HIV-1 Vpr Induces Apoptosis through Caspase 9 in T Cells and Peripheral Blood Mononuclear Cells*

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Karuppiha Muthumani, Daniel S. Hwang, Brijal M. Desai§, Donghui Zhang, Nathanael Dayes, Douglas R. Greene¶, and David B. Weiner§

From the Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104; the §La Jolla Institute for Allergy and Immunology, San Diego, California 92121; and ¶Viral Genomics, Philadelphia, Pennsylvania 19104

Human immunodeficiency virus, type 1 (HIV-1), vpr gene encodes a 14-kDa virion-associated protein, which exhibits significant effects on human cells. One important property of Vpr is its ability to induce apoptosis during infection. Apoptotic induction is likely to play a role in the pathogenesis of AIDS. However, the pathway of apoptosis is not clearly defined. In this report we investigate the mechanism of apoptosis induced by HIV-1 Vpr using a Vpr pseudotype viral infection system or adeno delivery of Vpr in primary human lymphoid cells and T-cells. With either vector, HIV-1 Vpr induced cell cycle arrest at the G2/M phase and apoptosis in lymphoid target cells. Furthermore, we observed that with both vectors, caspase 9, but not caspase 8, was activated following infection of human peripheral blood mononuclear cell with either Vpr-positive HIV virions or adeno-delivered Vpr. Activation of the caspase 9 pathway resulted in caspase 3 activation and apoptosis in human primary cells. These effects were coincident with the disruption of the mitochondrial transmembrane potential and induction of cytochrome c release by Vpr. The Vpr-induced signaling pathway did not induce CD95 or CD95L expression. Bcl-2 overexpressing cells succumb to Vpr-induced apoptosis. These studies illustrate that Vpr induces a mitochondria-dependent apoptotic pathway that is distinct from apoptosis driven by the Fas-FasL pathway.

The human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus subfamily of retroviruses. The HIV-1 genome consists of the gag, pol, and env genes, which are common to all members of the retrovirus family. In addition, HIV-1 also encodes the following six accessory genes: tat, vpr, vpu, nef, rev, and vif. Among these, vpr is one of the few accessory genes that is packaged in the viral particle in large amounts (1–4).

HIV-1 Vpr is a 96-amino acid 14-kDa protein that has been demonstrated to be a weak transactivator of the HIV long terminal repeat (2, 5). It has also been demonstrated that Vpr expression in primary CD4+ lymphocytes, T-cell lines, and other human cells inhibits their proliferation by inducing G2 cell cycle arrest (5–11). Vpr also induces apoptosis of monocytes (12) and neuronal cell lines (13, 14). Induction of apoptosis has been linked to regulation of nuclear factor-kB (15–17). Goh et al. (18) reported that Vpr function in cell cycle arrest is conserved among all primate lentiviruses, and the expression of HIV-1 is increased during the G2 phase of the cell cycle. Vpr may also indirectly enhance virus replication through effects on the host immune system (19). These studies illustrate the importance of Vpr in the virus life cycle.

Many investigators (15, 20–23) have proposed that apoptosis is a major pathogenic mechanism of the immune dysfunction caused by HIV-1 infection. Apoptotic cell death is a process characterized by cell shrinkage, loss of membrane integrity, DNA fragmentation, and formation of apoptotic bodies (24). The key apoptotic effectors in mammals are a family of cysteine-containing, aspartate-specific proteases called caspases (25). One important issue to be elucidated concerns the mechanism of activation of the caspase cascades. Yang et al. (26) reported that activating the signaling complex at the CD-95 (Fas/Apo-1) receptor leads to the autoproteolytic activating of caspase 8. Others (27) have identified events in apoptosis involving the release of cytochrome c from the mitochondria. Recently, Stewart et al. (28) reported that in vitro HIV-1 Vpr induced apoptosis and activation of caspase 3 suggesting that HIV utilized one of the major pathways of apoptosis. Recently, Jocotot et al. (17) reported that isolated HIV-1 Vpr protein when added to isolated intact mitochondria dissipate/neutraizes their membrane potential. However, it remains to be clarified through what pathway(s) HIV-1 Vpr as part of infectious virions induces apoptosis in primary cells.

The aims of the present study were, therefore, first to investigate the mechanism of apoptosis induced by HIV-1 Vpr in primary PBMCs and T-cells. To achieve this goal we used a HIV-1 pseudotype system and a novel adenoviral system that we developed to express HIV-1 Vpr or recombinant Vpr. We found that viral borne HIV-1 Vpr induces apoptosis in PBMCs as well as T-cells. Apoptosis was coincident with mitochondrial depolarization and activation of the caspase 9 pathway resulting in caspase 3 activation and the downstream effects of apoptosis. The caspase 8 pathway was not involved, demonstrating a lack of involvement of the Fas/FADD pathway. These find-
Viral titers were normalized for virus content by p24 ELISA. The viral 293 cells with agar overlay. The adenovirus vector concentration was gradient method. Titers of the stocks were tested by plaque formation in AB). PBMCs were incubated for 2 days with 5 H9262 (human colon carcinoma) cell lines were maintained in RPMI1640 medium supplemented with 10% FBS in a humidified atmosphere of 5% CO2, SW480 (human colon carcinoma) cell lines were maintained in RPMI1640 medium supplemented with 10% FBS in a humidified atmosphere of 5% CO2 and 95% air. Human PBMCs were isolated from healthy HIV- seronegative donors by Ficoll-Paque separation (Amersham Biosciences AB). PBMCs were incubated for 2 days with 5 µg/ml phytohemagglutinin (Sigma) before the addition of 5 units/ml human recombinant interleukin-2 (R & D Systems).

Construction and Generation of HIV-1 Virions Packaged with Vpr— Constructs containing Vpr were generated using overlap extension PCR at the indicated codons as described (30) and were cloned in pCDNA3.1 vector to make the replication-incompetent HIV-1 envelope infectious vector pNL4–3–HSA.R’ and pNL4–3.HAS.R’ was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program (31). This vector has been rendered vpr-deficient by introduction of frameshift mutations at amino acid 26 thus generating replication-incompetent virions capable of single round infections only by complementation. This system is well established for studying the biology of HIV infection (19, 23, 28, 31).

Virus Production and Infection—To prepare infectious viral stocks, HIV-1 pseudotyped particles (pNL4–3–HSA.R’/pNL4–3–HAS.R’) were generated by co-transfection with pVpr or without pVpr by the DOTAP Liposomal transfection method (BM GmbH, Germany) in 293 T-cells. Six hours post-transfection the medium was replaced with fresh Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. The supernatants were collected and ultracentrifuged at 48 h post-transfection, and the virus containing supernatants was centrifuged for 10 min at 12000 rpm to remove cells. The supernatants were passed through 0.4 µm pore size filters and ultracentrifuged at 50,000 rpm for 90 min. Supernatants were collected and resuspended in 0.1× Hank’s balanced salt solution, and viral titers were normalized for virus content by p24 ELISA. The viral titers were determined by infection of the human T-cell line Jurkat using the stock of the pseudotyped virus supernatant, virus titers were determined in range of 5–10 × 105 infectious units/ml. p24Gag antigen was measured by capture ELISA (Coulter, FL). Virus stocks were normalized for virus content by infection, and stocks were stored in the presence of 10% FBS in aliquots at –80°C until use.

For infection studies human PBMCs were isolated from normal, seronegative donors and infected by incubating with target cells with HIV-1 virus carrying Vprwt or Vpr at a concentration of 100 TCID50/106 cells/ml. Following infection the cells were washed three times with PBS and resuspended in growth medium. Mock infection was performed under the same conditions, except that the supernatants were generated from control/vector-transfected cells.

Construction and Generation of adCMV-vpr—The proviral construct Construction and Generation of adCMV-vpr—The proviral construct of HIV-1 strain 89.6 was used as a template for PCR amplification of Vpr. The Vpr open reading frame was cloned into the pAdCMV vector. Positive clones were identified and confirmed by DNA sequencing (32). Furthermore, the pAdCMV-vpr plasmid and adenoviral construct sub360 were linearized and were co-transfected into 293 cells using the DOTAP transfection agent (Roche Molecular Biochemicals). Plaques were propagated in 293 cells and tested for Vpr insertion into the viral genome by PCR and subsequent Southern blot analysis. Large quantities of Vpr adenovirus were purified by centrifugation in CsCl density gradient method. Titers of the stocks were tested by plaque formation in 293 cells with agar overlay. The adenovirus vector concentration was determined by measuring A260 and was expressed in optical particle units as described by Mittereder et al. (33).

Reovirus and Papillomavirus and Treatment—HIV-1 recombinant proteins were purified as described (19). The purity and specificity were determined by using SDS-PAGE which is 99% and further confirmed by Western blot analysis using anti-Vpr antibody. Highly purified (>95%) recombinant Vpr protein was prepared to study the effect of Vpr protein.

Flow Cytometry Analysis of Apoptosis and Cell Cycle Analysis—FACS analysis was performed to identify cells undergoing apoptosis as well as to determine the stage of cell cycle analysis. Human PBMCs (5 × 106) were infected with equal amounts of virions (Vprwt or Vpr–). These retroviruses encode as a specific cell-surface marker, the murine CD24 antigen, which allows for identification of infected target cells by FACS analysis. FACS was performed 48 h post-infection, gating on the CD24-positive cells. Titers of the stocks were tested by plaque formation in 293 cells. Equal numbers of cells from each group were collected for analysis. For cell cycle analysis, cells were harvested 48 h post-infection, washed with PBS three times, and stained with murine CD24 and propidium iodide (PI; 125 µg/ml) to analyze their DNA content using Cycle Test kit (BD Pharmingen). CD24-positive cells were gated, and the cellular DNA content was calculated. Fixed cells were then assessed with a FACSscan flow cytometer and analyzed with the ModFit LT program (BD Pharmingen). The percentage of cells in G0/M was assessed and compared with that of mock-infected or Vpr–infected cells.

For apoptosis quantification, the infected cells (1 × 106) were harvested 24 h post-infection and washed three times with PBS (pH 7.2). Apoptosis was analyzed using an annexin-V assay kit from BD Pharmingen. Data were analyzed by the CELL Quest program (BD Pharmingen).

Caspase Activation—Caspase 3 (CPP32, Yama, apopain), caspase 8 (FLICE, MACH, Mch-5), and caspase 9 (Mch6, ICE-LAP6) activity was determined as per the manufacturer’s instructions (MBL, Nagoya, Japan). Following addition of virus, CD24-positive (infected cells) were sorted by flow cytometry and the cells were lysed in cell lysis buffer (10 mM Tris-Cl, 10 mM NaHPO4, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate). The cell lysate (100 µg/100 µl protein) was incubated with the substrates DEVD-pNA (caspase 3), IETD-pNA (caspase 8), or LEHD-pNA (caspase 9) for 1–2 h at 37°C. The release of pNA activity was determined using a microplate reader reading at 405 nm (MBL, Nagoya, Japan).

The positive controls for this assay were 5 units of the recombinant human caspase 3, 8, or 9 depending on the assay. One unit of the recombinant caspase 3, 8, or 9 contains enzymatic activity that cleaves 1 nmol of caspase substrate DEVD-pNA, IETD-pNA, or LEHD-pNA per h at 37°C at the saturated substrate concentration. Negative controls included reactions performed in the presence of competitive inhibitors (DEVD-FMK, IETD-FMK, or LEAD-FMK for caspase 3, 8, or 9, respectively). These inhibitors carry the cleavage sites of the respective caspases (MBL, Nagoya, Japan).

Mitochondria Transmembrane Potential (ΔΨ)—Effect of Vpr on the mitochondria transmembrane potential (ΔΨ) was determined using the DePeisher (R & D Systems) assay kit, and staining was determined by both microscopic observation and flow cytometry. In brief, HeLa/CD4 cells were maintained in RPMI1640 medium supplemented with 10% FBS and seeded onto 4-cm2 Falcon and Biocoat culture slides (BD Pharmingen). Cells were infected with Vprwt or Vpr– virions, and 12 h post-infection the cells were washed with PBS two times and incubated with 1× reaction buffer containing DePeisher for 30 min at 37°C in a 5% CO2 incubator and observed immediately using fluorescence microscopy.

Analysis of membrane potential by FACS, human PBMC (1 × 106) were infected with either Vprwt or Vpr– virions. Twelve hours post-infection the cells were washed with PBS and stained with CD24 antibody for 1 h in FACS buffer, then washed, and resuspended in 1 ml of diluted DePeisher solution (1 µl of DePeisher to 1 ml of 1× reaction buffer). Cells were incubated at 37°C, 5% CO2 for 20 min, washed twice with PBS, and fixed with 0.5% formaldehyde for 5 min at 18–24°C and analyzed immediately by flow cytometry. CD24-positive cells were analyzed in FL-1 channel; the red aggregates were detected in the FL-2 channel, and the green monomer staining was detected using the FL-3 channel.

Determination of CD95 and CD95L—Single cell suspensions were washed in PBS (pH 7.2) containing 0.2% bovine serum albumin and 0.1% NaN3. Cells were incubated with anti-CD95, anti-CD95L antibody, and IgG control antibody for 60 min. Cells were washed with PBS and fixed with 2% paraformaldehyde and analyzed by fluorescence-activated cell sorter (BD Pharmingen). FITC, APC-conjugated mAbs, and soluble CD95L were purchased from BD Pharmingen, and human anti-CD95 antibody (ZB4) and anti-Fas antibody (CH-11) were from MBL, Nagoya, Japan. Data were analyzed using CELL Quest program (BD Pharmingen).

Quantification of Cytochrome c by Immunobssay—Cytochrome c distribution in apoptotic cells was determined by subcellular fractionation, extraction, and solubilization as described previously with minor modification (34). Briefly, 5 × 106 cells were harvested 96 h post-infection and washed with PBS. The cells were suspended in Buffer A (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM
Dithiothreitol, 250 mM sucrose, 1× protease inhibitor mixture) and homogenized by a Dounce homogenizer. Unbroken cells and nuclei were pelleted at 10,000 × g for 10 min at 4°C then removed. The supernatant was further centrifuged at 10,000 × g for 20 min. The supernatant was saved as a cytosolic fraction, and the precipitate was dissolved in buffer containing 0.5% (v/v) Nonidet P-40 and saved as the mitochondrial fraction. The cytosolic fraction was used to analyze cytochrome c release. Cytochrome c was adsorbed onto the plastic surface of 96-well plates and quantified as per the cytochrome c ELISA kit instructions (Oncogene Research Products, Cambridge, MA).

Localization of Cytochrome c—Cc-GFP-HeLa (29) cells were maintained in poly-t-lysine-coated glass cover slides at a density of 1 × 10⁵. Twenty four hours later, they were transfected with plasmid expressing HIV-1 antigen Vpr (pCpr) using DOTAP as described before (30). The pCDNA 3 vector-transfected cells were treated as mock-transfected. The culture medium was then replaced, washed with PBS, and fresh medium added. Twenty four hours post-transfection the cells were washed with PBS and fixed in 2% paraformaldehyde in PBS for 15 min. The fluorescence of GFP was detected by fluorescence microscopy, and images were analyzed.

Immunoblot Analysis—Equal numbers of cells were collected from mock-, Vprwt-, or Vprwt-infected culture. Cells were washed with ice-cold PBS, and the cells were lysed in protein lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3Vp4, 1 µg/ml leupeptin, and 1 µg/ml aprotinin). After a brief sonication, the lysates were clarified by centrifugation at 10,000 rpm, and protein content was measured by the Bradford method (Bio-Rad). Fifty µg of protein per lane was separated by 12% SDS-PAGE and blotted onto polyvinylidene difluoride transfer membrane. Immunoblot was carried out using the primary antibody (caspase 9/Mch6; anti-Fas, and anti-Bcl-2; cytochrome c) and re-immunoblotted with anti-actin (Calbiochem) antibody, which served as a loading control. The membranes were washed thoroughly using 1× Western re-probe buffer (Geno Tech, MO) and re-immunoblotted with anti-actin (Calbiochem) antibody, which recognizes the actin expression in cultured cells and serves as a positive control for gene expression as an internal standard.

Statistical Analysis—All of the experiments were performed at least three times. Results are expressed as mean ± S.E. Groups were compared using one- or two-tailed Student’s t test.

RESULTS

Productive HIV-1 Vpr Infection of Primary Cells Induced Apoptosis—Vpr was introduced into cells via infection with pseudotyped HIV-1 virions. These pseudotyped viruses carry CD24-HSA, which allows the detection of infected cells by fluorescence microscopy and flow cytometry (30). First, we gated on the CD24-positive cells as these represent the total infected cell population. There are several advantages for using the CD24 virons. (i) Uninfected cells can be eliminated from the complex cell background. (ii) Constant numbers of positive cells can be sorted out to allow the analysis of the effects of virus in cells that directly acquired the viral protein as the result of viral entry.

Previous studies from our laboratory and from other investigators using transient transfection showed that Vpr inhibits cell proliferation (35) and blocks the cell cycle at the G2/M phase transition (9, 22, 31). In the following experiment, we tested this observation by analyzing the effect of Vpr on the cell cycle in infected cells. To evaluate in a quantitative manner the levels of cytotoxicity, we measured cell death by FACS, as determined by cell staining with both propidium iodide (late apoptosis) and annexin-V (early apoptosis). The use of the intercalative dye propidium iodide permitted detection of cells with permeabilized membrane, caused by either necrosis or apoptosis-induced secondary necrosis, whereas annexin-V staining permits detection of early phase of apoptosis, namely phosphatidylserine exposure on the surface of the cell membrane. For cell cycle analysis, 48 h post-infection, CD24⁺ cells were further stained with propidium iodide to determine DNA content by flow cytometry in Fig. 1. Mock cells and cells infected with Vprwt virus served as negative controls. The cells infected with Vprwt virus showed a dramatic increase in the percentage of cells of the G2/M phase, 53.87%, as compared with mock-infected and Vprwt virion-infected cells (6.43 or 13.33%).

Productive HIV-1 Vpr Infection of Primary Cells Induced Apoptosis—Internucleosomal fragmentation of cellular DNA is a hallmark of apoptosis. This internucleosomal fragmentation of apoptosis has not been evaluated previously using pseudoviral Vpr. Annexin-V analysis 24 h post-infection revealed greater apoptosis in the Vprwt virion-infected cells (48.31%) compared with mock-infected (2.45%) or the Vprwt virion-infected cells (12.34%), see Fig. 2A. This induction of apoptosis was apparent when the cells were infected for 24 h but not before 12 h of infection. To assess the comparative potency of Vpr with a well characterized apoptotic ligand, we treated the cells with anti-Fas and assessed apoptosis in parallel with the Vpr-infected cells. Similar to the Vpr activation, the anti-Fas treatment caused apoptosis (Fig. 2B). However, the time frame of apoptosis was clearly different. Quantitation of apoptosis indicated that ~40% of the cells were positive within 6 h of anti-Fas treatment, whereas ~32% of the cells were positive within 24 h of Vpr virion infection. Furthermore, we evaluated the effects of HIV-1 Vpr on the incorporation of fluorescein into the 3’-OH of nicked chromosomal DNA (terminal dUTP nick-end labeling analysis), on the fragmentation of chromosomal DNA, and on the release of mono- and oligonucleosomes from chromosomes (Fig. 2C). Microscopic analysis of fluorescein-
dUTP-labeled cleaved DNA showed that infection of Jurkat T-cells with HIV-1 Vpr induced apoptosis. The level of cleavage was at least 5-fold higher in Vpr-positive virus than in the cells infected with HIV-1 Vpr-negative virus, indicating that the fragmentation was due specifically to the presence of Vpr. Taken together, these assays confirm that infection of cells with Vpr-positive virus induces apoptotic cell death. This death results in nucleosomal fragmentation consistent with caspase activation; however, the timing of apoptosis appears dissimilar to apoptosis induced by the activated Fas pathway.

Vpr Activates the Apoptosis Pathway by Activating Caspase 9—The hallmark of apoptosis is the activation of specific caspases, which initiate and propagate the apoptotic cascade. These are not yet clearly defined for Vpr. We next examined the effects of Vpr on the activation of relevant caspase pathways. Human PMBCs infected with Vprwt or VprH11002 virions as well as mock cells were lysed, and caspase-specific chromogenic substrates were mixed with the cell lysate, and the amount of substrate released was determined. The results indicate that infection with Vprwt induced a considerable increase in caspase 3 activity as compared with the VprH11002 virus as well as mock groups (Fig. 3). These data support a prior study (28) that also observed Vpr-induced caspase 3 activity. However, we were interested in examining the upstream activation of the caspase pathways. Importantly, we observed that there was no activation of the caspase 8 pathway by Vpr. All treated viruses exhibited the same background level of caspase 8 activity as mock-infected cells. These data suggested differences from the Fas pathway, which activates caspase 8.

In contrast to caspase 8, there was strong activation of caspase 9 by Vpr. The pattern of caspase 9 activation by Vpr was clearly different from that observed for CD95-induced apoptosis. Vpr−
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Virus caused little increase in caspase 9 activity compared with mock-infected cells. However, virus that carries Vprwt increased the caspase 9 level as compared with Vpr− virus. This activation was confirmed by the specific caspase 9 inhibitor (LEAD-FMK). Taken together these results clearly demonstrate that Vpr-induced apoptosis is mediated through the caspase 9 pathway.

Vpr-induced Apoptosis Occurs Independent of the CD95/CD95L Pathway—Several apoptosis triggering genes were shown to mediate apoptosis by increasing the expression of the CD95 receptor or inducing the CD95L molecule. Fas or TNF receptors engaged by Fas-L are a specific marker for the activation or triggering of apoptosis (36–38). Therefore, we monitored changes in the expression of the CD95 receptor as well as that of the CD95L after HIV infection. Human PBMC were infected with HIV-1 Vprwt or HIV-1 Vpr− virions. Cells were analyzed for Fas, Fas-L, and TNF expression after HIV-1 Vprwt infection. FACS analysis of infected cells indicated that expression of the CD95 receptor remained unchanged up to 24 h after infection. Examining CD95L expression, no up-regulation could be detected over a period of 24 h (Fig. 4A). Similarly secretion of TNF-α into the infected culture supernatants was not induced by HIV-1 Vpr− virus infection as detected using a highly specific ELISA (data not shown). To further corroborate this outcome, we used an antagonistic anti-CD95 antibody (ZB4) to block activation of the CD95 pathway. Human leukemia Jurkat T-cells were preincubated for 1 h at 4°C (150 ng/ml) with the ZB4 antibody, and treated cells did not undergo apoptosis following treatment with soluble CD95L (150 ng/ml), whereas using soluble rVpr (50 pg/ml) antibody-blocked cells died similarly to the cells that were not preincubated with ZB4 antibody (Fig. 4B). These data confirmed that Vpr-induced apoptosis was not mediated by direct binding and activation of Fas/FasL pathway.

In addition, the human colonic adenocarcinoma cell lines SW480 are Fas−/FasL(+) FADD-deficient cells. As demonstrated in Fig. 4C, adCMV-vpr-infected cells induced typical apoptosis in these cells. Therefore, the killing observed illustrates that Fas/FasL pathway is bypassed by Vpr. These results are in agreement with the caspase 8 data.

Involvement of Mitochondria during Vpr-mediated Cell Death: Mitochondrial Membrane Potential Disruption and Cytochrome c Activation—In the apoptotic signal transduction pathway, mitochondria play an essential role by releasing apoptogenic factors such as cytochrome c and apoptosis-inducing factor (38). Cytochrome c binds to Apaf-1, thus recruiting and activating one of the major caspases, caspase 9, that resides in the cytoplasm. Apoptosis-inducing factor is released during mitochondrial membrane potential (ΔΨ) loss and induces apoptotic changes in the nucleus in a caspase-independent manner.

In healthy cells, the transfer of electrons through the electron transport chain is accompanied by the translocation of protons out of the mitochondrial inner membrane, creating an electrochemical gradient and the membrane potential (ΔΨ) that provides energy for the phosphorylation of ADP to ATP. In apoptotic cells, because of the compromise of the inner membrane integrity, the electrochemical gradient will be disrupted, and cytochrome c as well as other molecules will be released from the mitochondria. Thus, measuring the ΔΨ disruption can provide critical information about the upstream apoptotic event. Jacotot et al. (17) using a synthetic HIV-1 Vpr on isolated mitochondria observed rapid dissipation of the mitochondrial transmembrane potential in cell-free purified mitochondria at micromolar doses inducing the release of apoptogenic proteins such as cytochrome c. The effects of Vpr on intact cell by infection with a viral particle have not been examined previously.

To elucidate further the mechanism of apoptosis induced by Vpr, we analyzed the transmembrane electropotential of mitochondria using pseudoviral infection with either Vprwt or Vpr− virions. We used a DePsipher lipophilic cation (5,5′,6,6′-tetra chloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide) to assess the membrane potential by both microscopic observation and flow cytometry. The assay uses a lipophilic cationic agent that can form an orderly arrangement in an electric field generating aggregates. The aggregated form has an orange-red fluorescent color compared with the monomeric form that is green. Thus, DePsipher agent will appear orange-red around normally functioning mitochondria in healthy cells and exhibit the exaggerated monomeric green form in apoptotic cells.

Both FACS as well as immunofluoroscopic microscopy were used to analyze membrane potential changes. For FACS analysis, CD4-positive HeLa cells were infected with Vprwt or Vpr− pseudotype viruses, and CD24-positive cells were analyzed in FL-1 channel. Furthermore, the CD24-positive population (R2 population) was reanalyzed for red and green color using both the FL-2 and FL-3 channels, respectively. The results revealed a significant percentage of the monomeric green cells in the
FIG. 4. Expression of Fas and Fas-L in human PBMC cells. A, cells (1 × 10⁶ cells) were infected with Vpr⁺/Vpr⁻ virions, and 2 days post-infection an equal number of cells (1 × 10⁶) were gated, and their expression of CD95 and CD95L was determined. These experiments were repeated three times, and similar results were obtained. B, Jurkat T-cells with either blocked (preincubated with the anti-CD95 antibody ZB4) or unblocked CD95 receptor were treated with rVpr (50 pg/ml) or as a positive control with soluble CD95-L (150 ng/ml) for 24 h. Apoptotic cells were quantified by FACS as described under “Materials and Methods.” Bars, mean ± S.E. of three independent experiments performed in triplicate; ***, p < 0.001; n.s not significant (Student’s t test). C, apoptotic effect of adCMV-vpr in SW480 cell lines 48 h post-infection. Apoptosis was determined by flow cytometry analysis of annexin V stained cells (see “Materials and Methods”). Cells were infected with mock (i), adCMV-lacZ (ii), or with adCMV-vpr (iii). Percentage of cells in the M1 channel refers to cells, which are annexin V-positive. The data shown are representative of three independent experiments.
extracts were prepared from mock- and Vprwt-infected cells after 12 h post-infection. At the cytosolic fraction was extracted and was used to analyze cytochrome type. Among the Vprwt-infected cells, there is a high proportion of cells indicating healthy mitochondria with aggregated phenotype. Among Vpr-infected cells, there are orange-red spots, in all cells indicating healthy mitochondria with aggregated phenotype. Among the Vprwt-infected cells, there is a high proportion of cells in which the cytoplasm is almost homogenously green indicating the exaggerated monomeric forms and no orange-red spots can be observed (Fig. 5i). Vpr-induced cytochrome c release in infected cells. The total cellular extracts were prepared from mock- and Vprwt-infected cells after 12 h post-infection. m, protein samples obtained from the membrane fraction; c, samples obtained from the cytosolic fraction. Fifty micrograms of the protein samples were separated by 15% SDS-PAGE and analyzed by Western blot with a polyclonal antibody against cytochrome c-Mch6 antibody, and blots were re-probed using anti-

Vpr is a promiscuous activator of host cell gene transcription. Accordingly, we examined if the expression level of pro-caspase 9/caspase 9 had been elevated by Vpr. Proteins were extracted from the cells infected with mock, Vprwt, and Vpr– viruses, and 50 μg of protein was loaded for each sample, and Western blot analysis was carried out. Analysis was performed using caspase 9/Mch6 antibody, and blots were re-probed using anti-actin antibody as a positive control antibody to illustrate that equal amounts of protein were loaded. A higher level of caspase 9 was detected in the Vpr–infected cells (Fig. 5C), and this indicates that Vpr not only triggers the activation of caspase 9 but increases its overall expression level as well likely providing a stronger apoptotic effect. The Vpr apoptotic effect is slowly building over the 24 h of infection. This transcription may come in to play in HIV-induced apoptosis.

To evaluate the role of apoptogenic factors released by mitochondria in HIV-1 Vpr-mediated cell death, cytosolic fractions prepared from uninfected or Vprwt– or Vpr– infected cells and were analyzed using specific antibodies and ELISA for the presence of cytochrome c, a 15-kDa protein that is involved in caspase activation. Fig. 5D (i), which shows that the cytosolic fraction of Vpr-positive infected cells at 12 h post-infection contains more cytochrome c than Vpr-negative and uninfected cells, indicates that mitochondria was the target for Vpr-mediated effect. In addition we fractionated the cells into cytosolic
and membrane-bound fractions to detect cytochrome c by immunoblotting. The membrane-bound fraction contained mitochondria (34). The results are presented in Fig. 5D (ii). In control cells cytochrome c was present in mitochondria. After infection, the cytochrome c in the mitochondria fraction was decreased, and this was accompanied by the presence of cytochrome c in the cytosol. We next utilized, HeLa cells that stably express cytochrome c-GFP predominantly in the mitochondria to confirm the cytochrome c release from mitochondria. Immunocytochemistry of HIV-1 Vpr-transfected cells revealed a punctate distribution of cytochrome c-GFP compared with control vector-transfected cells (Fig. 5D (iii)). This finding is consistent with mitochondrial membrane potential distribution and confirms the involvement of mitochondria in Vpr-induced apoptosis.

**Bcl-2 Significantly Reduces, but Does Not Prevent, Apoptosis Induced by Vpr**—Because apoptosis plays such an important role in immune cells, many studies have examined the function of apoptosis regulatory molecules in this process. A particular member of the Bcl-2 family has attracted much attention. Among these the Bcl-2 family of proteins confer protection against many apoptotic stimuli that are involved in the regulation of mitochondria permeability during apoptosis. Recently Jacotot et al. (17) reported that HIV-1 Vpr may interact with the intermembrane face of the adenine nucleotide translocator to induce mitochondrial membrane permeabilization in isolated mitochondria. Furthermore, they showed that preincubation of recombinant Bcl-2 protein with isolated mitochondria prevented the Vpr-induced matrix-induced swelling at the inner mitochondrial membrane permeabilization. We next sought to evaluate the functional role played by Vpr in context of Bcl-2 overexpression in infected cells. For these studies we used Jurkat cells (Bcl-2 overexpressing cells) infected with HIV-1 Vpr wt or HIV-1 Vpr H11002 virions. Western blot analysis revealed that HIV-1 Vpr wt-infected cells exhibited a 1-fold decrease in Bcl-2 expression compared with mock or HIV-1 Vpr H11002 (Fig. 6A). In contrast, levels of actins were equivalent in both cells.

Jurkat (Bcl-2 overexpressing cells) cells were exposed to recombinant Vpr protein. Surprisingly, Bcl-2 failed to abrogate Vpr-mediated apoptotic cell death. This was true at multiple dilutions of Vpr (5–10 pg). Fig. 6B shows the dose-response studies of rVpr performed with Jurkat cells treated with different concentrations of recombinant Vpr protein. Thus, HIV-1 Vpr seems to be able to trigger apoptosis and effectively by-passes mitochondrial protection by Bcl-2 in intact cells.

The *adCMV-vpr-mediated Effect on Apoptosis and Caspase Activation*—In order to study further this aspect of Vpr biology, we engineered a modified adenovirus to carry the HIV-1 vpr gene product in order to deliver Vpr into primary cells. We used
adCMV-vpr to study the apoptotic activity of Vpr in the absence of other HIV gene products in a viral model. We tested the Vpr-mediated effect on cell cycle arrest, apoptosis, and caspase activation. An identical adenovirus carrying the LacZ protein was used as a control in these studies. To address whether the adCMV-delivered vpr gene can be expressed in human cells, human PBMC were infected adCMV-vpr particles. Human PBMC were infected with 10^10 adCMV-vpr or adCMV-lacZ particles, and the expression of Vpr protein in the infected cells was examined 48 h post-infection (Fig. 7A). The cells infected with adCMV-vpr produced a 14-kDa protein (Fig. 7A, lane b) which matches the size of baculovirus-purified HIV-1 Vpr protein (Fig. 7A, lane a), detected with the anti-Vpr polyclonal antibody. There was no Vpr protein expression in adCMV-lacZ-infected cells (Fig. 7A, lane c).

Furthermore, 48 h post-adenoviral infection, cells were harvested, fixed, and stained for DNA content with propidium iodide to determine DNA content by flow cytometry. Mock cells and cells infected with adCMV-lacZ virus served as negative controls. The cells infected with adCMV-vpr virus showed a dramatic increase in the percentage of cells in the G2/M phase at 48 h post-infection compared with mock- and adCMV-lacZ-infected cells (Fig. 7B).

Flow cytometry analysis was used to measure annexin V binding as an apoptosis-specific signal by gating on the PI-negative (non-necrotic) population. adCMV-vpr infection strongly induced apoptosis as compared with adCMV-lacZ-infected groups. Apoptosis was significantly more common in adCMV-lacZ-infected cells compared with mock cultures illustrating a background level of apoptosis by adenovirus (Fig. 7C). However, adCMV-vpr apoptosis was severalfold higher.

We next examined the effects of adCMV-vpr on the activation of relevant caspase pathways. Human PBMCs were infected with adCMV-lacZ, adCMV-vpr, or mock-infected, and the cells were lysed, and protein concentration was determined. Caspase-specific chromogenic substrates were mixed with cell lysate, and the amount of substrate released was determined. Importantly, we observed that there was no activation of the caspase 8 pathway by adCMV-vpr (Fig. 7D (i)). In contrast, caspase 9 activity was present in adCMV-vpr-infected cells and not in adCMV-lacZ or mock groups (Fig. 7D, (ii)). All infected cells exhibited the same background level of caspase 8 activity as mock cells. Cytosolic fraction of adCMV-vpr infected at 12 h post-infection contains more cytochrome c than mock cells (data not shown). This result supports that activation by Vpr was occurring through the caspase 9 pathway. The pattern of caspase 9 activation by adCMV-vpr was highly reproducible and allows analysis of the Vpr effects without the complication of other HIV antigens. These results are consistent with the hypothesis that HIV-1 Vpr leads to apoptosis by inducing caspase 9.

**DISCUSSION**

The loss of functional immune cells is a hallmark of AIDS. Induction of apoptosis following viral infection of a cell is generally viewed as an attempt by the host cell to limit virus replication. In fact, many viruses carry their own anti-apoptotic genes or up-regulate anti-apoptotic cellular genes, which...
can block the premature death of infected cells. This strategy facilitates a persistent infection or prolongs the survival of infected cells to maximize the production of viral progeny. On the other hand, it has also been suggested that apoptotic cell death may be part of the cycle of HIV-1 replication. Thus, like other animal viruses, HIV-1 may have developed strategies to modulate the apoptotic cell death of its target cells. Research into the biology of the accessory genes of HIV-1 constitutes an active area of investigation. The vpr accessory gene is a potent regulator of the host cell. One dramatic function of Vpr is its effect on host cell proliferation, which results in transcription changes in host cells (35). This growth arrest occurs in the G2/M phase of the cell cycle (6, 7, 10, 31). Cell cycle arrest may be at least temporally linked to the induction of apoptosis (10). The apoptotic cascade induced by Vpr is not understood in detail; however, recent studies have helped to provide a scaffold.

**Fig. 7. Adeno delivered Vpr-induced cell cycle arrest, apoptosis, and caspase activation.** A. immunoblot analysis of human PBMC (1 × 10⁶) was infected with adCMV-vpr and adCMV-lacZ. Cells were lysed, and 50 μg of total proteins from each group were then separated by 12% SDS-PAGE and analyzed by Western blotting analysis using polyclonal anti-Vpr antibody raised in rabbit. Lane A, positive control rVpr protein (10 μg) purified in baculovirus; lane B, adCMV-vpr; and lane 3, adCMV-lacZ. FACS analysis either by annexin V-FITC and PI staining quantified apoptotic and G2 arrest cells. B, PI staining and counting PI-stained nuclei with a subdiploid DNA content. C, counting annexin V-FITC-positive and PI-negative cells. D, caspase 8 and caspase 9 activity in infected cells after 48 h post-infection. Measurements were performed by the fluorometric cleavage assay as described under "Materials and Methods." All the experiments were performed three times with similar results. Bars, mean ± S.E. of three independent experiments performed in triplicate.
of information for consideration. Stewart et al. (28) have reported that Vpr activates caspase 3. The relationship of this downstream activator of apoptosis and Vpr biology requires further work. Most recently, by studying Vpr as a free protein, a direct effect on isolated mitochondria was observed, and protein Vpr induced mitochondria destabilization resulting in the induction of the apoptotic cascade in vitro (17, 39). However, these studies did not test the effects of viral borne Vpr on primary cells that are the actual targets of HIV infection. Furthermore, the link between caspase 3 activation and the destabilization of the mitochondrial pathway by either of the upstream caspase pathways 8 or 9 was not determined. Finally analysis of the direct relationships between Vpr cell cycle arrest, apoptosis induction, caspase activation, and mitochondria destabilization in primary lymphocytes by viral borne Vpr has not been investigated previously.

Accordingly, we first directly tested caspase activation induced by viral delivered HIV-1 Vpr in PBMCs. We delivered Vpr protein into target cells by infection using pseudotyped viruses. Compared with previous transfection studies, the infection model more closely resembles the natural process in which Gag-associated protein Vpr and other viral proteins as well as genomic RNA are introduced into target cells. In addition, infection allows delivery of viral proteins into cells that are difficult to transfect, such as human PMBCs.

There are two major pathways for induction of apoptosis, the Fas-Fas ligand pathway and the mitochondrial pathway. In both cases, a hallmark and downstream target of both of these apoptotic pathways is the activation of caspase 3. Therefore, we examined caspase 3 activation in cells infected with pseudotyped viruses that carry Vprwt or VprΔ51. In cells infected with virions that carry Vprwt, caspase 3 was dramatically elevated. These data confirmed a prior study which illustrated that HIV-1 Vpr apoptosis involves activation specifically of caspase 3 (28). We subsequently tested both caspase 8 and caspase 9 activity. Caspase 8 activity is a downstream indicator of activation of the Fas-Fas ligand apoptotic pathway, whereas caspase 9 is a downstream indicator of the mitochondrial apoptotic pathway. We found that infection Vprwt or VprΔ51 viruses failed to induce caspase 8 activity. This suggested that in PBMCs HIV-1 Vpr-induced apoptosis is not through the Fas death receptor pathway. There are several additional links of evidence that collectively illustrate that Vpr acts independently of the caspase 8 Fas/FADD pathway.

Some HIV genes including env and nef were reported to activate the CD95 receptor pathway by increasing the CD95 expression or inducing the CD95L. Therefore, we investigated whether this mechanism also involves Vpr. Flow cytometric analysis of infected cells neither increased CD95 expression nor induced CD95L. In addition, these results were further corroborated using the antagonistic anti-CD95 antibody ZB4. Preincubation with this antibody blocked CD95L-induced apoptosis, whereas Vpr-mediated cell death was unaffected. Finally we tested the ability of Vpr to induce apoptosis of a Fas/FADD-deficient cell line SW480 (40). SW480 was effectively induced to undergo apoptosis by Vpr. Thus, Vpr is able to trigger apoptosis independent of the CD95 receptor pathway. Besides the caspase cascades initiated by death receptors, another cascade that is essentially controlled by mitochondria is also important in apoptosis. The natural pro-caspase 9 activation pathways involve the release of cytochrome c from mitochondria, which form a complex with Apaf-1. The complex that activates the mitochondrial apoptosis pathway consists of cytochrome c released from the mitochondria (Apaf-2), the human homolog of Ced-4 (Apaf-1), and the caspase 9 proenzyme (Casp9) (Apaf-3). The Apaf-1-cytochrome c complex recruits pro-caspase 9 and induces its auto-activation by aggregation (41, 42). Inhibitors such as XIAP (X-linked inhibitor of apoptosis), c-IAP-1 (cellular inhibitor of apoptosis protein-1), and c-IAP-2 (43, 44) also regulate the activity of caspase 9. Recently, a new protein Smac has been identified that activates the caspase 9 pathway by eliminating IAP inhibition (45). In order to elucidate whether Vpr as a viral protein asserts its apoptotic effect through the mitochondria, or if it by passes mitochondria and acts directly on the downstream pro-caspase 9, we measured the transmembrane potential of the mitochondria during viral infection. The disruption of transmembrane potential, which indicates the disruption of the membrane integrity, is one of the earliest phenomena in the mitochondrial pathway of apoptosis. We observed that HIV-1 viral borne Vpr disrupts the mitochondrial membrane potential, indicating that Vpr induces apoptosis through mitochondria disruption. By making the mitochondria membrane-permeable, Vpr would facilitate the release of apoptotic proteins such as cytochrome c, which ultimately affects caspase 9 and later cleaves caspase 3. During the course of this study, Jocotot et al. (17) reported that a synthetic Vpr could destabilize isolated purified mitochondria. Here we observed the release of cytochrome c was induced by Vpr in whole cells, thus extending these original observations.

Because the Bcl-2 protein family regulates mitochondrial potential and early apoptotic changes, we have further investigated the expression of Bcl-2 and Vpr-induced apoptosis. Vpr markedly reduced the expression level of Bcl-2 proteins, as assessed by Western blot assay. These proteins inhibit the release of caspases and caspase-activating factors from apoptotic mitochondria (46). However, Bel-2 overexpression did not inhibit the ability of Vpr to induce apoptosis. This result is interesting as it suggests that either the enhanced breakdown of Bel-2 or through another mechanism Vpr can overcome this block to apoptosis.

In summary, we observe that HIV-1 Vpr induces apoptosis through the caspase 9-dependent mitochondrial pathway in primary immune cells. Vpr as an apoptotic protein targets the mitochondrial apoptotic pathway resulting in its destabilization as follows: release of cytochrome c which likely aggregates Apaf-1 and induces a heightened level of caspase 9 activity, and heightened levels of caspase 3 activity leading ultimately to the autodestruction of infected targets of HIV. Furthermore, investigation into the role of the caspase 9 apoptotic pathway in HIV pathobiology is warranted, as it is interesting to speculate that inhibition of this pathway may be exploited as an approach to novel therapeutics.

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