr+− virus containing the wild-type 89.6 or mutant Vpr (namely A59P, H71C, HXB2) in the transfer vector and lacking Vpr in the packaging vector (left side: fluorescent staining for the TUNEL assay; right side: phase contrast photomicrograph of the same slide section). *(Continued)*

the Vpr transgene and containing (Vpr−+) or lacking (Vpr−−) virion-encapsidated Vpr. The Vpr++ virus containing the Vpr 89.6 transgene, along with Vpr in the packaging construct, exhibited prominent induction of apoptosis. The Vpr+− virus containing Vpr 89.6 in the transduction vector but lacking Vpr in the packaging vector, also showed a significant induction of apoptosis, suggesting that the presence of Vpr in the packaging vector (i.e., only virion-encapsidated Vpr) does not significantly influence the level of transgene induction of neuronal apoptosis. In contrast, Poon and coworkers have previously reported that the level of cell-cycle arrest in T-cells was lower for virion-associated Vpr alone when compared to *de novo* Vpr expression from the wild-type virus (Poon *et al*, 1998; Stewart *et al*, 1999). Also, in a later study, the same group reported that virions containing Vpr in the absence of a viral genome induced apoptosis as efficiently as virions containing both a vector genome and Vpr (Poon *et al*, 1998; Stewart *et al*, 1999). It is important to note that due to aggregating growth patterns of the mature neurons, precise quantitation of apoptosis is difficult. Nonetheless, the levels of apoptosis induced in the present study were dramatically different between the Vpr+− and Vpr−− viruses, by at least 100-fold.

To begin to ascertain the molecular mechanisms involved in intracellularly expressed, Vpr-induced apoptosis in neurons, gene microarrays were used to analyze the regulation of apoptotic genes in human neurons transduced with Vpr+− viral vectors containing the Vpr 89.6 transgene alone. Our findings indicate that the Vpr 89.6 virus vector acted to induce apoptosis via an up-regulation of the components in the mitochondrial apoptotic pathway, by affecting Bcl-2-related proteins, when compared to neuronal cells treated with β-gal Vpr−− virus. Bad (Bcl-2-associated death promoter) translocates to mitochondria from the cytoplasm and forms a pro-apoptotic complex with Bcl-xL. This complex in turn promotes the release of cytochrome c. Released cytochrome c induces apoptosis, apparently through effects of the VDAC (voltage dependent anion channel) (Shimuzu *et al*, 1999; Desagher *et al*, 1999; Shostak *et al*, 1999; Kroemer and Reed, 2000; Jacotot *et al*, 2001). In the present study, the microarray analyses demonstrated an up-regulation of Bad induced by intracellular Vpr from lentivirion vectors.



(D)

Figure 3 (Continued) (**D**) TUNEL assay staining for neurons transduced with Vpr+− virus containing Vpr in the transfer vector only and lacking Vpr in the packaging construct. Top and middle panels show TUNEL staining for the control viruses; β-gal virus lacking Vpr in the transfer vector and containing virion-encapsidated Vpr from the packaging construct, and β-gal virus lacking Vpr in both the transfer vector and the packaging construct (left side: fluorescent staining for the TUNEL assay; right side: phase contrast photomicrograph of the same slide section).

Prominent up-regulation of mRNA expression was also observed for the IL-2 receptor α, IL-4 receptor α, and TRAF2. We have shown previously that HIV-1 Vpr plays a role in potent activation of mononuclear phagocytic cells and initiates erythrophagocytosis (Kulkosky *et al*, 1999). The up-regulation of interleukin receptors is significant in this regard and hence, it is possible that transducing marrow cells with Vpr results in increased receptor display lowering the threshold for activation in the marrow cultures, thereby signaling mononuclear phagocytes to initiate erythrophagocytosis. However, one study has attempted to demonstrate that Vpr has an antiproliferative effect and suppresses the expression of IL-2, IL-10, IL-12, TNFα, and IL-4 (Ayyavoo *et al*, 1997). Our ongoing studies seek to define whether there is an up-regulation of a broad cytokine profile in the neuronal cells transduced with Vpr+− virus vector.

Interestingly, an up-regulation of NF-κB mRNA upon treatment with Vpr transgene-containing virus was also demonstrated. NF-κB exerts a central role in regulating the expression of a number of proinflammatory and viral genes, including survival genes such as the inhibitor of apoptosis (IAP) (Finkel, 2001). The up-regulation of NF-κB may be a mechanism by which the induction of apoptosis by Vpr is partially counteracted. A previous study has shown that Vpr can either induce or inhibit apoptosis in T-cells, depending upon T-cell receptor-mediated activation of apoptosis (Ayyavoo *et al*, 1997). This group's findings suggest that Vpr regulates apoptosis in T-cells via suppression of NF-κB through the induction of IκB, an inhibitor of NF-κB.

The Vpr 89.6 transgene-containing virions slightly down-regulated expression of IAP-2, IL-12, Rb 1, and the TNF receptor 1-associated death domain (TRADD) mRNA, after transduction of the neuronal

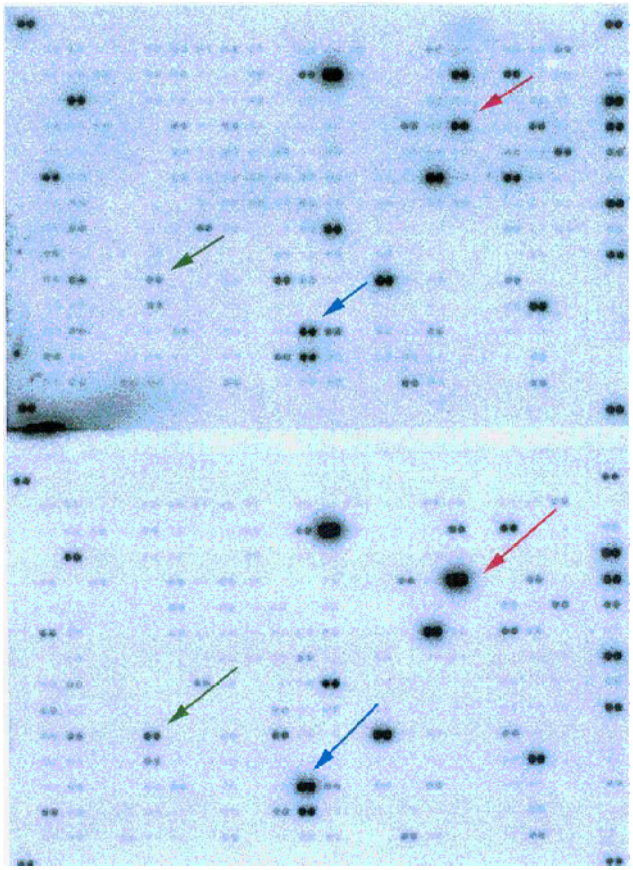


Figure 4 Human apoptosis gene microarray of neurons transduced with HIV-1 β-gal (Vpr−−) virus (top panel) and Vpr+− virus (containing the wild-type Vpr 89.6 in the transduction vector only—bottom panel). TRAF2 up-regulation (red arrow), IL-2 receptor up-regulation (blue arrow), and Bad up-regulation (green arrow) was observed in neurons transduced with Vpr 89.6+− virus (bottom panel), as compared to β-gal (Vpr−−)-transduced cells (top panel).

cell cultures. Though, the low-levels of down-regulation may not be functionally relevant.

Overall, the results suggest that HIV-1 Vpr expressed intracellularly induces apoptosis in human neurons, most likely through specific mitochondrial components involved in programmed cell death. These include Bcl-2-related proteins, Bad and Bik, which initiate the release of cytochrome c, thus inducing apoptosis. Another component activated through cytochrome c is caspase-9, which in turn activates caspase-3 (Budihardjo *et al*, 1999; Ferri *et al*, 2000; Finkel, 2001). Both caspase-9 and caspase-3 were modestly up-regulated at the mRNA level in our system. It should be noted that initiation of apoptosis by apoptotic proteins do not fully rely on *de novo* synthesis of protein and therefore up-regulation of their mRNAs might not always be expected to be mechanistically critical. Additionally, the neuronal cells showed an up-regulation of TNFR1 and FasL, which are pro-apoptotic stimuli leading to an activation of caspase-8 in the caspase cascade (Boldin *et al*, 1995; Budihardjo *et al*, 1999; Ferri *et al*, 2000). Furthermore, in a recent study, Vpr-mediated thymocyte apoptosis in transgenic mice expressing Vpr was independent of Fas-Fas ligand pathway, and rather involved Bcl-X, Bax, and caspase-1 for induction of apoptosis (Yasuda and Miyao, 2001). Recently, Jacotot and colleagues have proposed that the mitochondrial adenine nucleotide translocator (ANT) is a novel Vpr target (Jacotot *et al*, 2001). Furthermore, they argue that Vpr targets ANT by passing through the voltage-dependent anion channel (VDAC), based on studies with a VDAC inhibitor (Jacotot *et al*, 2001). Thus, the major pathway of intracellular Vpr-induced apoptosis probably occurs through a mechanism involving the mitochondria. Clearly, the developing microarray technologies will be critical in the functional analyses of diverse viral infection interactions with differentiated cellular gene expression.

There has been much debate regarding HIV-1 infectivity of neurons. Sharpe and coworkers have demonstrated that the neuron is a major target in the viral infection by the immune neurotropic retrovirus of mice (Sharpe *et al*, 1984). It has been hypothesized that this retroviral disease of neurons is likely caused by indirect consequences of viral infection of glia or endothelial cells. Sharpe *et al*. have argued against this hypothesis by demonstrating that spongiform degeneration of the neurons is a result of direct but low-level viral gene expression in the neurons. HIV-1-seropositive patients, some with ADC, were demonstrated, with co-labeling experiments to harbor some infected neurons and astrocytes (Nuovo *et al*, 1994). These studies also suggest that HIV-1 commonly exists in the CNS of asymptomatic patients. Recent reports have suggested that only certain neuronal cell populations may become infected *in vivo*. However, strict localization in some studies involving human tissue has been difficult (Canto-Nogues *et al*, 2001). In a study using mixed embryonic rat brain cultures as a model to examine physiologic effects of extracellular Vpr, researchers found that Vpr had no effect on primary cortical neurons, but 200 picograms per milliliter of Vpr induced cell-death in hippocampal neurons and astrocytes, suggesting that differential toxic effects of extracellular Vpr are observed in different subtypes of neurons (Huang *et al*, 2000). Furthermore, Nuovo *et al* (1994) observed that the level of HIV-1-infected neurons showed considerable variability in the same tissue section. It is questionable as to what level the virus infects and replicates in the different cell-types of the nervous system and, thus, this potential mechanism probably does not fully explain the pathogenesis of ADC, because it is unlikely that every neuronal cell that drops out during the evolution of ADC does so as a result of direct HIV-1 infection.

Of note, it has been suggested that there are differences in neurological disease progression between pediatric and adult patients due to the immaturity of the cells of the CNS in fetal brains. Immature

neuronal and glial cells are considerably more susceptible to viral replication and may represent some of the major targets and reservoirs for HIV-1 in the developing nervous system. Localization of simian immunodeficiency syndrome (SIV) in fetal brain by *in situ* hybridization and immunohistochemistry revealed virus-positive cells in the cortical plate region of the animal brains. Based on the location, the cells infected were most likely neurons and/or astrocytes in the macaque fetal brain (Lane *et al*, 1996). As well, a recent study has demonstrated HIV-1-infected neurons selectively in the cortical region of HIV-1-infected children with encephalopathy (Canto-Nogues *et al*, 2001).

Additionally, there have been reports that a human cerebral cortical neuronal cell-line (HCN-1A) has properties of immature undifferentiated cells of neuronal origin and lacks surface CD4 protein (Truckenmiller *et al*, 1993), as has been reported for mature NT2 neurons in our study (Patel *et al*, 2000). Furthermore, NT2 neurons express CXCR4 receptors on their surface (Lavi *et al*, 1997; Glabinski and Ransohoff, 1999; Hesselgesser and Horuk, 1999). Thus, a mechanism other than CD4 viral entry is likely responsible for low-level HIV-1 infection of neurons (Ensoli *et al*, 1995). Additionally, viral replication was enhanced three-fold in the neuronal HCN-1A cell-line by nerve growth factor and fibroblast growth factor (Mizrachi *et al*, 1994), thus suggesting a potentially more robust viral replication *in vivo* as compared to *in vitro*.

One possible complexity of the neuron system utilized in the present studies is that since the neuronal cultures had some basal undifferentiated cells, on top of which the neurons attach themselves, there may be the release of certain factors from these cells, such as complement moieties, as reported by Speth and colleagues for human astrocytes (Speth *et al*, 2001). The release of complement factors in the brain have been reported to modulate cytokine expression, induce nerve growth factor, and activate signal transduction pathways (Speth *et al*, 2001). Thus, the potential contributions of the basal underlying cells will require future analysis.

In conclusion, HIV-1 Vpr delivered intracellularly, as well as extracellularly (Patel *et al*, 2000), is involved in the potent induction of apoptosis of human neurons. This may be a critical molecular mechanism in the still enigmatic entity of HIV-1-induced encephalopathy.

Materials and methods

HIV-1 Vpr molecular clones: Construction of Vpr wild-type and Vpr mutant transfer vectors

To generate HIV-1 vector constructs containing the wild-type Vpr 89.6 gene, as well as the Vpr mutants A59P, H71C, and HXB2 genes, the respective Vpr fragments were cloned into the transfer vector of a three plasmid HIV-1 expression system (Naldini *et al*, 1996; Zufferey *et al*, 1997). The Vpr fragments were generated from digestion of pSLXCMVVpr by restriction endonucleases BamHI and XhoI (Kulkosky *et al*, 1999). The vector, pHR'CMVVpr, was generated from cloning these fragments into the transfer vector, pHR'CMV-lacZ, after excision of the lacZ fragment. Mutant Vpr A59P (alanine substituted at position 59 to proline), Vpr H71C (histidine substituted at position 71 to cysteine) and Vpr HXB2 (frameshift mutation at position 78) are mutations in the helical domain II or the basic amino acid-rich domain of the carboxy terminus of Vpr (Kulkosky *et al*, 1999).

Viral supernatants and HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA)

Replication-defective retroviral particles were generated by transient cotransfection of 293T human kidney cells using the calcium-phosphate transfection method (Promega). The three plasmid expression system was used to generate retroviral particles with simultaneous cotransfection on 100-mm plates of 293T cells with 12 μg of transfer vector containing and lacking the Vpr *orf* (pHR'CMVVpr or pHR'CMVlacZ), 16 μg of packaging construct (pCMVR8.2: with Vpr in the packaging construct; or pCMVΔR8.91, without Vpr in the packaging construct), and 10 μg of a vesicular stomatitis virus (VSV) envelope-encoding plasmid (pMD.G). Thus, four types of viral supernatants were generated: Vpr++, made from simultaneous triple transfection of pHR'CMVVpr, pCMVΔR8.2 and pMD.G; Vpr+−, made from simultaneous triple transfection of pHR'CMVVpr, pCMVΔR8.91 and pMD.G; Vpr−+, made from simultaneous triple transfection of pHR'CMVlacZ, pCMVΔR8.2 and pMD.G; Vpr−−, made from simultaneous triple transfection of pHR'CMVlacZ, pCMVΔR8.91 and pMD.G.

For Vpr++ and Vpr+−, the respective mutant viruses were also generated by substituting the transfer vector pHR'CMVVpr with the transfer vector containing the respective mutant Vpr (Vpr A59P or Vpr H71C or Vpr HXB2). Viral supernatants were collected 72 h posttransfection and HIV-1 p24 antigen ELISAs were performed. Input of viral vector supernatants were then strictly normalized before transduction of the human neurons.

Growth and differentiation of human NT2 cells

NT2 cells were grown in Dulbecco's modified Eagle's medium (DMEM-HG) containing 10% fetal bovine serum, 1% penicillin–streptomycin, and 1% glutathione. For the present experiments, NT2 cells were grown on chamber slides (Falcon) and transductions were administered to undifferentiated as well as mature, differentiated neuronal cultures following daily microscopic evaluation for morphological changes occurring in the cells. Mature, differentiated

neurons were induced from undifferentiated NT2 cells through an extensive differentiation, replating, and neuronal harvesting protocol (Patel *et al*, 2000). Differentiation was achieved with treatment of 10 μM retinoic acid for a period of 5 to 6 weeks after which neurons were harvested by selective trypsinization (Replates 1 and 2). At this stage, to prevent the undifferentiated cells from overgrowing the post-mitotic neurons, mitotic inhibitors (10 μM 5-fluoro-2′-deoxyuridine, 10 μM uridine, 1 μM cytosine β-D-arabinofuranoside) were added to the culture medium. After further enrichment by treatment with conditioned medium consisting of growth factors (complete DMEM collected at the end of Replate 1) essential for the neurons and selective trypsinization, the cells were replated once more (Replate 3) to obtain 90 to 95% pure neuronal cultures (Pleasure *et al*, 1992; Patel *et al*, 2000; Pleasure and Lee, 1993; Naldini *et al*, 1996).

Immunostaining and immunoflourescence microscopy

The transduced neuronal cultures were immunostained for Vpr expression using a standard protocol. The cells were incubated with a dilution of 1:200 of rabbit anti-Vpr polyclonal IgG (kindly provided by N. Landau, Salk Institute), and after several washes with a 1:200 dilution of secondary antibody, Cy2-linked goat anti-rabbit antibody. Mature neurons were also stained for morphology using MAP2 antibody for microtubules. Immunoflourescence microscopy was performed using an Olympus System microscope, Model BX60.

DNA fragmentation and terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end-labeling (TUNEL) assay

DNA fragmentation occurs at a later stage in apoptosis and this assay utilizes the generated 3′ free hydroxyl groups at the ends of the fragmented DNA, to be labeled with fluorescein dUTP in the presence of TdT (Li *et al*, 1995). TUNEL assays were performed using the *In Situ* Cell Death Detection Kit, TMR Red (Boehringer-Mannheim). Cell samples were fixed with 2% paraformaldehyde solution in 1× phosphate-buffered saline (PBS), pH 7.4 for 60 min at room temperature. Slides were rinsed with 1× PBS and incubated in permeabilization solution consisting of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min at 4°C. Cells were washed twice with 1× PBS prior to labeling. TUNEL reaction labeling was performed by using the specified amounts of the "Label Solution," and "Enzyme Solution," and incubated for 60 min in the dark, at 37°C in a humidified chamber. Results were analyzed by fluorescence microscopy. An Olympus System Microscope Model BX60 with Fluorescence Attachment BX-FLA was utilized. Of note, the mature neurons tend to form clumps and these aggregating growth patterns make strict quantitation of apoptotic cell numbers difficult, as compared to a flat layer of cells such as the undifferentiated NT2 cells.

Human apoptosis gene microarray

The array consists of 198 different apoptosis-related DNA templates affixed onto charged nylon membranes (Sigma-Genosys, Woodlands, TX). Briefly, total cellular RNA was extracted from samples of Vpr 89.6+− and control Vpr−− transduced mature neurons and used to generate ^{32}P-labeled cDNAs. The labeled cDNA were hybridized onto the arrays and phosphorimaging was used to quantitate the gene expression levels. Specifically, the cDNAs were generated using an array-specific primer provided by the kit and radiolabelled using α-^{32}P-CTP (NEN). The filters were hybridized overnight at 68°C and washed according to the manufacturer's instructions. The data were captured using a Molecular Dynamics phosphorimager. For data analysis, the human genomic DNA spotted at the four corners were used to normalize the signal between the β-gal (Vpr−−) virus-treated samples and the Vpr 89.6+− virus-treated samples.

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