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Human Immunodeficiency Virus Type 1 Nef Potently Induces Apoptosis in Primary Human Brain Microvascular Endothelial Cells via the Activation of Caspases

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The lentiviral protein Nef plays a major role in the pathogenesis of human immunodeficiency virus type I (HIV-1) infection. Although the exact mechanisms of its actions are not fully understood, Nef has been shown to be essential for the maintenance of high-titer viral replication and disease pathogenesis in in vivo models of simian immunodeficiency virus infection of monkeys. Nef has also been suggested to play a pivotal role in the depletion of T cells by promoting apoptosis in bystander cells. In this context, we investigated the ability of extracellular and endogenously expressed HIV-1 Nef to induce apoptosis in primary human brain microvascular endothelial cells (MVECs). Human brain MVECs were exposed to baculovirus-expressed HIV-1 Nef protein, an HIV-1-based vector expressing Nef, spleen necrosis virus (SNV)-Nef virus (i.e., SNV vector expressing HIV-1 Nef as a transgene), and the HIV-1 strain ADA and its Nef deletion mutant, ADAΔNef. We observed that ADA Nef, the HIV-1 vector expressing Nef, and SNV-Nef were able to induce apoptosis in a dose-dependent manner. The mutant virus with a deletion in Nef was able to induce apoptosis in MVECs to modest levels, but the effects were not as pronounced as with the wild-type HIV-1 strain, ADA, the HIV-1-based vector expressing Nef, or SNV-Nef viruses. We also demonstrated that relatively high concentrations of exogenous HIV-1 Nef protein were able to induce apoptosis in MVECs. Gene microarray analyses showed increases in the expression of several specific proapoptotic genes. Western blot analyses revealed that the various caspases involved with Nef-induced apoptosis are processed into cleavage products, which occur only during programmed cell death. The results of this study demonstrate that Nef likely contributes to the neuroinvasion and neuropathogenesis of HIV-1, through its effects on select cellular processes, including various apoptotic cascades.

Nef (negative factor) is a 27-kDa accessory protein that plays a major role in the pathogenesis of HIV-1 infections (36, 40). Although the exact mechanisms of its actions are not fully understood, Nef has been shown to be essential for the maintenance of high-titer viral replication and disease pathogenesis in in vivo models of simian immunodeficiency virus (SIV) infection of monkeys (54). Nef has also been suggested to play a pivotal role in the depletion of T cells during HIV-1 infection (5). It is of interest that macaques infected with SIV with a deletion in *nef* do not rapidly develop AIDS-like symptoms, and this inability of SIV with a deletion in Nef to induce AIDS-like symptoms in these animals has been attributed to a drastic reduction in apoptotic death of cytotoxic T lymphocytes (CTLs) and $CD4^+$ T cells (54). Actually, careful analyses of previous reports suggest that pathogenesis of SIV with a deletion in *nef* occurs but is quite slow in progression (45).

Nef in vitro has been shown to deplete T cells during HIV-1 infection and also enhances virion infectivity via interference with signal transduction pathways and down-modulation of major histocompatibility complex class I molecules and CD4 receptors (76, 98). In addition, Nef accelerates rapid endocytosis and degradation of major histocompatibility complex class I molecules, resulting in a reduction of epitope density and evasion of CTL lysis (17). In a recent study, Sol-Foulon et al. (97) reported that HIV-1 Nef induces the up-regulation of the expression of DC-SIGN, a dendritic cell (DC)-specific lectin which modulates clustering of DCs with T lymphocytes for the initiation of immune responses (97). It was shown in that study that the up-regulation of DC-SIGN expression significantly increased clustering of DCs to T cells. It was suggested that this phenomenon could affect the potency of DCs to activate lymphocytes, thereby leading to increased viral replication (97).

The activities of this lentiviral protein, as observed in vitro, have led to the suggestion that Nef allows HIV-1 to avoid immune surveillance, via either active or passive mechanisms (17). These mechanisms become especially important in examining the neuropathological abnormalities of the brains of HIV-1-infected individuals. These abnormalities include neuronal apoptosis and dropout, which most likely result in the central nervous system (CNS) damage associated with AIDS dementia complex (3). More than 50% of untreated HIV-1-infected patients develop CNS disorders, including neoplasms,

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chronic HIV-1 encephalopathy, and opportunistic infections (84). Blood-brain barrier (BBB) leakage has been observed more frequently in neurologically symptomatic than in asymptomatic HIV-infected patients, although the exact pathophysiological mechanisms causing the altered BBB permeability during HIV-1 infection are not yet fully understood (93, 94).

In this context, we examined the role of Nef in the induction of apoptosis in primary human brain microvascular endothelial cells (MVECs). We utilized, in a complementary fashion, both extracellular and endogenously expressed HIV-1 Nef to induce apoptosis in MVECs. Most of the previous studies utilizing extracellular Nef have employed bacterially expressed Nef protein, which lacks the N-terminal myristoylation (7, 48). Since it has been suggested that the cytopathic effects of Nef are due to its being targeted to the plasma membrane and other cellular membranes via the N-terminal myristoyl group (18, 19), we used baculovirally expressed Nef protein, which is known to be myristoylated. In these experiments, complementary treatment protocols for Nef were developed and utilized. Thus, the present studies evaluated the effects of baculovirally expressed HIV-1 Nef protein, HIV-1-based Nef virus (i.e., an HIV-1-based vector expressing Nef), spleen necrosis virus (SNV)-Nef virus (i.e., a SNV vector expressing HIV-1 Nef as a transgene), and the HIV-1 strains ADA and its Nef deletion mutant, ADAΔNef, in an in vitro culture system of primary human brain MVECs. As MVECs are a major cellular component of the human BBB, it was hypothesized that HIV-1 Nef may alter the integrity of the BBB during infection.

MATERIALS AND METHODS

Primary human brain MVECs. Primary human brain MVECs were purchased from Cell Systems (Kirkland, Wash.). The cells were initially seeded into 75-cm^2 flasks in Endothelial Cell Basal Medium-2 supplemented with endothelium growth factors (Biowhittaker, Walkersville, Md.). Cells were incubated at 37°C with 5% CO_2 in a humidified environment. The purity of the cells was assessed by immunostaining for von Willebrand's factor (factor VIII), as described previously (70, 71).

Recombinant HIV-1 Nef protein. Baculovirally expressed Nef protein was generated in our laboratory, as reported previously (1). Briefly, a consensus Nef-coding sequence, isolated from pLconsNef (National Institutes of Health AIDS Reagent Program), was cloned into a baculovirus transfer vector, pAcGHLT-A (BD PharMingen, San Diego, Calif.) at the EcoRI site to produce a glutathione *S*-transferase (GST)–Nef fusion product. The recombinant vector was cotransfected into Sf9 insect cells with linearized baculovirus DNA to generate the recombinant virus. The viral supernatant was collected, amplified, and used to infect Sf9 cells for Nef protein expression. Protein purification was carried out by applying soluble protein extracted from infected Sf9 cells to glutathione agarose beads (Amersham Pharmacia, Piscataway, N.J.), which were equilibrated with 1× phosphate-buffered saline (PBS). After extensive washing with PBS, the Nef protein was cleaved from the fused GST. The fractions containing Nef protein were pooled and further purified by passage through benzamidine Sepharose. This step removed the bovine thrombin from the recombinant protein. The purity of Nef protein was confirmed by Western blotting with Nef monoclonal antibody. As a negative control, recombinant GST was utilized, after production via the same protein expression system as was used to create Nef.

Construction of Nef-expressing vectors. To generate an HIV-1-based vector containing the Nef consensus sequence gene, the Nef fragment was cloned into the transfer vector of a three-plasmid HIV-1 expression system (73). The Nef fragment was excised from pLconsNef with EcoRI restriction endonuclease. The fragment was subcloned into the EcoRI site of the expression vector pBluescript SK I (Stratagene Corporation). The Nef fragment was then generated by digesting the pBluescript with the restriction endonucleases BamHI and XhoI. The vector, pHR′CMVNef, was generated by cloning this fragment into the transfer vector pHR′CMVLacZ after removal of the LacZ fragment with BamHI and XhoI.

The SNV-Nef vector was generated by cloning the *nef* open reading frame into the SNV transfer vector pZP35 via blunt-ended ligation (78, 79). The Nef fragment was generated as indicated above, and the pZP35 was linearized with SmaI restriction endonuclease. The 3′ and 5′ ends of both the pZP35 and the Nef fragment were blunt ended by using the DNA polymerase I large fragment minikit (Promega, Madison, Wis.). The Nef fragment was subsequently ligated into the pZP35 vector to generate the SNV-Nef vector. As negative controls, both HIV-1 and SNV vectors expressing LacZ as a transgene were utilized (69, 78).

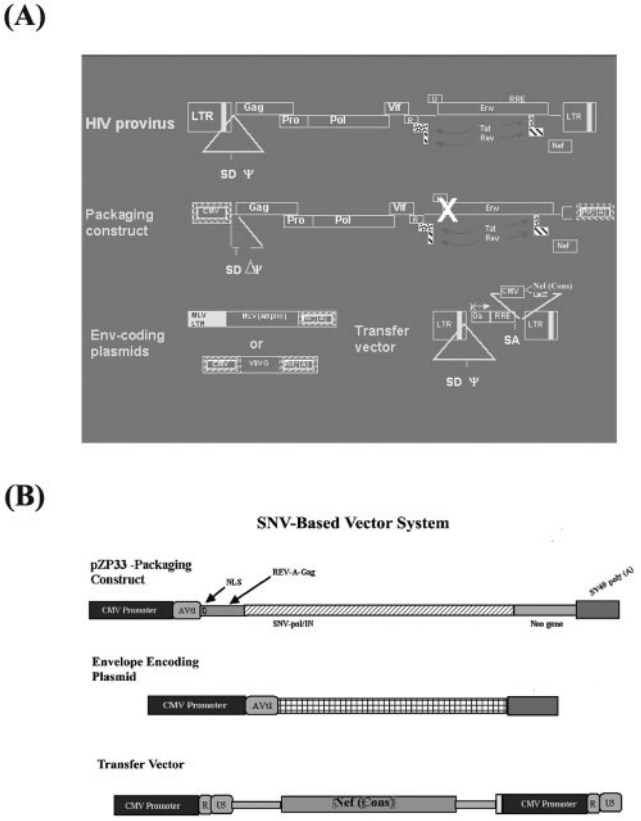

FIG. 1. Schematic representations of the triple-vector plasmid systems employed for the recombinant HIV-1 Nef, SNV-Nef, and LacZ viruses. Replication-competent retroviral particles were generated by triple transfection of transfer vector (A) pHR′CMVNef (HIV-1-based vector expressing Nef) or (B) SNV-Nef (SNV-based vector expressing Nef) or pHR′CMVLacZ, the packaging construct pCMVΔR8.2, and the VSV envelope-encoding plasmid pMD.G.

HIV-1- and SNV-based viral vectors and HIV-1 virions. The various components of the HIV-1-based vector system, as well as the SNV-Nef vector, used in this study are depicted in Fig. 1. The plasmids for HIV-1 strains ADA and ADAΔNef were kindly provided by Mario Stevenson (University of Massachusetts). The HIV-1-derived vectors were obtained by transient transfection of 293T cells with plasmids encoding viral proteins, pCMVΔR 8.2, the gene transfer vector pHR′CMVNef, and an envelope-encoding vesicular stomatitis virus (VSV) glycoprotein plasmid (pMD.G). Lentiviral particles, harvested after transfection, were utilized for infection experiments. SNV-Nef retroviral particles were generated by transient transfection of 293T cells with packaging construct pZP33, the gene transfer vector pZP35-Nef, and the VSV envelope glycoprotein-encoding plasmid (pMD.G) (78). The HIV-1 ADA and ADAΔNef viral particles were also obtained by transient transfection, as described above.

Exposure of primary human brain MVECs to HIV-1 Nef protein and virions. Low-passage primary human brain MVECs were trypsinized and seeded into four-well chamber slides. The cells were allowed to attach overnight before being exposed to recombinant HIV-1 Nef protein at concentrations ranging from 1 to 1,000 ng/ml. The quantities of HIV-1-based Nef virus, HIV-1 strain ADA, and its deletion mutant ADAΔNef utilized ranged from 1 to 10 ng of p24 antigen equivalents per ml. The amount of SNV-Nef virus used ranged from 10 to 1,000 CFU/ml. Following treatment with the protein and viruses, the cells were incubated for 48 h at 37°C with 5% CO_2 in a humidified environment.

TUNEL assay. To study apoptotic cell death, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were performed on treated and untreated cells with the in situ cell death detection system, TMR Red (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Briefly, cells were washed twice with 1× PBS and fixed for 10 min with acetone at room temperature. The fixed cells were washed three times with 1× PBS, and 50 μl of TUNEL reaction mixture was added to each well. Cells treated with the TUNEL mixture were incubated at 37°C in a humidified environment for 1 h. On completion of incubation, the treated cells were washed four times with blocking buffer (PBS, 0.1% Triton X-100, 0.5% bovine serum albumin) and evaluated by fluorescence microscopy. As positive controls, MVECs were treated with DNase (Life Technologies, Gaithersburg, Md.) at a concentration of 1.0 μg/ml and incubated for 10 min at room temperature to induce DNA strand breaks. Untreated cells, GST-treated cells, and cells transduced with LacZ-expressing vectors were used as negative apoptosis controls.

Semiquantitative apoptosis determinations. For semiquantitative measurements of apoptosis, images generated with a charge-coupled device array camera (RT Color; Diagnostic Instruments, Inc., Sterling Heights, Mich.) were subjected to fluorescence brightness value determinations on a monochromatic scale in light of red, green, and blue values (ASCII numbers 0 to 255). Apoptosis was defined based on a red monochromatic scale in the range of 0 to 255. Blank values were subtracted from the averages of seven random values from different cells.

Human apoptosis gene microarrays. The Clontech Atlas cDNA Expression Human Apoptosis Array was used in this study. Total cellular RNA was extracted from third-passage human brain MVECs with an Ultraspec RNA isolation kit (Biotecx Laboratories, Inc., Houston, Tex.), according to the manufacturer's instructions. After the RNA extraction, cDNA probe mixtures were synthesized by reverse transcribing the respective RNA with the cDNA synthesis primer mix provided by the manufacturer and [α-^{32}P]dATP (Perkin-Elmer Sciences, Inc, Boston, Mass.). Each radioactively labeled probe mix was then hybridized to separate Atlas arrays overnight at 68°C. After high-stringency washes, as suggested by the manufacturer, the hybridization patterns were analyzed by autoradiography and quantified with a phosphorimager (Molecular Dynamics). The relative expression levels of a given cDNA from RNA obtained from MVECs treated with recombinant HIV-1 Nef protein or the HIV-1 vector expressing Nef were assessed by comparing the signal obtained with a probe of RNA from treated MVECs to that obtained with a probe of control RNA.

Protein assays and Western blot analyses. MVECs were analyzed for the activation of various caspases and poly(ADP-ribose) polymerase (PARP). Cells were either exposed to recombinant HIV-1 Nef protein or GST or infected with the HIV-1 vector expressing Nef or LacZ and incubated for 48 h at 37°C with 5% CO_2 in a humidified environment. Cells were then harvested and disrupted with mammalian cell lysis buffer (Pierce Biotechnologies, Rockford, Ill.). Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce Biotechnologies). Approximately 25 μg of each protein preparation was resolved on sodium dodecyl sulfate–12% polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences) by electroblotting. Membranes were washed in PBS containing 0.01% Tween 20 (Sigma-Aldrich, St. Louis, Mo.) and then blocked for nonspecific proteins with PBS-based blocking buffer (Pierce Biotechnologies). The membranes were probed with specific monoclonal antibodies against caspase-3 (Oncogene, San Diego, Calif.), caspase-9 (Cell Signaling Technologies, Beverly, Mass.), and PARP (BD PharMingen) as primary antibodies and horseradish peroxidase-labeled goat anti-rabbit or anti-mouse immunoglobulin G (heavy plus light chains) as secondary antibodies. All three antibodies recognized both the pro- and active forms of the respective proteins. The protein-antibody complexes were visualized by incubating the membranes with the Supersignal West Fermto Western blotting detection system (Pierce Biotechnologies) and subsequently exposing them to BioMax MS autoradiographic film (Kodak, Rochester, N.Y.).

RESULTS

HIV-1 Nef virions express Nef intracellularly in MVECs. Human brain MVECs transduced with recombinant HIV-1-based Nef virus and SNV-Nef virus (i.e., HIV-1- and SNV-based vectors expressing HIV-1 Nef as a transgene, respectively) were immunostained for the intracellular expression of Nef. The results are depicted in Fig. 2. It was observed that Nef, as a transgene, was efficiently integrated and expressed in the cells by both vectors, although the levels of transduction and expression may be modestly less with HIV-1 compared to SNV vectors.

HIV-1 Nef induces apoptosis in human brain MVECs. HIV-1 Nef has been reported to inhibit apoptosis processes by interacting with cellular kinases and thereby repressing proapoptotic signaling in HIV-1-infected cells (76). For example, it has been reported that Nef protects HIV-1-infected cells against CD95 and tumor necrosis factor alpha (TNF-α) receptor-mediated death signaling by inhibiting the apoptosis signal-regulating kinase-1 (34). Furthermore, Nef has also been reported to repress signaling by Bad, a proapoptotic member of the Bcl-2 protein family whose expression is induced by HIV-1 (105).

In this study, we investigated the effects of recombinant HIV-1 Nef protein and intracellular Nef delivered through lentiviral and oncoretroviral delivery systems into MVECs. It was observed that treatment of MVECs with extracellular Nef at low concentrations (i.e., 10 ng/ml) resulted in a moderate amount of programmed cell death; however, at higher concentrations (i.e., 50 and 100 ng/ml), there was substantial induction of apoptosis, as illustrated in Fig. 3. Similarly, TUNEL assays demonstrated that MVECs transduced with recombinant HIV-1-based Nef and SNV-Nef viral vectors exhibited dose-dependent programmed cell death, as depicted in Fig. 4 and 5, respectively.

To further confirm the proapoptotic potential of Nef, we infected MVECs with the HIV-1 strain ADA (CCR5-tropic) and the mutant with a deletion in Nef, ADAΔNef. The results of the TUNEL assays are depicted in Fig. 6 and 7. As illustrated (Fig. 6), HIV-1 ADA at 10 ng of p24 antigen equivalents per ml induced substantial levels of apoptosis in MVECs, whereas the quantity of apoptosis induced by its ADAΔNef, was quite low (Fig. 7). However, at higher levels of ADAΔNef (i.e., 100 ng of p24 antigen equivalents of virus per ml), there was a substantial increase in the number of apoptotic cells (Fig. 7). These results show that both endogenously expressed and extracellular HIV-1 Nef induced apoptosis in MVECs. The results also reveal that Nef may play an important but not sole role in the induction of programmed cell death by HIV-1 in MVECs.

Strict quantitation is difficult in these assay systems, but in evaluating multiple assays they can be reasonably described semiquantitatively; HIV-1-based vector Nef virus was the most potent in inducing apoptosis, followed by recombinant Nef protein and then SNV-Nef (Table 1). Similarly, HIV-1 ADA also induced substantial levels of apoptosis in MVECs. Thus, cell-free and intracellularly expressed HIV-1 proteins may disrupt the BBB via induction of programmed cell death.

Analyses of MVECs transduced with HIV-1 Nef by human apoptosis gene microarrays. Induction of apoptosis by HIV-1 Nef occurs through unknown mechanisms. Therefore, to elucidate the molecular mechanisms involved in Nef-induced apoptosis in MVECs, we used targeted gene microarrays to investigate the up-regulation of apoptosis-related genes in MVECs transduced with HIV-1-based vector Nef virus and baculovirally expressed recombinant HIV-1 Nef protein. The human apoptosis gene microarray generated with MVECs transduced with HIV-1-based Nef virus (i.e., a HIV-1-based vector expressing Nef as a transgene) revealed a substantial up-regulation of the mRNA levels of proapoptotic genes. The genes

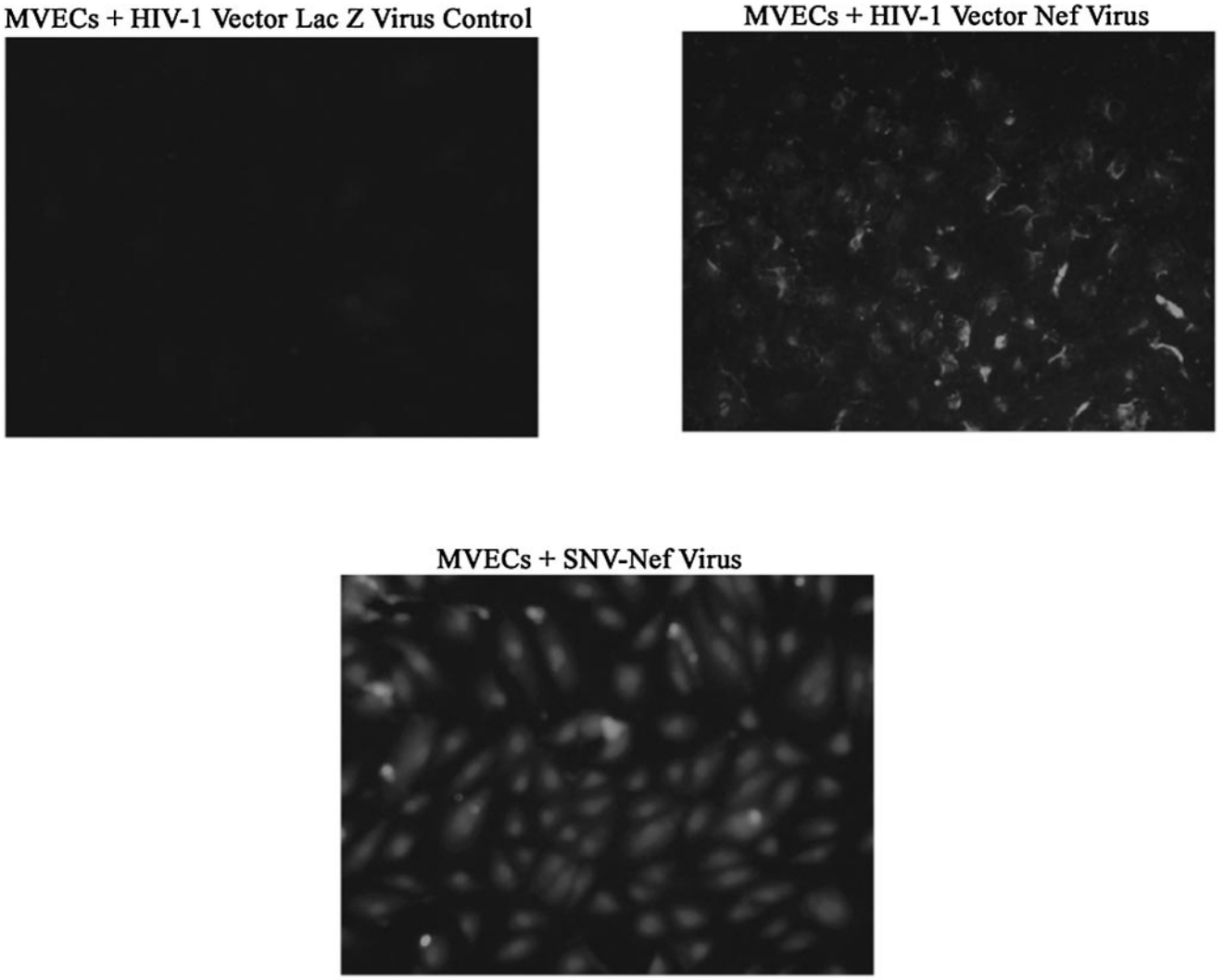

FIG. 2. MVECs transduced with HIV-1 Nef and SNV-Nef vector virions expressing Nef. Primary isolated human brain endothelial cells were transduced with either HIV-1 LacZ, HIV-1 Nef, or SNV-Nef vector virions and then cultured on two-well chamber slides for 48 h. The cells were fixed with 2% paraformaldehyde and incubated with primary antibodies against Nef, and later with Cy2-conjugated secondary antibodies, after permeabilization. These results are representative of those from experiments performed in triplicate and repeated at least twice.

up-regulated include those for caspase-6, -8, -9, and -10, as well as those for DAXX, TNF receptor 12 (TNFR 12), tumor proteins p53 and p73, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) 3, and MAPK 7, and other apoptosis-related genes, compared with the HIV-1 LacZ-treated control. Since these genes have been implicated in both the mitochondrial and Fas/FasL apoptotic pathways, increases in their mRNA expression levels suggest a possible involvement of the mitochondria in HIV-1 Nef-induced apoptosis in MVECs (Fig. 8 and Table 2). Since in the present study we observed up-regulation of mRNA expression levels of genes involved in both the Fas/FasL and the mitochondrial apoptotic pathways, it could be strongly suggested that HIV-1 Nef may utilize more than one pathway in inducing apoptosis in MVECs.

Nef induces PARP cleavage. Apoptosis or programmed cell death is triggered by a variety of stimuli, including cell surface receptors such as Fas, the mitochondrial response to stress, and cytotoxic T cells. It is considered to be one of the major mechanisms for the depletion of $CD4^+$ T lymphocytes during HIV-1 infection (77). There are two major pathways involved in apoptosis: the mitochondrial apoptotic pathway and the Fas/Fas ligand (also known as CD95 and APO-1) apoptotic pathway (4, 13, 20, 52, 92). Both pathways involve several representatives of the caspases, a class of cysteine proteases that are involved in apoptosis. The caspases convey the apoptotic signal in a proteolytic cascade, with caspases cleaving and activating other caspases that then degrade other cellular targets, leading to cell death (52, 92). The caspases at the upper end of the cascade include caspase-8 and caspase-9. Caspase-8 is the initial caspase involved in the response to receptors with a death domain such as Fas (52, 92). PARP is a 116-kDa chromatin-associated enzyme which is involved in DNA repair. PARP is recognized as a substrate for numerous caspases and as an important mediator of apoptosis. The degradation of PARP from the 116-kDa protein to approxi-

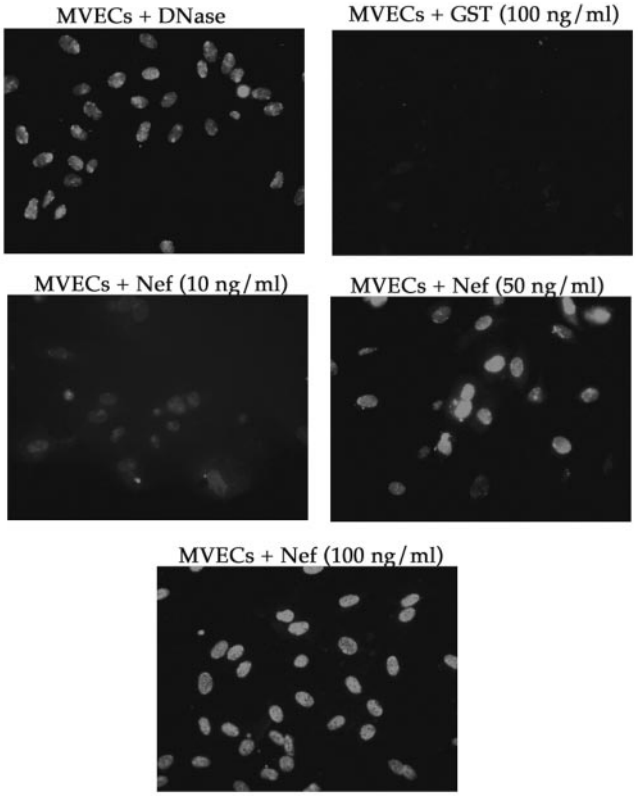

FIG. 3. Recombinant HIV-1 Nef protein-mediated apoptosis of human brain MVECs. Representative TUNEL staining of MVECs exposed to GST- and baculovirally expressed HIV-1 Nef protein is shown. MVECs were exposed to either GST protein or HIV-1 Nef protein expressed from Sf9 insect cells for 72 h. The concentrations of protein used were 10, 50, and 100 ng/ml. As a positive control, MVECs were exposed to DNase at a concentration of 10 μg/ml for 10 min at room temperature. TUNEL assays were performed with the in situ cell death detection kit, TMR Red (Roche Diagnostics). The cells were analyzed by fluorescence microscopy (Olympus System microscope, model BX60, with fluorescence attachment BX-FLA). These results are representative of those from experiments performed in triplicate and repeated at least twice.

mately 89- and 24-kDa cleavage products is one of the classical indicators of apoptosis (60).

To test whether the caspase mRNAs detected via the microarrays act at the translational level to induce apoptosis in MVECs, we analyzed apoptosis by using PARP cleavage via Western blot analyses. It was observed that MVECs transduced with the HIV-1 vector expressing Nef resulted in the cleavage of the PARP protein into the 116-kDa fragment and a smaller 85-kDa fragment, which were not detected in the LacZ virus-treated controls (Fig. 9A). Similar results were obtained when cells were exposed to extracellular soluble Nef protein (Fig. 9A). Thus, Nef-induced apoptosis correlated with the biochemical cleavage of PARP, a known substrate of caspase-3.

Nef induces caspase activation in MVECs. Since PARP is known to be cleaved by caspase-3 and -9 (52, 92) and the microarray data in this study showed a substantial up-regulation of the expression of caspase-9 (Table 2) and a modest increase in the level of caspase-3 (results not illustrated), we investigated whether these caspases undergo cleavage.

After either exposure extracellular soluble Nef or transduction with HIV-1 Nef-expressing virions, cells were analyzed for caspase activity. Western blot analyses utilizing specific antibodies revealed the expression of caspase-3, which was cleaved into 17- to 19-kDa fragments when the cells were either exposed to extracellular Nef or transduced with Nef-expressing HIV-1 vector particles (Fig. 9B). Similar results were obtained for caspase-9 (Fig. 9C), which was cleaved into 48- and 37-kDa cleavage products. Pretreatment of cells with specific caspase-3 and -9 inhibitors (Ac-DEVD-CHO and Z-LEHD-FMK, respectively) completely eliminated the cleavage of both caspases (Fig. 9B and C). Thus, the Western blot analyses corroborated the microarray data, and both studies showed the involvement of caspase-3 and -9 in HIV-1 Nef-induced apoptosis in human brain MVECs.

DISCUSSION

One of the hallmarks of HIV-1 infection of the CNS is the development of AIDS dementia complex, which is characterized by diffuse motor, sensory, and cognitive dysfunctions (6, 10). It has been shown that monocytes/macrophages are the CNS-based-cells that are most commonly and productively infected with HIV-1 (25, 33, 55a, 65, 68, 104). However, certain studies have demonstrated that low-level viral replication occurs in MVECs, astrocytes, and neurons (8, 11, 42, 69–71). HIV-1 infection also leads to a decline in the number of $CD4^+$ T lymphocytes, achieved either through direct cytopathic effects (103) or the activation of apoptotic cascades (110).

The molecular mechanisms involved in apoptosis of CNS-

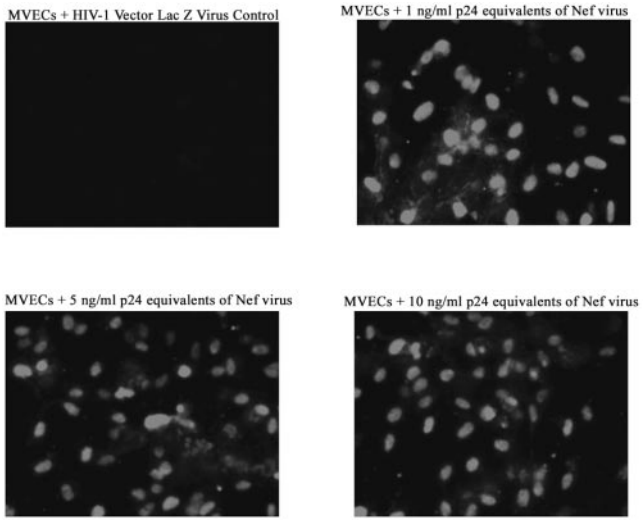

FIG. 4. Nef expressed from the retroviral vector pHR′CMVNef is sufficient to induce apoptosis in MVECs in a dose-dependent manner. Representative TUNEL staining of MVECs infected with HIV-1-based vectors expressing either Nef or LacZ for 72 h, after which cells were subjected to TUNEL staining, is shown. The amount of virus used varied from 1 to 10 ng of HIV-1 p24 antigen equivalents per ml. TUNEL assays were performed with the in situ cell death detection kit, TMR Red (Roche Diagnostics). The cells were analyzed by fluorescence microscopy (Olympus System microscope, model BX60, with fluorescence attachment BX-FLA). These results are representative of those from experiments performed in triplicate and repeated at least twice.

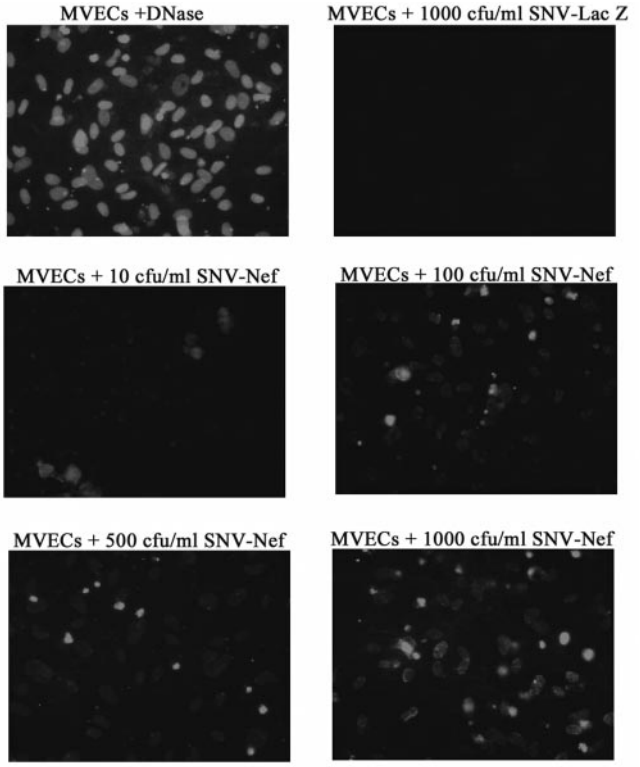

FIG. 5. HIV-1 Nef expressed from the spleen necrosis viral vector, SNV-Nef, induces apoptosis in MVECs in a dose-dependent manner. Representative TUNEL staining of SNV-LacZ and SNV-Nef$^+$ vector virion-treated MVECs, infected for 72 h, is shown. The quantities of virions used to infect cells were 10, 100, 500, and 1,000 CFU/ml. As a positive control, MVECs were exposed to DNase at a concentration of 10 μg/ml for 10 min at room temperature. TUNEL assays were performed with the in situ cell death detection kit, TMR Red (Roche Diagnostics). The cells were analyzed by fluorescence microscopy (Olympus System microscope, model BX60, with fluorescence attachment BX-FLA). These results are representative of those from experiments performed in triplicate and repeated at least twice.

based cells are not completely understood. During HIV-1 infection, cells can release specific viral products (82, 107, 113), which may induce apoptosis in bystander cells. In CNS-based cells, it has been shown that HIV-1 infection can lead to the production and release of proinflammatory cytokines (9, 26, 83, 114), which are neurotoxic. It is believed that induction of apoptosis in the CNS occurs through the direct cytopathic effects of viral proteins and/or indirectly via the release of soluble cellular factors (35). It has been shown in primary brain cultures that HIV-1 infection induces apoptosis of neurons and astrocytes at 1 to 2 weeks after peak virus production (91). Also, the fact that in the brain tissues of HIV-1-infected individuals most apoptotic cells are not localized in close proximity to HIV-1 infected cells (91) suggests that soluble cellular factors and/or free viral proteins may induce apoptosis in those cells.

Several HIV-1 proteins, such as Tat, gp120, Vpr, Vpu, and intact viral particles (55, 80, 81, 109), have been shown to induce apoptosis in both infected and uninfected cells. The role of Nef in inducing apoptosis is controversial for certain cell types (2, 34, 57, 75, 85). For example, Nef has been reported to favor cell survival through the down-modulation of HLA-I molecules that allow CTLs to detect viral peptides, but at the same time preserving HLA-I molecules that inhibits cell killing by natural killer (NK) cells. Furthermore, Nef has also been reported to protect infected cells by repressing death signaling from "within" by the proapoptotic Bcl-2 family of proteins (34, 105) and by up-regulating FasL expression in infected cells, which induces apoptosis in attacking CTLs. Nevertheless, it has been recently reported that Nef induces apoptosis in CD4$^+$ T cells (50). We also reported recently that Nef in combination with ethanol induced apoptosis in MVECs (1). In the present study, we wished to assess the ability of Nef to induce apoptosis in CNS-based cells in detail, by studying molecular mechanisms involved in Nef-induced apoptosis in CNS-based cells.

We investigated the potency of Nef in inducing apoptosis in the context of the virus by using both lentiviral and oncoretroviral delivery systems, where Nef was expressed from the transfer vector, packaged in the virion vectors, and delivered

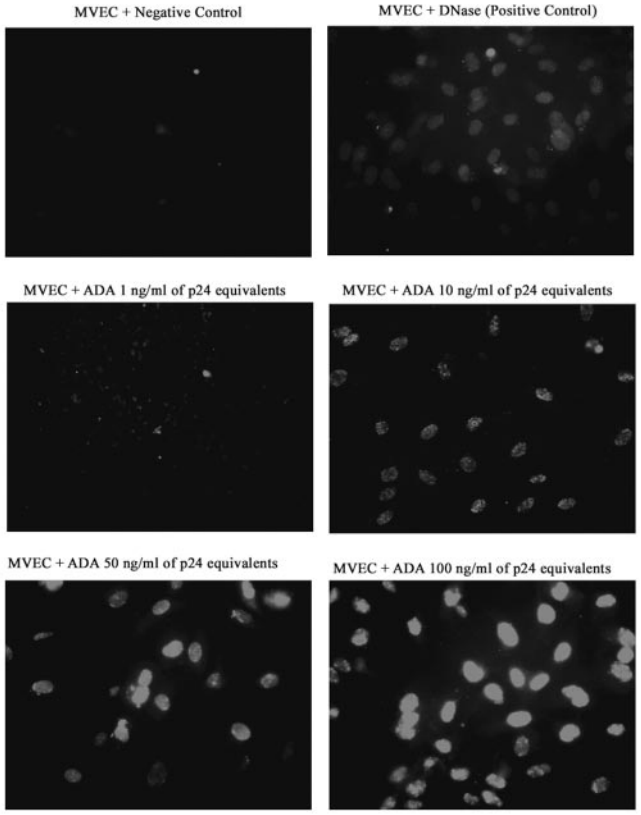

FIG. 6. HIV-1 R5 strain ADA induces apoptosis in MVECs in a dose-dependent manner. Representative TUNEL staining of HIV-1 ADA-treated MVECs is shown. Cells were infected with HIV-1 ADA for 72 h, after which cells were subjected to TUNEL staining. The amount of virus used ranged from 1 to 100 ng of HIV-1 p24 antigen equivalents per ml. As a positive control, MVECs were exposed to DNase at a concentration of 10 μg/ml for 10 min at room temperature. TUNEL assays were performed with the in situ cell death detection kit, TMR Red (Roche Diagnostics). The cells were analyzed by fluorescence microscopy (Olympus System microscope, model BX60, with fluorescence attachment BX-FLA). These results are representative of those from experiments performed in triplicate and repeated at least twice.

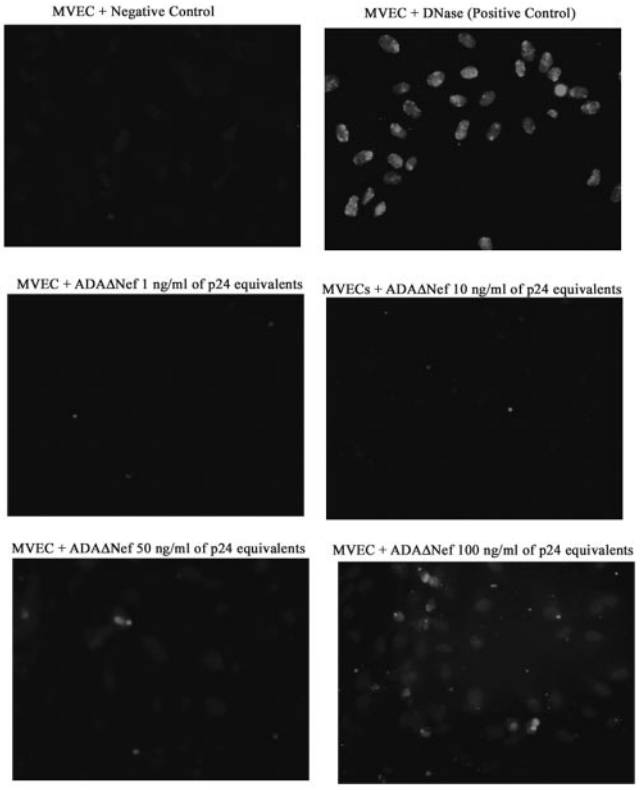

FIG. 7. An HIV-1 R5 strain with a deletion in the Nef gene (ADAΔNef) induces apoptosis in MVECs at lower levels than wild-type HIV-1 ADA. Representative TUNEL staining of HIV-1 ADAΔNef-treated MVECs is shown. Cells were infected with HIV-1 ADAΔNef for 72 h, after which they were subjected to TUNEL staining. The amount of virus used ranged from 1 to 100 ng of HIV-1 Gag p24 equivalents per ml. As a positive control, MVECs were exposed to DNase at a concentration of 10 μg/ml for 10 min at room temperature. TUNEL assays were performed with the in situ cell death detection kit, TMR Red (Roche Diagnostics). The cells were analyzed with fluorescence microscopy (Olympus System microscope, model BX60, with fluorescence attachment BX-FLA). These results are representative of those from experiments performed in triplicate and repeated at least twice.

into the cellular genome for integration. The results indicated the efficient expression of Nef protein within MVECs. We also investigated the ability of the extracellular recombinant Nef protein expressed in and purified from Sf9 insect cells to induce apoptosis in MVECs.

Apoptosis assays utilizing TUNEL staining indicated that intracellularly expressed Nef induced significant levels of apoptosis in a dose-dependent manner. Similarly, dose-response experiments with baculovirally expressed extracellular Nef showed that this cell-free lentiviral protein could induce considerable apoptosis in MVECs. Cells exposed to the average level of soluble Nef protein (10 ng/ml) normally detected in the sera of HIV-1-infected patients (31) showed substantial levels of apoptosis. Furthermore, the TUNEL staining clearly demonstrated that MVECs exposed to Nef protein and those transduced with HIV-1-based vector Nef virus revealed cytoplasmic shrinkage and nuclear fragmentation (results not illustrated), with higher doses of Nef producing elevated levels of nuclear fragmentation and apoptotic bodies. It is not clear precisely how extracellular Nef induces programmed cell death in brain MVECs, whether by entering cells or at the membrane level. In addition, it was observed that there was an increase in trypan blue dye uptake by the treated cells, confirming the increasing cell death observed in primary isolated MVECs exposed or transduced with HIV-1 Nef or SNV-Nef viral vectors (not illustrated). As such, Nef induces apoptosis in primary human brain MVECs in a dose-dependent manner. We also observed that infection of cells with the HIV-1 strain ADA resulted in substantial induction of apoptosis. In contrast, exposure of cells to the mutant strain with a deletion in Nef, ADAΔNef, resulted in markedly reduced apoptosis. These results clearly demonstrate that both extracellular and intracellularly expressed Nef are capable of killing MVECs through apoptosis. This is consistent with other reports which suggest that HIV-1 proteins, such as Nef, gp120, Tat, and Vpr, can initiate apoptotic cascades in CNS-based cells (1, 80, 100, 109).

Nef inhibits (i.e., protects infected cells) and induces apoptosis in bystander cells (34, 105). The N terminus of the Nef protein has been implicated in this bystander cell death (48). Studies by Okada et al. (75) have shown that soluble Nef protein, in conjunction with Nef antibodies, causes the death of a wide range of uninfected lymphoid tissues (74, 75). Similarly, transgenic animal models developed to study the in vivo expression of HIV-1 proteins in the host systems (21–23, 37, 43, 51, 99) were found to undergo lymphocyte depletion, as well as most of the organ dysfunction commonly found in AIDS patients. It is important to note that these animals required only expressed Nef in the target cells in order to manifest these

TABLE 1. Semiquantitative determination of HIV-1 Nef-induced apoptosis in primary human brain MVECs

Treatment	Red-green-blue values
Nef protein (ng/ml)	
10	60.25
50	142.0
100	207.0
HIV-1-based vector Nef virus (ng of p24 antigen equivalents per ml)	
1	134.5
5	185.3
10	233.5
SNV vector-based Nef virus (CFU/ml)	
10	22.8
100	44.0
500	49.0
1,000	97.0
HIV-1 strain ADA (ng of p24 antigen equivalents per ml)	
1	8.0
10	66.8
50	151.8
100	189.5
HIV-1 strain ADAΔNef (ng of p24 antigen equivalents per ml)	
1	3.0
10	3.5
50	18.0
100	86.50

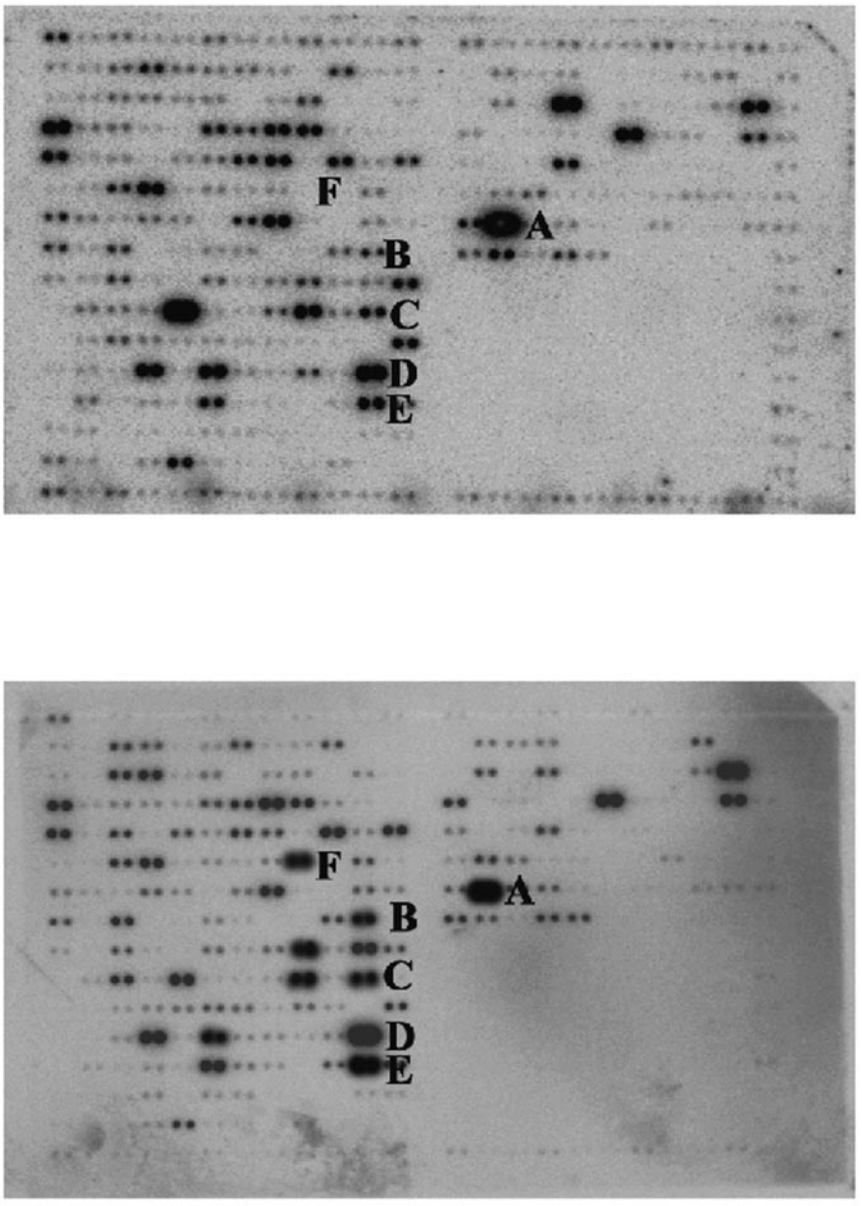

FIG. 8. Human apoptosis gene microarrays of MVECs transduced with HIV-1 LacZ virus (top panel) and HIV-1-based vector Nef^+ virus (bottom panel). These results are representative of those from two independent experiments.

changes. Therefore, the observation in the present study that extracellular soluble Nef induced apoptosis in MVECs is consistent with the bystander effect hypothesis (27), which implicates a viral protein or virally stimulated cellular factors released into the medium as mediators of apoptosis (24, 72). Since Nef is synthesized early during initial viral infection and it has been shown that Nef produced by recombinant plasmids is secreted into culture medium (31, 50, 61), it is possible that intracellular expression of Nef in this study resulted in the release of the protein into the culture, which in turn may have caused the bystander effects. Hence, it could also be said that the induction of apoptosis in MVECs by intracellular Nef is also consistent with the bystander effect hypothesis (27).

Although the TUNEL assays in the present study demonstrated clearly that Nef induces apoptosis in MVECs, the exact mechanisms were not known. Therefore, to begin to elucidate the molecular mechanisms involved, targeted gene microarrays and Western blotting were employed to analyze the regulation of apoptotic genes in MVECs exposed to soluble Nef protein and also those transduced with an HIV-1 viral vector containing the Nef transgene alone. These were compared with MVECs exposed to GST and LacZ virus (i.e., a HIV-1 vector expressing LacZ), respectively. We observed that Nef induced apoptosis in MVECs by up-regulating the expression of the components of both the Fas/Fas ligand (also known as CD95 and APO-1), and the TNF/TNFR 1 signaling pathways, plus the mitochondrial apoptotic pathways.

The components of the Fas/Fas ligand that were found up-regulated included DAXX, TNFR-12 (also known as TRAMP, LARD, APO3, DR3, and TNFR 25), and caspase-8 and -10.

TABLE 2. cDNA microarray analyses of human brain MVECs transduced with HIV-1 vector expressing Nef

Up-regulated gene product	Fold increase
CDC-like kinase (CLK 3)	2.0
PICTAIRE protein kinase 2 (apoptosis related)	1.5
Mitogen-activated protein kinase 7	1.7
MAPK/ERK 3	2.0
Tumor protein p73	2.5
Tumor protein p53	1.5
Glutathione *S*-transferase-like protein	1.5
Caspase-6	2.5
Caspase-8	1.5
Caspase-9	3.0
Caspase-10	2.0
Death-associated protein 6 (DAXX)	2.5
Secreted apoptosis-related protein 2	2.5
Death-associated protein kinase 1	1.8
Tumor necrosis factor receptor superfamily, member 12	2.5
Insulin-like growth factor 1	1.7
Insulin-like growth factor binding protein 6	1.6

Receptors in the TNF receptor family are associated with the induction of apoptosis, as well as inflammatory signaling. Binding of FAS to oligomerized FasL on another cell leads to the formation of a death-induced signal complex (DISC) composed of Fas, Fas-associated death domain protein, and caspase-8 or -10 (12). Recruitment of procaspase-8 or -10 to DISC results in its activation, and it then cleaves and activates downstream caspases and a variety of cellular substrates, including PARP, leading to cell death (49, 52, 92). TNFR 12 or DR3 shows close sequence similarity to TNFR 1 (15). Also, the responses that it triggers are similar to those of TNFR 1, namely, nuclear factor κB (NF-κB) activation and apoptosis. TNFR 12 triggers apoptosis through TNFR1-associated death domain (TRADD), Fas-associated death domain protein, and caspase 8 and activates NF-κB through TRADD, TNF receptor-associated factor 2, and receptor interacting protein (15, 46).

In addition to the caspase activation cascade, induction of the Fas/FasL pathway may also involve the activation of c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) (112). After Fas/FasL ligation, an adaptor protein called DAXX is recruited by Fas and then interacts with apoptosis signal-regulating kinase 1, activating JNK/SAPK and p38 MAPK by phosphorylation (14, 47). This eventually results in the activation of caspases and subsequent apoptosis.

Our results demonstrate up-regulation in the expression of DAXX, TNFR 12 (DR3), and caspase-8 and -10. Since these genes have been implicated in the Fas/FasL and the TNF/TNFR apoptotic pathways (12, 15, 46), the results suggest that both extracellular and intracellularly expressed HIV-1 Nef induces apoptosis in human brain MVECs, most likely through components of the Fas/FasL and the TNF/TNFR apoptotic pathways. These results are fully consistent with the results of other studies which have shown that Nef increases the expression of Fas ligand, resulting in Fas signaling and apoptosis in bystander cells (28–31, 85, 108). However, some studies have demonstrated that extracellular Nef protein induces apoptotic cytolysis in both lymphoid and myeloid cells of murine and human origins independently of the Fas/FasL apoptotic pathway (74–76). The apparent discrepancy between our study and those studies may be explained by the fact that different cell

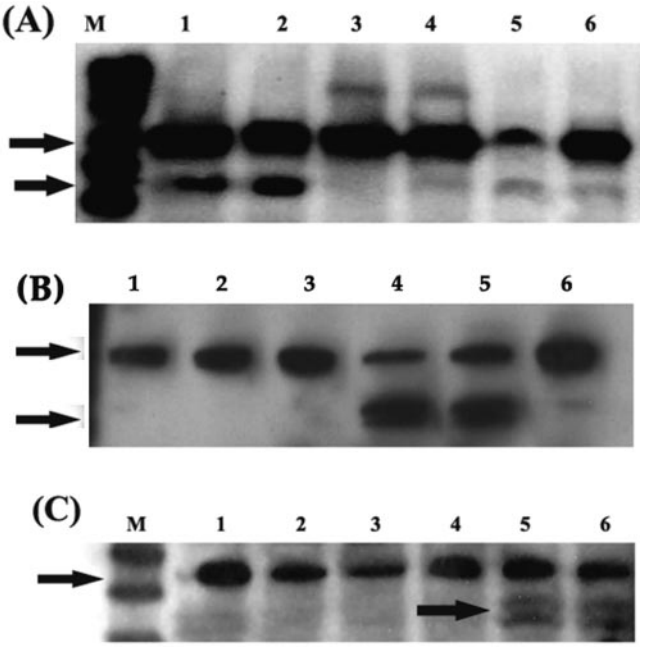

FIG. 9. Western blotting analyses of PARP (A), caspase-3 (B), and caspase-9 (C) during HIV-1 Nef-induced apoptosis in MVECs. Cells were either left untreated, exposed to 100 ng of GST protein per ml, transduced with 10 ng of p24 antigen equivalents of HIV-1-based vector LacZ virus per ml, treated with 100 ng of recombinant Nef protein per ml, transduced with 10 ng of p24 antigen equivalents of HIV-1-based vector Nef virus per ml, or pretreated with 100 nM specific caspase inhibitor and then transduced with HIV-1-based vector Nef virus. Twenty-five micrograms of total protein extract was loaded in each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Caspase-3 and -9 and PARP were analyzed by Western blotting with specific caspase-3, caspase-9 and PARP monoclonal antibodies. These results are representative of those from experiments repeated at least twice. (A) PARP. Lanes: M, protein marker; 1, MVECs treated with 100 ng of recombinant Nef protein per ml; 2, MVECs transduced with 10 ng of p24 antigen equivalents of HIV-1-based vector Nef virus per ml; 3, untreated MVECs; 4, MVECs exposed to 100 ng of GST protein per ml; 5, MVECs pretreated with 100 nM broad-spectrum caspase inhibitor Z-VAD-FMK (BD Phar Mingen) and then transduced with HIV-1-based vector Nef virus; 6, MVECs transduced with 10 ng of p24 antigen equivalents of HIV-1-based vector LacZ virus per ml. The upper arrow depicts 116-kDa PARP, and the lower arrow depicts approximately the 85-kDa cleaved PARP. (B) Caspase-3. Lanes: 1, untreated MVECs; 2, MVECs exposed to 100 ng of GST protein per ml; 3, MVECs transduced with 10 ng of p24 antigen equivalents of HIV-1-based vector LacZ virus per ml; 4, MVECs treated with 100 ng of recombinant Nef protein per ml; 5, MVECs transduced with 10 ng of p24 antigen equivalents of HIV-1-based vector Nef virus per ml; 6, MVECs pretreated with 100 nM of Ac-DEVD-CHO (BD PharMingen), a specific caspase 3 inhibitor, and then transduced with HIV-1-based Nef virus. The top arrow depicts 35-kDa intact caspase-3, and the bottom arrow depicts the 17- to 19-kDa cleaved caspase-3 fragment. (C) Caspase-9. Lanes: M, protein marker; 1, untreated MVECs; 2, MVECs exposed to 100 ng of GST protein per ml; 3, MVECs transduced with 10 ng of p24 antigen equivalents of HIV-1-based vector LacZ virus per ml; 4, MVECs pretreated with 100 nM Z-LEHD-FMK, a specific caspase 9 inhibitor (BD PharMingen), and then transduced with HIV-1-based Nef virus; 5, MVECs treated with 100 ng of recombinant Nef protein per ml; 6, MVECs transduced with 10 ng of p24 antigen equivalents of HIV-1-based vector Nef virus per ml. The top arrow depicts 45-kDa pro-caspase-9, and the bottom arrow depicts 35- and 37-kDa cleaved caspase-9 fragments.

types were studied. In addition, whereas our study employed endogenously expressed Nef and N-terminal myristoylated extracellular Nef, Okada et al. (74–76) utilized nonmyristoylated Nef. Nonetheless, our results indicate that apoptosis via triggering of the Fas/FasL pathway may not be common to all cell types that are made to undergo Nef expression and could very well be dependent on the cell type, culture conditions, and nature of the stimulus.

Other apoptosis-related genes found to be up-regulated upon exposure of MVECs to HIV-1 Nef were those for caspase-3, -6, and -9. Western blot analyses revealed the cleavage products of caspase-3 and -9 and PARP, which are detected only in cells undergoing apoptosis. Caspase-9 (also known as ICE-LAP6, Mch-6, or Apaf-3) is a 45-kDa protein that is involved in the mitochondrial apoptotic pathway (52, 92). Upon apoptotic stimulation, cytochrome *c* released from mitochondria associates with procaspase-9 and Apaf-1 in the presence of dATP to form a proapoptotic complex. This complex promotes the self-cleavage of procaspase-9 into a large active subunit (35 or 17 kDa) and a small 10-kDa subunit (56, 58). Once activated, caspase-9 initiates a caspase cascade involving caspase-3, -6, and -7, which act by themselves to cleave cellular targets such as PARP, leading to cell death (16, 63, 67, 86). As well, caspase-8 activated via the Fas/FasL and the TNF/TNFR apoptotic pathways can stimulate proteolytic cleavage of Bcl-2-interacting protein, which initiates cytochrome *c* release in the mitochondria (38), leading to the activation of caspase-9 and -3.

In addition to the above-described proteins, our studies also revealed prominent up-regulation of the mRNA expression of MAPK 7, ERK, MAPK, and the tumor suppressor proteins p53 and p73. The tumor suppressor protein p53 is involved in the mitochondrial apoptotic pathway (88) and plays a role in apoptosis by down-modulating the mitochondrial membrane potential and promoting the release of cytochrome *c* (90). p53 also leads to cell cycle arrest and induces apoptosis by regulating at the transcriptional level several genes, including proapoptotic *Bax* of the Bcl-2 family (59, 66, 102), the gene for the cell cycle inhibitor p21, and the DNA repair gene GADD45 (53). The transcriptional regulation by p53 of *Bax* and the other proapoptotic genes, according to Soengas et al., is dependent on Apaf-1/caspase-9 activation involved in the mitochondrial pathway (96). p53 can also induce apoptosis by means of a direct signaling pathway involving the expression of p53AIP1, which has been reported to regulate the mitochondrial apoptotic pathway (64). Thus, the up-regulation of p53 mRNA levels in this study was important in that it showed that Nef-induced apoptosis in MVECs most likely occurred via specific mitochondrial elements associated with cell death.

Unlike p53, MAPK and MAPK/ERK have not been directly linked to the mitochondrial apoptotic pathway. Also, the relationship between the activation of MAPK cascades and induction of apoptosis is still not clear and may vary among different cell types (58, 101, 106). However, it has been reported that overexpression of MAPK results in increased expression and activity of p53, causing a permanent growth arrest and apoptosis (32).

Nef possesses the capacity to regulate numerous transcriptional activities by targeting and controlling various *src* family kinase, MAPK, and p53 activities. However, most reports regarding Nef-mediated transcriptional activation of p53 and MAPK pathways have been with T cells, and these analyses have been less than conclusive. Most of those studies suggest that Nef has the capacities to both inhibit and enhance T-cell activity (62, 95). From work with MOLT-4 cells, Greenway et al. (41) reported that Nef binds to p53 to protect the cells against p53-mediated apoptosis. Similarly, other studies (39, 44) have suggested that Nef binds to p53, Raf-1, and MAPK to destabilize these proteins and inhibit their transcriptional activation. Nef has been shown to bind and inhibit Lck and MAPK activity (39). In contrast, Schrager et al. (89) demonstrated in primary $CD4^+$ T cells that the expression of Nef specifically increases activity of the ERK/MAP kinase cascade. Similarly, Robichaud and Poulin (87), using the U251MG human astrocytoma cell line, reported that Nef preferentially enhanced MAPK and JNK activation. Furthermore, it has been demonstrated that HIV-1 Nef, Rev, and Tat could be potential substrates that may activate MAPK cascades (111). Thus, it appears that the regulation of the transcriptional activities of various kinases and p53 by Nef is quite complex and that whether or not Nef inhibits or enhances these activities may depend on the cell type and possibly on nature of the Nef protein involved. Nonetheless, there is evolving support for Nef as a positive regulator for p53 and MAPK.

Based on the evidence presented regarding the involvement of caspase-3 and -9, p53, p73, and MAPK cascades, it is clear that Nef-induced apoptosis in human brain MVECs involves certain components of the mitochondrial apoptotic pathways. In summary, the present studies strongly suggest that the HIV-1 protein Nef can potently induce apoptosis in human brain MVECs. As well, these data demonstrate, for the first time, that Nef-induced apoptosis in MVECs may result from a combination of several intracellular signaling pathways.

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