Lentiviral delivery of HIV-1 Vpr protein induces apoptosis in transformed cells

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Most current anticancer therapies act by inducing tumor cell stasis followed by apoptosis. HIV-1 Vpr effectively induces apoptosis of T cells after arrest of cells at a G_2/M checkpoint. Here, we investigated whether this property of Vpr could be exploited for use as a potential anticancer agent. As a potentially safer alternative to transfer of genes encoding Vpr, we developed a method to efficiently introduce Vpr protein directly into cells. Vpr packaged into HIV-1 virions lacking a genome induced efficient cell cycle arrest and apoptosis. Introduction of Vpr into tumor cell lines of various tissue origin, including those bearing predisposing mutations in p53, XPA, and hMLH1, induced cell cycle arrest and apoptosis with high efficiency. Significantly, apoptosis mediated by virion-associated Vpr was more effective on rapidly dividing cells compared with slow-growing cells, thus, in concept, providing a potential differential effect between some types of tumor cells and surrounding normal cells. This model system provides a rationale and proof of concept for the development of potential cancer therapeutic agents based on the growth-arresting and apoptotic properties of Vpr.

HIV-1 Vpr is a 96-aa protein that is expressed after HIV-1 infection. A number of functions have been ascribed to Vpr, including induction of G_2 arrest followed by apoptosis in T cells as well as other human cells (1–6). Vpr was found to be a strong inducer of apoptosis (5, 6), although in some reports Vpr was shown to inhibit apoptosis (7, 8). Vpr also is found in HIV-1 virions (9–11), and virion-associated Vpr can induce cell cycle arrest after infection in the absence of new Vpr expression (12). Here we demonstrate apoptosis in a wide range of target cells and investigate whether the cytostatic and apoptotic effects of Vpr could be used to develop potential model strategies for cancer therapeutics.

Because of the potential safety issues involving gene transfer and constitutive expression of a gene with potent cell-arresting and apoptotic effects, we devised a strategy to transiently introduce Vpr protein into cells in the absence of a viral genome. Recently, we demonstrated that Vpr packaged within HIV-1 virions, in the absence of *de novo* protein synthesis, was sufficient to induce G_2 arrest (12). We examined whether virion-associated Vpr was also capable of inducing apoptosis. This approach afforded a system in which we could examine the effects of Vpr without permanently introducing a viral genome into the host cells' genomic DNA.

Materials and Methods

Cells. HeLa and 293T cells were maintained in DMEM plus 10% BCS. SW480, XP12BESV, HT1080.ATCC, and HT1080.GTG were maintained in DMEM plus 10% FCS. SupT1 cells were maintained in Iscove's modified Dulbecco's medium (Iscove's) plus 10% FCS. LNCap cells were maintained in RPMI medium 1640 plus 10% FCS. Normal human diploid fibroblasts (ATCC CRL2076) were maintained in Iscove's plus either 10% FCS or 1%-depleted FCS. Iscove's plus 1%-depleted FCS was prepared as follows: Iscove's plus 10% FCS was sequentially passaged onto fresh cells six times. After the sixth passage the media was diluted 1:10 with fresh Iscove's without FCS, thus producing media containing 1%-depleted FCS. All cells were maintained at 37°C and 5% CO_2.

Viral Constructs and Production of Viral Stocks. The retroviral vectors, pHR'Thy and pHR'Vpr, were produced by cloning the murine *thy 1.2* and HIV-1 *vpr* genes, respectively, into pHR'-CMV-lacZ (13). By oligo-directed mutagenesis, the C-terminal 64 aa of Vpr were deleted from the packaging plasmid pCMVΔR8.2 (14), resulting in pCMVΔR8.2ΔVpr. HR'Thy(Vpr−) and HR'Vpr viral stocks were produced by cotransfection of pHR'Thy or pHR'Vpr, pCMVΔR8.2ΔVpr, and pCMVVSV-G. The HR'Thy(Vpr+) viral stock was produced by cotransfection of pHR'Thy, pCMVΔR8.2, and pCMVVSV-G. Virions devoid of viral RNA were produced by cotransfection with either pCMVΔR8.2 or pCMVΔR8.2ΔVpr, and pCMV-VSV-G, resulting in RNA-(Vpr+) and RNA-(Vpr−), respectively. HIV-$1_{NL4-3\text{-}thy}$env(-) was generated by cotransfection of pCMVVSV-G and NLthyΔBgl (15). All viral stocks were produced by calcium phosphate-mediated transfection (16) of 293T cells. Virus was collected 48 and 72 h posttransfection and concentrated (5, 15).

Infections and Flow Cytometry. Cells were infected and analyzed by flow cytometry for both cell cycle content and apoptosis (5). All stained cells were analyzed on a FACScan II (Becton-Dickinson) and acquired by the CELL QUEST software package.

γ-Irradiation of HeLa Cells. HeLa cells were exposed to 4,000 rads of γ-irradiation from a ^{137}Cs source.

Western Blot Analysis. Concentrated virus stocks were lysed in 2× loading buffer and subjected to electrophoresis on SDS-15% polyacrylamide gels (12). Western blot analysis was performed with anti-HIV-1 hyperimmune plasma (kindly provided by P. Krogstad and Y. Bryson, University of California, Los Angeles) or anti-Vpr polyclonal antibody (kindly provided by N. Landau, Salk Institute for Biological Studies, La Jolla, CA) and developed with the enhanced chemiluminescence assay (Amersham Pharmacia).

Results

HIV-1 Vpr is packaged into the virion through an association with the structural Gag protein, p6 (17, 18). Therefore, by using a packaging plasmid expressing Gag and Vpr, we can generate virions that contain Vpr protein but do not encode Vpr within

Abbreviation: VSV, vesicular stomatitis virus.

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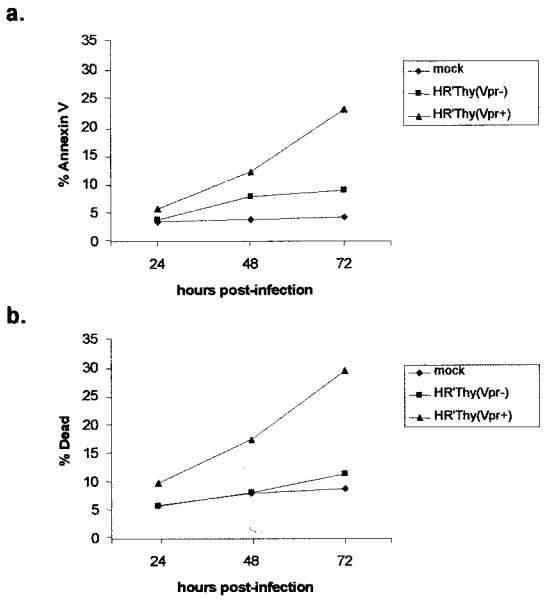

Fig. 1. Virion-associated Vpr is sufficient to induce apoptosis. (*a*) HeLa cells (5×10^4) were infected and analyzed by annexin V staining at 24, 48, and 72 h postinfection. Concurrent Thy 1.2 analysis revealed that 66.2% and 81.7% of cells were infected with HR'Thy(Vpr−) and HR'Thy(Vpr+), respectively. The *y*-axis represents the percent of cells that are annexin V-positive and 7AAD-negative and thus undergoing apoptosis. Results are for mock (◆)-, HR'Thy(Vpr−) (■)-, and HR'Thy(Vpr+) (▲)-infected cells. The data shown are representative of eight independent experiments. (*b*) The percent of dead cells in the cultures was determined at the same time as the cells were analyzed for annexin V staining. The *y*-axis represents the cumulative number of dead cells within the culture. Symbols are as described for *a*.

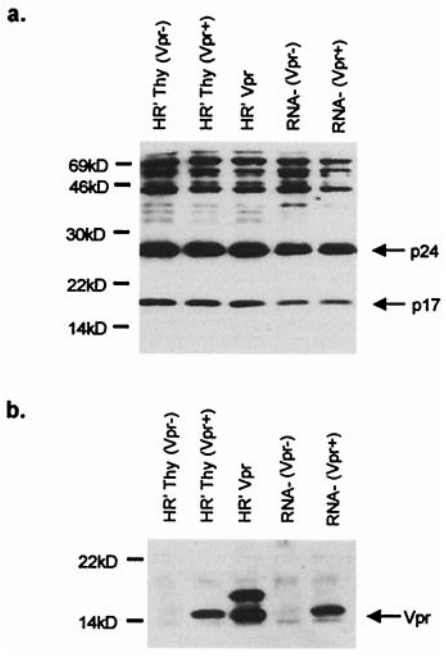

Fig. 2. Vpr is packaged into virions. (*a*) Concentrated virus preparations were analyzed for the presence of Gag. Blots were probed with a human hyperimmune sera. The Gag species, p24 and p17, are indicated by arrows. (*b*) Concentrated virus preparations were subject to Western blot analysis and probed with an anti-Vpr antibody. pHR'Vpr produces wild-type Vpr and Vpr containing an N-terminal influenza hemagglutinin tag caused by the presence of a second initiation codon before Vpr. The protein doublet in the HR'Vpr lane is caused by the detection of both forms by the anti-Vpr sera. The arrow indicates wild-type Vpr.

the genome. The genome of the HIV-1 retroviral vector, HR'Thy, which expresses the murine *thy 1.2* gene, was copackaged to facilitate identification of infected cells. Thus, HR'Thy(Vpr−) and HR'Thy(Vpr+) were produced by cotransfection of pHR'Thy, a packaging plasmid either encoding a truncated *vpr* gene (pCMVΔR8.2ΔVpr) or wild-type *vpr* gene (pCMVΔR8.2), respectively, and a third vector expressing the vesicular stomatitis virus envelope, pCMVVSV-G.

At 24, 48, and 72 h postinfection, HeLa cells were analyzed for cell survival and apoptosis. Analysis at 72 h postinfection of cells infected with virions containing Vpr [HR'Thy(Vpr+)] revealed that they arrested and underwent apoptosis whereas those infected with virions minus Vpr [HR'Thy(Vpr−)] behaved similarly to mock-infected cells [compare 22.9% annexin V-positive cells in the HR'Thy(Vpr+)-infected culture versus 8.8% and 4.2% in the HR'Thy (Vpr−) and mock-infected cultures, respectively (Fig. 1*a*)]. Analysis of the dead cell population confirmed that Vpr packaged into virions efficiently induced cell death (Fig. 1*b*). At 72 h postinfection, 29.4% of cells infected with virions bearing Vpr were dead compared with 8.7% and 11.1% of cells either mock-infected or infected with Vpr-minus virions, respectively. The percent of dead and apoptotic cells reported reflect only that measured at a single time point. However, when the data is examined in a cumulative fashion we observe that virtually all of the cells infected with the Vpr-bearing virus, HR'Thy(Vpr+), died by apoptosis. Thus virion-associated Vpr is sufficient to induce apoptosis.

Virion-Associated Vpr Within Virions Lacking a Viral Genome Induces Apoptosis. Another concern over the use of retroviral vectors for gene delivery is the possibility of insertional mutagenesis. Therefore, we modified the above strategy to deliver Vpr within virions

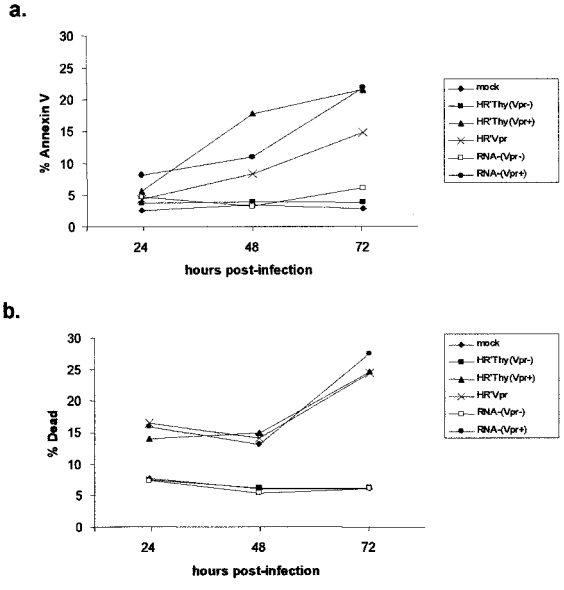

Fig. 3. Vpr packaged into an empty virion is sufficient to induce apoptosis. (*a*) 5×10^3 infected HeLa cells were analyzed by annexin V staining at 24, 48, and 72 h postinfection. DNA analysis at 48 h postinfection revealed that 27.7%, 31.0%, 80.6%, 95.9%, 27.7%, and 85.9% of mock-, HR'Thy(Vpr−)-, HR'Thy(Vpr+)-, HR'Vpr-, RNA-(Vpr−)- and RNA-(Vpr+)-infected cells, respectively, were in the G_2 phase of the cell cycle, indicating the presence of 1.6×10^4 to 4.8×10^4 infectious units per infection. Results are for mock (◆)-, HR'Thy(Vpr−) (■)-, HR'Thy(Vpr+) (▲)-, HR'Vpr (×)-, RNA-(Vpr−) (□)-, and RNA-(Vpr+) (●)-infected cells. The data shown are representative of four independent experiments. (*b*) The percent of dead cells in the cultures was determined at the same time as the cells were analyzed for annexin V staining. Symbols are as described for *a*.

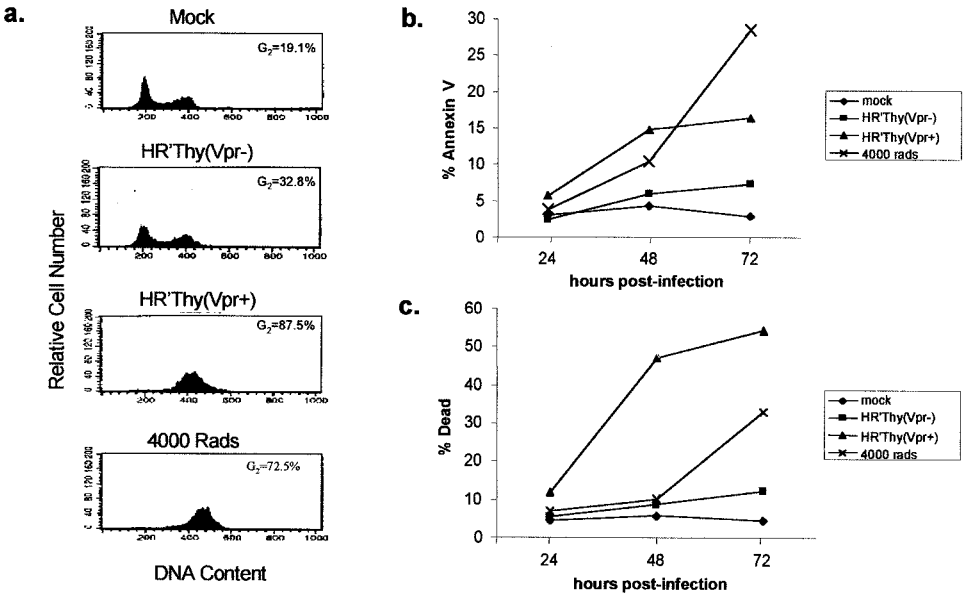

Fig. 4. Virion-associated Vpr results in more cell death than γ-radiation. (*a*) HeLa cells (5×10^4) were infected with 1.7×10^4 infectious units or irradiated with 4,000 rads of γ-irradiation, and analyzed for DNA content at 24 h postinfection. Relative cell numbers are indicated on the *y*-axis, and DNA content is indicated on the *x*-axis. Percent of cells in G_2 is indicated at the upper right of each panel. (*b*) 5×10^3 HeLa cells were analyzed for annexin V staining at 24, 48, and 72 h postinfection. Results are for mock (◆)-, HR'Thy(Vpr−) (■)-, HR'Thy(Vpr+) (▲)-infected cells, and γ-irradiated (×) cells. (*c*) The percent of dead cells in the cultures was determined at the same time as the cells were analyzed for annexin V staining. Symbols are as described for *b*.

devoid of viral RNA. Virions that lacked the viral genome and carried Vpr [RNA-(Vpr+)] were produced by cotransfection with pCMVΔR8.2, which expresses the viral structural proteins and wild-type Vpr, and the vesicular stomatitis virus (VSV)-G expressing plasmid, pCMVVSV-G. In addition, virus lacking both Vpr and a genome [RNA-(Vpr−)] was produced by cotransfection with pCMVΔR8.2ΔVpr, which encodes a truncated *vpr* gene, and pCMVVSV-G.

Western blot analysis of the viral stocks demonstrated the presence of the Gag protein, p24, and p17 (Fig. 2*a*). As expected, virions made in the presence of wild-type Vpr [RNA-(Vpr+)] contained Vpr whereas those made in the absence of Vpr [RNA-(Vpr−)] did not (Fig. 2*b*).

Virions containing Vpr in the absence of a viral genome induced apoptosis as efficiently as virions containing both a vector genome and Vpr [Fig. 3*a* compare RNA-(Vpr+) (●) with

Table 1. Cell cycle arrest and apoptosis mediated by HIV-1 Vpr in transformed cells

Cell name	Cancer origin	Phenotype	Virus	G_1/G_2¶	Annexin V‖
HeLa	Cervical carcinoma	p53 null*	HR'Thy (Vpr−)	1.7	2.4
			HR'Thy (Vpr+)	0.3	14.3
SW480	Colon adenocarcinoma	p53 mt†	HR'Thy (Vpr−)	1.0	28
			HR'Thy (Vpr+)	0.35	40.6
HT1080.ATCC	Fibrosarcoma	p53 wt‡	HR'Thy (Vpr−)	1.95	4.3
			HR'Thy (Vpr+)	0.75	20
HT1080.6TG	Fibrosarcoma	p53 mt‡	HR'Thy (Vpr−)	1.18	8.8
			HR'Thy (Vpr+)	0.61	25.4
SupT1	T cell lymphoma	Unknown	HR'Thy (Vpr−)	1.67	4.0
			HR'Thy (Vpr+)	0.41	8.9
LNCap	Prostate carcinoma	p53 wt§	HR'Thy (Vpr−)	8.4	13.6
			HR'Thy (Vpr+)	2.64	28.3
HCT116	Colon carcinoma	hMLH−‡	Mock	1.3	9.2
			HR'Thy (Vpr+)	0.3	18.0
XP12BESV	Xeroderma pigmentosum, complementation group A	XPA−‡	HR'Thy (Vpr−)	1.54	7.3
			HR'Thy (Vpr+)	0.89	28

Cells were either mock-infected or infected with HR'Thy (Vpr−) or HR'Thy (Vpr+). Cell cycle arrest and apoptosis were monitored on day 2 (HT1080.6TG, HCT116) or day 3 (HeLa, SW480, HT1080.ATCC, SupT1, LNCap, XP12BESV) postinfection, determined by the time point at which maximal apoptosis was observed. wt, wild type; mt, mutant; null, unstable. The data are representative of two independent experiments.

*The phenotype of the cell line was determined in ref. 28.

†The phenotype of the cell line was determined in ref. 29.

‡The phenotypes of the cell lines were determined as described in the text.

§The phenotype of the cell line was determined in ref. 30.

¶The ability of Vpr to mediate cell cycle arrest is expressed as the ratio of Thy 1.2-positive cells in G_1 versus Thy 1.2-positive cells in G_2.

‖The ability of Vpr to induce apoptosis is expressed as the percentage of Thy 1.2-positive cells that stain positive for annexin V and negative for 7-AAD.

HR'Thy(Vpr+) (▲)]. As expected, control cells infected with virions without Vpr [HR'Thy(Vpr−) (Fig. 3*a*, ■) or RNA-(Vpr−) (Fig. 3*a*, □], did not undergo apoptosis. Interestingly, infection with virus containing Vpr either in the presence [HR'Thy(Vpr+)] or absence [RNA-(Vpr+)] of the viral RNA resulted in a more rapid induction of apoptosis than that induced by infection with virus that expresses Vpr *de novo* (HR'Vpr) (Fig. 3*a*). Quantitation of the dead cell populations confirmed the results of the apoptosis analysis (Fig. 3*b*).

Exogenously added soluble Vpr has been reported to increase HIV-1 infection (19) and to affect the differentiation of rhabdomyosarcoma cells (20). To control for the effects, if any, of exogenous Vpr, we produced genome-minus virions containing Vpr without the VSV-G envelope. Consistent with our previously published results (12), infection of HeLa cells with non-VSV-G-enveloped virions did not result in G_2 arrest or apoptosis (data not shown). This result indicates that viral entry, presumably resulting in delivery into the cell of virion-associated Vpr, was required to induce apoptosis.

The Kinetics of Vpr-Induced Cell Death Is More Rapid Than γ-Radiation-Induced Apoptosis. Thirty to 60 grays (3,000–6,000 rads) of γ-radiation often are used to treat cancer in the clinical arena. Therefore, we compared the efficiency of apoptosis induced by Vpr versus a clinically relevant dose of γ-radiation. HeLa cells infected with virions containing Vpr arrested at the G_2/M border as efficiently as cells receiving 4,000 rads of γ-radiation (Fig. 4*a*). Annexin V analysis revealed that Vpr-containing virions and γ-radiation both induced apoptosis efficiently. However, Vpr-induced apoptosis appears to occur more rapidly (Fig. 4*b*), resulting in greater cumulative cell death by 72 h posttreatment (Fig. 4*c*).

HIV-1 Vpr Induces Cell Cycle Arrest and Apoptosis in Multiple Tumor Cell Lines. Many tumor cells are refractory to induction of cell cycle arrest and/or apoptosis by standard chemotherapeutic agents. Resistance often is associated with mutations in tumor suppressor genes. p53, in particular, has been shown to be required for certain cell cycle arrest and apoptotic pathways (21–23). We examined Vpr-induced cell cycle arrest and apoptosis in cell lines that varied in their p53 phenotype status or were deficient in DNA repair mechanisms, which are predisposing to malignancy (Table 1). Virions bearing Vpr induced G_2 arrest and apoptotic cell death regardless of p53 phenotype status. We also examined two matched fibrosarcoma cell lines differing only in p53 genotype. HT1080.ATCC contains two wild-type p53 alleles and HT1080.6TG contains two mutant p53 alleles (24). Virion-associated Vpr was equally proficient in inducing G_2 arrest and apoptosis in these cells regardless of the presence or absence of p53. Similarly, introduction of Vpr into tumor cell lines defective in different DNA repair mechanisms, such as mismatch repair (hMLH1) (25) and nucleotide excision repair (xeroderma pigmentosum, complementation group A) (26), resulted in G_2 arrest and apoptosis.

Vpr-Induced Apoptosis Preferentially Targets Rapidly Dividing Cells. The effective use of cancer therapeutic agents such as radiation and chemotherapy depend on the differential effects induced in tumor cells versus normal cells. The increased sensitivity of tumor cells to genotoxic agents is likely caused by the increased and unregulated growth of these cells, thereby increasing their susceptibility to apoptosis (22). HIV-1 Vpr's cytotoxic effects correlate with induction of G_2 arrest, suggesting that Vpr-induced cell death requires entrance into G_2 (5, 6). Because the half-life of Vpr is approximately 20 h (27), we predicted that stationary or slowly dividing cells would be less susceptible to apoptosis induced by virion-associated Vpr relative to rapidly dividing cells.

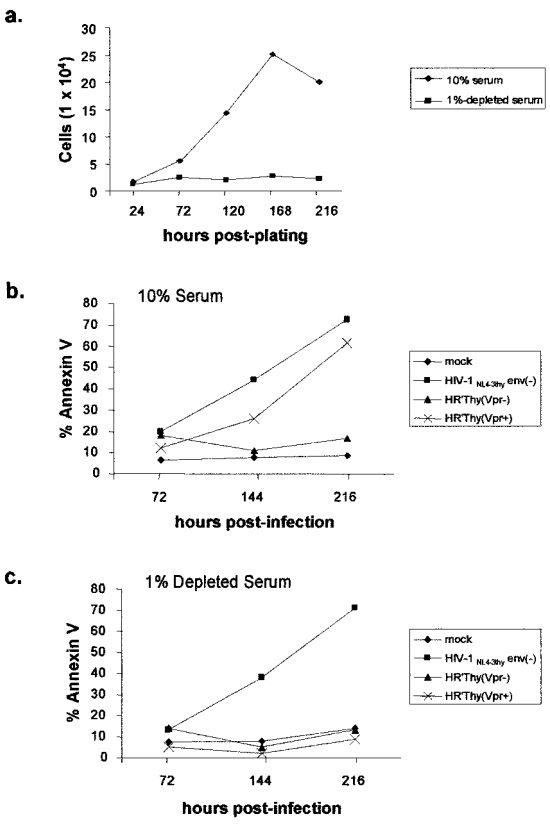

Fig. 5. Vpr preferentially kills rapidly dividing cells. (*a*) Human fibroblasts were grown in Iscove's plus 1%-depleted FCS or 10% FCS. Cells (2×10^4) were plated on day 0 and counted on days 1, 3, 5, 7, and 9. (*b*) Normal human fibroblasts were grown in 10% FCS and mock-infected (◆) or infected with HIV-$1_{NL4-3Thy}$env(-) (■), HR'Thy(Vpr−) (▲), or HR'Thy(Vpr+) (×). At 144 h postinfection, infection with HIV-$1_{NL4-3Thy}$env(-), HR'Thy(Vpr−), and HR'Thy(Vpr+) resulted in 63%, 66%, and 71% Thy 1.2-positive cells, respectively. At 72, 144, and 216 h postinfection, Thy 1.2-positive cells were analyzed for annexin V staining. (*c*) Normal human fibroblasts were grown in 1% depleted FCS and mock-infected (◆) or infected with HIV-$1_{NL4-3Thy}$env(-) (■), HR'Thy (Vpr−) (▲), or HR'Thy(Vpr+) (×). At 144 h postinfection, infection with HIV-$1_{NL4-3Thy}$env(-), HR'Thy(Vpr−), and HR'Thy(Vpr+) resulted in 48%, 65%, and 82% Thy 1.2-positive cells, respectively. At 72, 144, and 216 h postinfection, Thy 1.2-positive cells were analyzed for annexin V staining.

We tested this prediction by growing normal human diploid fibroblasts in either media containing 10% bovine FCS or 1%-depleted FCS. Cells grown in 10% FCS continued to divide while the growth rate of cells grown in 1%-depleted FCS was reduced (Fig. 5*a*).

Cells were infected with virus bearing the VSV-G envelope and expressing *de novo* synthesized Vpr [HIV-$1_{NL4-3Thy}$env(-)], with virions containing virion-associated Vpr only [HR'Thy(Vpr+)], or with virions lacking Vpr [HR'Thy(Vpr−)]. Infection of normal diploid fibroblasts grown in either 10% FCS or 1%-depleted FCS with each of these three viruses resulted in efficient infection as evidenced by Thy 1.2 staining (Fig. 5*b*, legend). Analysis of annexin V-positive cells indicated that continuous *de novo* production of Vpr after infection with HIV-$1_{NL4-3Thy}$env(-) resulted in apoptosis of virtually all cells infected regardless of their rate of division (Fig. 5 *b* and *c*, compare 10% serum and 1%-depleted serum), reinforcing the concept that continuous expression of an apoptotic gene would be detrimental to normal cell viability. In marked contrast, infection of slowly dividing fibroblasts grown in 1%-depleted FCS with virions bearing Vpr but without *de novo* production

[HR'Thy(Vpr+)] resulted in only 8.6% annexin V-positive cells 216 h postinfection, similar to cells infected with virions without Vpr [HR'Thy(Vpr−)] (Fig. 5*c*). Infection of rapidly dividing fibroblasts grown in 10% FCS with the same Vpr-containing virus [HR'Thy(Vpr+)] resulted in 61.5% annexin V-positive cells (Fig. 5*b*), indicating that rapidly replicating cells are more sensitive to incoming Vpr delivered via virions.

Discussion

In summary, we demonstrate that Vpr is capable of inducing cell cycle arrest and apoptosis in many cancer cell lines, including those defective for some tumor suppressor genes and DNA repair genes. We further show that delivery of the Vpr protein, rather than a gene encoding Vpr, can be accomplished by taking advantage of the ability of Vpr to be packaged into virions.

Apoptosis induced by Vpr contained within virions depends on the presence of the VSV-G envelope to allow entry of virions into cells. Furthermore, virions can be produced without the presence of an infectious genome within the virion particle. The latter strategy minimizes the potential for insertional mutagenesis. If needed, one approach to further enhance safety could be to deliver Vpr in the presence of inhibitors of HIV-1 replication including reverse transcriptase and protease inhibitors. Inclusion of such inhibitors would prevent reverse transcription and viral maturation, respectively, but as we demonstrated previously, would have no effect on virion-induced cell cycle arrest (12).

Successful treatment of malignancies in humans depends on the ability of anticancer therapies to preferentially target tumor cells, stop their replication, and kill them. We show that apoptosis induced by Vpr delivered in virions depends on continued cell division, thus providing a potential differential effect on tumor cells relative to surrounding normal cells. It remains to be tested whether virion-associated Vpr can, in fact, preferentially target tumor cells *in vivo*. Recent studies have suggested that some types of cancer cells may not replicate more rapidly than surrounding tissue. In contrast, some types of cancer do grow more rapidly than the normal surrounding tissue, suggesting that they may be good targets for therapy. Notably, induction of apoptosis by virions lacking a viral genome can be achieved by multiplicities of infection less than one, suggesting that virions can be produced in amounts suitable for testing in *in vivo* models.

The mechanism of HIV-1 Vpr-induced G_2 arrest and apoptosis remains to be elucidated, although many features of the arrest are similar to that induced by DNA damaging agents (15). Developing a further understanding of the targets of Vpr in specific signal transduction pathways that lead to cell cycle arrest and apoptosis will be critical for developing the most effective approaches to use this property of Vpr for cancer therapeutics. There are a number of approaches, including those described here, using gene or protein delivery, which may be feasible for some types of cancer. The HIV-1 vectors described here serve as the basis for future development of more specific targeting strategies underway in multiple laboratories. Together with Vpr's preferential effects on rapidly growing cells, vector-specific targeting strategies will provide even greater specificity for tumor cells. Thus, our results provide a proof of concept for the potential adaptation of the unique properties of Vpr in the setting of cancer.

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