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StpC-based gene therapy targeting latent reservoirs of HIV-1

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Abstract

The ability of HIV-1 to form latent reservoirs presents a major obstacle to eradication. One approach to elimination of the latent reservoir is induction therapy, whereby cells harboring latent virus are activated and therefore initiate virus replication. We have constructed a lentiviral vector encoding *Herpesvirus saimiri* subgroup C saimiri transformation-associated protein (StpC), which has been shown to modulate HIV-1 replication, under the control of a cytomegalovirus promoter in order to determine the ability of StpC to upregulate latent HIV-1. We have included a suicide gene, herpes simplex virus thymidine kinase (TK), under the control of the HIV-1 long terminal repeat (LTR) promoter. We hypothesized that upon StpC expression in latently infected cells induction of virus replication and subsequent production of viral transactivators of the LTR will activate expression of the *tk* gene, sensitizing the cells to the nucleoside analogue ganciclovir (GCV). Transduction of the latently infected cell line J1.1 resulted in increased virus replication. In the presence of GCV transduced cells exhibited decreased HIV-1 replication, inhibition of cell proliferation, and increased apoptosis. This prototype vector serves as a proof of concept of the utility of gene-based induction agents and suicide genes as a new method for targeting reservoirs of latent HIV-1.

Keywords: HIV-1; Latency; *Herpesvirus saimiri*; StpC; Suicide gene; Lentiviral vector

1. Introduction

There are nearly 40 million people in the world living with human immunodeficiency virus (HIV). The 3.1 million deaths due to HIV/AIDS reported in 2005 were outnumbered by 4.9 million new infections, illustrating the continuing catastrophic nature of this epidemic. Current antiretroviral drugs against HIV type 1 (HIV-1) have been very successful in reducing viral burden in infected individuals by interrupting virus replication at several key steps. Initial analysis of the kinetics of viral decay after highly active antiretroviral therapy (HAART) suggested that eradication may be possible after a few years of uninterrupted treatment (Cavert et al., 1997; Perelson et al., 1997). However, a latent viral reservoir of quiescent cells remains that is not susceptible to current drug therapy because these cells are not supporting productive viral replication. The latent reservoir is replication competent and retains the ability to replenish viral load upon activation or interruption of HAART (Chun et al., 1997b). The majority of the latent reservoir is composed of resting $CD4^+$ T lymphocytes bearing a memory phenotype (Brenchley et al., 2004; Chun et al., 1997a). Due to the long half-life of this reservoir (∼44 months), it has been estimated that complete eradication with current treatment protocols would take over 60 years (Finzi et al., 1999). Multiple studies have resulted in the recovery of replication-competent HIV-1 from cells isolated from HIV-positive patients after years of virally-suppressive HAART, confirming the stability of the reservoir (Chun et al., 1997b; Finzi et al., 1997, 1999; Wong et al., 1997) [reviewed in Blankson et al., 2002].

The inability of combination drug therapy to target HIV-1 reservoirs has inspired the search for novel strategies to overcome the obstacle to virus eradication. One approach to eradication of the latent reservoir is induction therapy, whereby cells harboring latent virus are activated and therefore initiate viral replication. Previous induction strategies include treatment with cytokines (Chun et al., 1998; Scripture-Adams et al., 2002; Wang et al., 2005), caspase inhibitors (Scheller et al., 2002), the phorbol ester Prostratin (Biancotto et al., 2004; Korin et al., 2002; Kulkosky et al., 2001), deacetylase inhibitors (Demonte et al., 2004; Lehrman et al., 2005; Ylisastigui et al., 2004), and recombinant HIV-1 proteins (Fujinaga et al., 1995; Kinter et al., 2003; Lin et al., 2003; Tobiume et al., 2002).

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A novel approach to induction of latent HIV-1 is the *Herpesvirus saimiri* (HVS) protein StpC (saimiri transformation-associated protein subgroup C). HVS is a gamma-herpesvirus which persists in its natural host, the squirrel monkey, without disease symptoms, but causes acute lymphoma in other non-human primates [reviewed in Fickenscher and Fleckenstein, 2001]. Human T cells transformed by HVS exhibit continuous growth in culture in the absence of stimuli while retaining many properties of normal T cells, making them a useful system for studying T cell biology (Biesinger et al., 1992; Broker et al., 1993) [reviewed in Tsygankov, 2005]. These transformed cells were also found to support productive infection with HIV-1 (Nick et al., 1993) and therefore provide a unique model for the study of HIV-1. Transformation of T cells by HVS results from the combined expression of two proteins that are unique to this herpesvirus, StpC and Tip (tyrosine kinase interacting protein) (Duboise et al., 1998), the former of which has been shown in our laboratory to enhance HIV-1 replication when ectopically expressed in lymphocytes and the human T cell line MOLT 4 (Henderson et al., 1999; Raymond et al., 2004). It is of interest to note that several other members of the gamma-herpesvirus family, including human herpes virus 8 (HHV-8) and Epstein-Barr virus (EBV), also encode proteins which modulate HIV-1 replication (Caselli et al., 2003, 2005; Pati et al., 2003; Romano et al., 1997). StpC has been shown previously to associate with TRAFs (Lee et al., 1999; Sorokina et al., 2004), bind the signaling molecule Ras (Jung and Desrosiers, 1995), and activate NF-κB (Lee et al., 1999; Merlo and Tsygankov, 2001; Sorokina et al., 2004). NF-κB has been shown to be important in HIV-1 transcription and induction from latency (Brooks et al., 2003; West et al., 2001), making StpC a potential candidate for induction therapy.

It has been suggested that induction of virus replication in latently infected cells will likely lead to cell death in most cases due to cytopathic effects of virus production, and presumably when used in conjunction with HAART new infection of other cells will be prevented (Chun et al., 1998). However, it seems possible and even likely that, based on the proposed model for establishment of the latent reservoir (Blankson et al., 2002), proliferation of infected cells may result in some cells reverting back to resting state, in which case the problem of a latent reservoir has not been resolved. We have constructed an induction vector containing a suicide gene in order to kill activated cells that are infected with HIV-1, therefore eliminating the possibility of reversion to resting state. Suicide genes code for proteins that alone have little or no effect on the host cell, but upon introduction of an otherwise innocuous pro-drug the gene product metabolizes the pro-drug to a toxic compound. A well-characterized examples of this approach is the herpes simplex virus (HSV) type 1 thymidine kinase (TK) gene (Moolten, 1986). The HSV *tk* gene product is an enzyme that phosphorylates nucleoside analogues such as ganciclovir (GCV) allowing further phosphorylation by cellular kinases and subsequent incorporation into growing DNA chains, which results in double stranded DNA breaks and apoptosis (Tomicic et al., 2002). Ganciclovir has been used extensively for treatment of cytomegalovirus (CMV) infections, blocking virus replication by incorporation in virus DNA by viral DNA polymerase. Ganciclovir triphosphate is also a substrate for cellular DNA polymerase and can be incorporated into host cell DNA during replication, initiating apoptosis in the cell (Thust et al., 2000; Tomicic et al., 2002). The use of suicide vectors as a potential treatment for HIV-1 infection has been previously considered: human $CD4^+$ cell lines H9, CEM, U937, and HUT-78 engineered to express the HSV-TK gene under the transcriptional control of the HIV-1 long terminal repeat (LTR) promoter undergo cell death upon infection with HIV and subsequent exposure to GCV or ACV (Caruso and Klatzmann, 1992; Miyake et al., 2001). Full length transcription from the HIV-1 LTR is significantly reduced in the absence of Tat (Lassen et al., 2004; Miyake et al., 2001), therefore, placing the *tk* gene under the LTR promoter permits the gene to be expressed primarily in cells with actively replicating HIV-1, allowing selective killing (Caruso and Klatzmann, 1992; Marcello and Giaretta, 1998; Miyake et al., 2001).

The potential utility of suicide vectors for eliminating latently infected cells has not been addressed. We have constructed a vector containing StpC under the control of the CMV immediate early promoter and HSV TK under the control of the HIV-1 LTR promoter. We show that upon expression in latently infected cell lines StpC induced virus replication which in turn activated the expression of the suicide gene and sensitized the transduced cells to treatment with GCV.

2. Materials and methods

2.1. Cell lines and culture

J1.1 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: J1 from Dr. Thomas Folks (Perez et al., 1991). J1.1 cells are a latently infected cell line in which latency is maintained at the transcriptional level, originally cloned by limiting dilution from Jurkat cells infected with the LAV strain of HIV-1 (Butera et al., 1994; Perez et al., 1991). J1.1 cells and Jurkat cells (ATCC, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% FBS, PenStrep (l00 U/ml and 100 μg/ml), and gentamicin (10 μg/ml). 293T cells (ATCC, Manassas, VA) were cultured in DMEM supplemented as above for RPMI 1640. Cell cultures were incubated at 37 °C with 5% CO_2.

2.2. Preparation of vectors

The pStpC-TK transfer plasmid was prepared by removing the *tk* gene from pMOD-TK:Sh (Invivogen, San Diego, CA) by restriction enzyme digestion and inserting into pCE, which has been described previously (Hasham and Tsygankov, 2004). The StpC-PGK-Pac cassette was transferred from pCSPP (Hasham and Tsygankov, 2004) and inserted following the CMV promoter. The resulting plasmid was designated pStpC-TK (CMV-*stpC*-LTR-*tk*) and contains a puromycin *N*-acetyl transferase gene under the transcriptional control of the PGK promoter to allow for selection of transduced cells by culture in medium containing 0.5–1.0 μg/ml puromycin.

2.3. Transfection and transduction of cell lines

Lentiviral vectors were produced as described previously (Hasham and Tsygankov, 2004; Naldini et al., 1996). This transduction protocol has been shown to yield high transduction efficiency in Jurkat cells (Hasham and Tsygankov, 2004). Briefly, a packaging plasmid encoding HIV *gag-pol* and *rev* under the control of the CMV promoter and an envelope plasmid encoding the vesicular stomatitis virus glycoprotein (VSV G) under the control of the CMV promoter were transfected along with the pStpC-TK transfer plasmid into 293T cells using ProFection calcium phosphate (Promega, Madison, WI). Supernatants were removed 60 h after transfection, passed through a 0.4 μm filter, and added immediately to J1.1 cell cultures. Twenty-four hours post-transduction J1.1 cultures were replated in medium containing puromycin to select transduced cells. Previous results have shown that viability of untransduced J1.1 cells cultured in medium containing 1 μg/ml puromycin is reduced to <5% in less than 7 days, compared to J1.1 cells cultured in medium alone, which remained 96–100% viable (data not shown). Unless otherwise noted, all transduced cells were cultured in selection medium for a minimum of 5 days before use in experiments, and transduced lines were propagated in selection medium.

2.4. Quantitation of cell number, HIV-1 p24, and apoptosis

Total number of live cells was determined by trypan blue exclusion. HIV-1 p24 antigen was quantitated using the HIV-1 p24 antigen capture assay (SAIC, Frederick, MD). Apoptosis of cells cultured in GCV was determined using an ELISA protocol described previously (Salgame et al., 1997). This assay detects cytoplasmic nucleosomes which are released from the nucleus following DNA fragmentation, an early event in apoptosis. Capture antibodies (murine antibody specific for the N-terminus of histone H2B) and biotinylated detection antibodies (murine antibody specific for the nucleosome subparticle composed of histones H2A, H2B, and DNA) for the apoptosis ELISA were kindly provided by Dr. M. Monestier (Temple University, Philadelphia, PA) and Dr. P. Salgame (University of Medicine and Dentistry of New Jersey, Newark, NJ).

2.5. PAGE and Western blot analysis

Total protein was isolated from transduced cell cultures and 25 μg was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. StpC and GAPDH were detected by polyclonal antibodies and HRP-conjugated secondary antibodies (Santa Cruz Biosciences, Santa Cruz, CA), and developed using ECL Plus Detection Reagent (Amersham Biosciences, Piscataway, NJ).

2.6. Infection of Jurkat cells

Supernatants from J1.1-StpC-TK cells cultured with and without 500 μM GCV for 96 h were passed through a .45 μm filter to remove any cells and used to infect Jurkat cells that had been activated with TNF-α (2.5 ng/ml) for 24 h. After 4 h the Jurkat cells were centrifuged and the culture medium aspirated to remove any unbound virus and GCV in the supernatant. Jurkat cells were plated in fresh medium and incubated for 96 h, at which point level of p24 in cell culture supernatants was determined and DNA was isolated from the cell cultures.

2.7. PCR and real-time PCR

DNA was isolated from cell cultures using DNeasy Tissue Kit (Qiagen, Valencia, CA). Real-time PCR was used to quantitate HIV-1 and β-globin sequences. Reactions were completed in a LightCycler (Roche, Mannheim, Germany) with 30 ng DNA, 400 nM final concentration each primer (HIV-1: IDT, Coralville, IA; β-globin: Sigma Genosys, The Woodlands, TX), 10 μl SYBR Green JumpStart Taq Ready Mix (Sigma, St. Louis, MO) and water to a total volume of 20 μl. Cycling conditions for HIV-1 primers were 95 °C for 3 min, followed by 45 cycles of 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 10 s with a single fluorescence acquisition. Cycling conditions for β-globin primers were the same except for the annealing temperature which was 62 °C. HIV-1 primer sequences were as follows: HIV-1 sense 5′-TTg Tgg CAA AgA Agg gCA CAT AgC-3′, HIV-1 antisense 5′-Tgg CTC Tgg TCT gCT CTg AAg AAA-3′. β-Globin primer sequences were published previously (Cole et al., 2001).

2.8. Statistical analysis

Statistical significance of differences between treated samples and untreated controls was determined using Student's *t*-test. *p*-Values were calculated for two tails. Error bars represent the standard deviation of calculated means. Asterisks represent a statistically significant change in experimental sample value compared to control ($^{*}p < .05$, $^{**}p < .01$, $^{***}p < .005$). All results shown are representative of three separate experimental replicates with the exception of Fig. 4B, which is representative of two separate experimental replicates.

3. Results

3.1. StpC reactivates J1.1 cells from latency

We have shown previously that StpC expression enhances HIV-1 replication (Henderson et al., 1999; Raymond et al., 2004), and therefore we wanted to determine if StpC is a feasible candidate for induction therapy. J1.1 cells were transduced with a vector coding for StpC under the control of the CMV promoter and HSV TK under the control of the HIV-1 LTR promoter, resulting in the cell line J1.1-StpC-TK. The transfer plasmid used for production of the lentiviral vector is represented in Fig. 1. HIV-1 p24 was upregulated approximately 1.4- to 2.3-fold in J1.1-StpC-TK cells (Fig. 2) illustrating the ability of StpC to activate HIV-1 in latently infected cell lines.

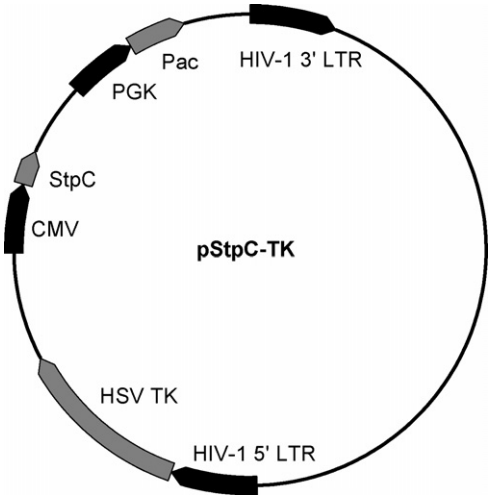

Fig. 1. Diagram of the pStpC-TK transfer plasmid. HSV TK expression is under the transcriptional control of the HIV-1 LTR promoter, and StpC under the CMV promoter. The PGK-Pac cassette allows selection of transduced cells in puromycin.

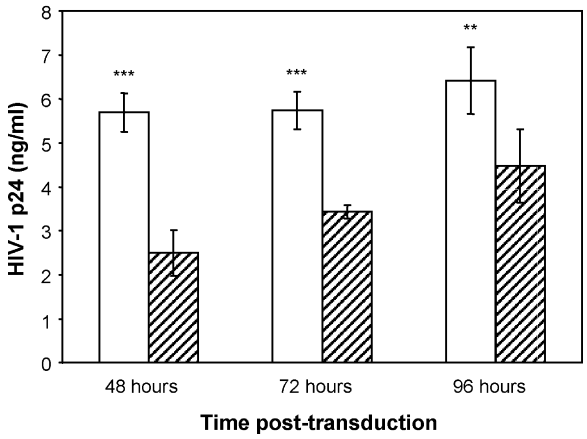

Fig. 2. Transduction with StpC-TK reactivates HIV-1 in J1.1 cells. J1.1 cells were transduced with a lentiviral vector containing pStpC-TK. HIV-1 p24 in cell culture supernatants of J1.1-StpC-TK cells (open bars) and J1.1 cells (hatched bars) was quantitated 48, 72, and 96 h post-transduction by ELISA. $^{**}p < .01$, $^{***}p < .005$.

3.2. GCV inhibits proliferation and reduces HIV-1 p24 in transduced cells

In order to determine the effect of GCV on proliferation and HIV-1 p24 production in transduced cells, J1.1-StpC-TK cells were plated in medium containing various concentrations of GCV. After 96 h in culture, J1.1-StpC-TK cells showed reduced proliferation at all GCV concentrations above 25 μM (Fig. 3A). Cell number of J1.1-StpC-TK cells cultured in 500 μM GCV was decreased by approximately 50% compared to J1.1-StpC-TK cells cultured in medium alone. In contrast, J1.1 cells exhib-

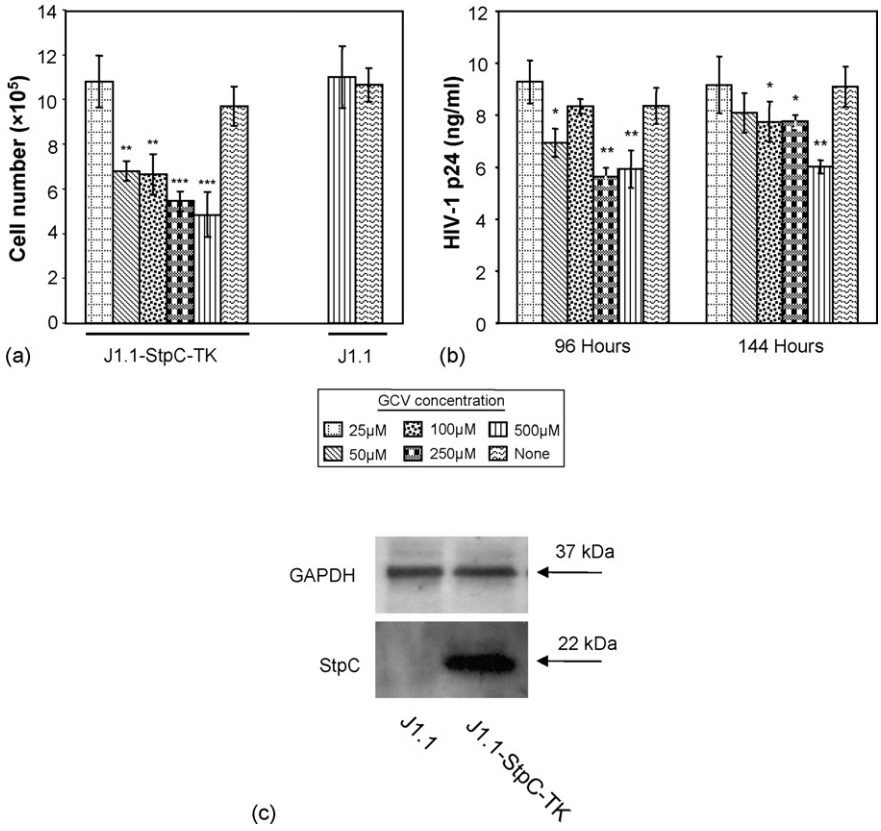

Fig. 3. GCV inhibits proliferation and HIV-1 p24 production in transduced cells. J1.1 and J1.1-StpC-TK cells were plated at 4×10^5 cells/ml in increasing concentrations of GCV. (A) Cell number of transduced and control cells was determined by trypan blue exclusion 96 h after plating. (B) HIV-1 p24 was quantitated in supernatant of J1.1-StpC-TK cells cultured in the presence of GCV for 96 and 144 h. (C) Whole cell lysates of transduced and parental J1.1 cells were separated by SDS-PAGE and StpC and GAPDH were detected by Western blot. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .005$.

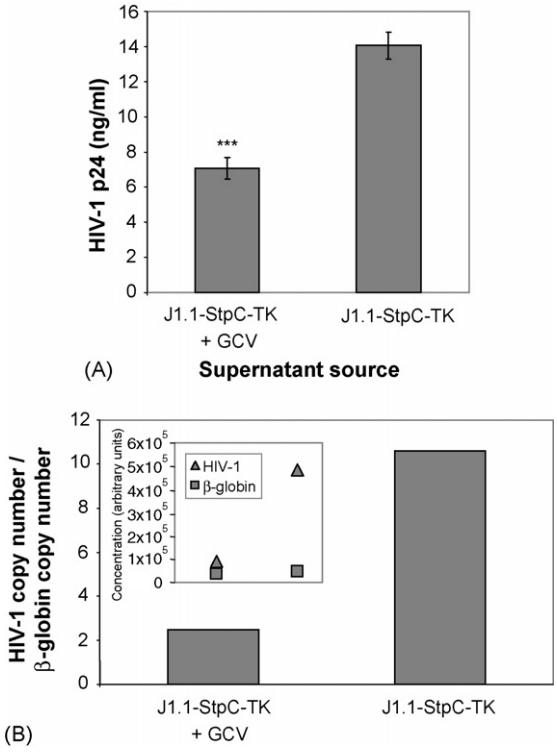

Fig. 4. J1.1-StpC-TK cells have reduced HIV-1 production when cultured in the presence of GCV. (A) HIV-1 p24 in cell culture supernatants of infected Jurkat cells was quantitated 96 h after infection. $^{***}p < .005$. (B) Real-time PCR analysis of ratio of HIV-1 to β-globin sequences and calculated copy concentration (inset) in infected Jurkat cells.

ited no alteration in proliferation at the highest concentration of GCV at this time point (Fig. 3A). By 96 h HIV-1 p24 concentration was reduced by approximately 25% in the two highest concentrations of GCV, and by 144 h p24 concentration was reduced in the three highest concentrations of GCV (Fig. 3B). This reduction was over 30% in cells cultured in 500 μM GCV. StpC expression in transduced cells was verified by Western blot (Fig. 3C).

3.3. HIV-1 virus production is reduced in transduced cell cultures containing GCV

In order to confirm that the reduction of p24 corresponded to a reduction in the number of infectious virions produced, activated Jurkat cells were infected with filtered supernatants from J1.1-StpC-TK cells cultured with and without GCV. Jurkat cells infected with supernatants from J1.1-StpC-TK cells cultured in medium containing GCV showed a 50% reduction in p24 production compared with those infected with supernatants from J1.1-StpC-TK cells cultured in medium alone (Fig. 4A). Activated Jurkat cells mock-infected with medium alone had no p24 in the culture supernatant (data not shown). These results were confirmed by real-time PCR analysis comparing the ratio of HIV-1 to β-globin sequences in the DNA of infected Jurkat cells (Fig. 4B). We would expect that the ratio of HIV-1 sequences to β-globin sequences would be lower in Jurkat cells that were infected with supernatant from J1.1-StpC-TK cells cultured in

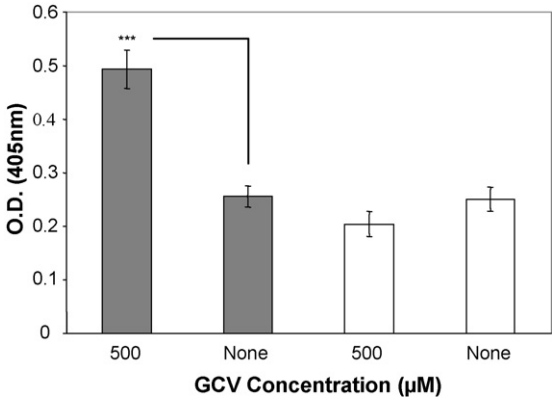

Fig. 5. GCV induces apoptosis in transduced cells with no effect on control cells. 4×10^5 J1.1-StpC-TK cells (shaded bars) or J1.1 cells (open bars) were plated in media with or without 500 μM GCV and incubated for 96 h. 4×10^5 cells from each sample were then analyzed for apoptosis by ELISA. $^{***}p < .005$.

GCV compared to those infected with supernatant from J1.1-StpC-TK cells cultured in medium alone. We observed a four-fold decrease in HIV-1 sequences per β-globin sequence in the Jurkat cells infected with supernatants from J1.1-StpC-TK cells cultured in medium containing GCV compared to those infected with supernatant from Jl.l-StpC-TK cells cultured in medium alone, confirming the decrease in infectious virions produced when J1.1-StpC-TK cells are cultured GCV.

3.4. Ganciclovir induces apoptosis in J1.1-StpC-TK cells

GCV has been shown to initiate apoptosis in CHO cells expressing TK (Tomicic et al., 2002), therefore we wanted to ascertain if induction of apoptosis occurred in J1.1-StpC-TK cells cultured in GCV. Transduced cells cultured in GCV for 96 h were analyzed for apoptosis using an ELISA assay described previously (Salgame et al., 1997). J1.1-StpC-TK cells cultured with 500 μM GCV exhibited a nearly two-fold increase in apoptosis compared to background of those cultured in medium alone (Fig. 5). However, J1.1 cells cultured in identical concentrations of GCV had no increase in apoptosis compared to Jl.l cells in medium alone (Fig. 5).

4. Discussion

The ability of HIV-1 to form stable latent reservoirs presents a major obstacle to the eradication of the virus with current available therapy. Although the advancement of HAART has greatly reduced plasma viral load in many individuals, long-term treatment is associated with multiple side effects, and the latent viral reservoir is not affected. The resistance of these reservoirs to combination drug therapy has motivated studies of many induction strategies intended to activate virus replication. However, targeting reservoirs of latent HIV-1 has proven to be a formidable challenge. Although many agents have been shown to induce expression of latent HIV-1, their potential therapeutic benefit is unknown. Current in vitro therapeutic strategies targeting latent HIV-1 reservoirs could encounter problems when applied in vivo. Treatment with cytokine combinations may result in

immune complications associated with non-specific activation of large numbers of cells, both infected and uninfected. Also, the release of pro-inflammatory mediators in response to cytokine treatment is likely to lead to severe complications. It is hypothesized that after induction of latent HIV-1 infected cells will die from the cytopathic effects of virus production and, when used in combination with HAART, new infection of cells will be prevented. However, it seems possible and even likely that, as is observed in initial infection, some activated cells would not die from cytopathic effects, but would instead revert to a resting state, in which case the latent reservoir has not been eliminated. Proposed induction strategies thus far do not address these problems. The work presented here has focused on the construction of a vector designed to both induce latent reservoirs and sensitize them to drug therapy. Whereas systemic treatment with induction agents could lead to widespread non-specific activation of multiple cell types, gene-based induction could be designed for targeted expression in specific cell types.

Previous work in our laboratory has shown that StpC enhances HIV-1 replication during acute infection (Henderson et al., 1999; Raymond et al., 2004). Therefore, we wanted to evaluate the ability of StpC to induce HIV-1 replication in latently infected cells. We have constructed an induction vector containing *H. saimiri stpC* under the human cytomegalovirus promoter. In order to specifically eliminate these cells, we included a suicide gene, HSV *tk*, under the control of the LTR promoter. We hypothesized that upon StpC expression in latently infected cells induction of virus replication and the subsequent production of viral transactivators of the LTR will activate expression of the *tk* gene, thus sensitizing the cells to treatment with the nucleoside analogue GCV. Placing the suicide gene under the control of the LTR promoter limits initiation of apoptosis to infected cell populations expressing the suicide gene and cultured in the presence of the GCV, with minimal effects in uninfected populations or those cultured without GCV.

For our experiments we used J1.1 cells (Butera et al., 1994; Perez et al., 1991). These cells produce very low levels of HIV-1 in culture, but can be stimulated to produce high levels of virus by treatment with PMA or TNF-α. Perez et al. (1991) reported an approximately seven-fold increase in HIV-1 p24 production in response to incubation with 1000 U/ml TNF-α for 48 h. This concentration of TNF-α is higher than that used in other induction studies using primary cells (2.5 ng/ml) (Chun et al., 1998; Ghose et al., 2001), which may explain the magnitude of the increase. We have observed an approximately four-fold increase in p24 production in J1.1 cells in response to incubation with 50 U/ml (5 ng/ml) TNF-α for 48 h (data not shown). In the data presented here, we have shown that StpC can also induce HIV-1 replication from the latently infected cell line Jl.l, and that coordinated expression of a suicide gene can sensitize transduced cells to drug therapy.

J1.1-StpC-TK cells expressed a high level of StpC and exhibited increased HIV-1 replication compared to parental J1.1 cells. The addition of GCV to the culture medium of the J1.1-StpC-TK cells resulted in a reduction of HIV-1 p24 in culture supernatants, inhibition of cell proliferation, and increased apoptosis. The reduction of viability in J1.1-StpC-TK cells cultured in GCV as determined by trypan blue exclusion was between 30% and 50% depending on the concentration of GCV used, with the highest inhibition being observed at 500 μM GCV. Miyake et al. (2001) reported a 90% reduction in viability by MTT assay in H9, CEM, and U937 cells transduced with a LTR-TK vector, infected with HIV-1 and incubated in 10 μg/ml (39.18 μM) GCV for 5 days, with nearly complete loss of viability using 100 μg/ml (391.8 μM). The reduction observed in these cells compared to that which was observed in the J1.1-StpC-TK cells could be explained by differences in cell lines used: the transduced H9, CEM, and U937 cells were supporting productive HIV-1 infection at the time of the assay. Therefore, the level of viral proteins available to bind the LTR was likely higher in these cells than in J1.1-StpC-TK cells, even after induction. This hypothesis is supported by the observation that transduced H9 cells infected with HIV-1 mixed 1:1 with uninfected transduced H9 cells (total 2×10^5 cells/well) had approximately 75 ng/ml HIV-1 p24 at 48 h after plating (Miyake et al., 2001), compared to approximately 5.75 ng/ml observed in J1.1-StpC-TK cells (plated at 5×10 cells/well) at the same time point (Fig. 2). As the process of cell death by TK phosphorylation of GCV is dual-dose dependent, meaning it is dependent both on the amount of TK being expressed and the concentration of nucleoside analogue used (Borrelli et al., 1988; Caruso and Klatzmann, 1992), the increased level of TK in the H9 cells renders them sensitive to lower levels of GCV than if less TK was present.

Previous reports have shown that the HIV-1 LTR contains binding sites for many cellular factors (Karn, 1999; Pereira et al., 2000); therefore, the possibility of activation of the LTR by host cell transactivators in the absence of virus reactivation must be considered. Although it has been shown that the LTR promoter has some basal transcription in the absence of Tat (Caruso and Klatzmann, 1992), multiple studies have reported that transcription from the HIV-1 LTR in the presence of Tat is dramatically increased (Caruso and Klatzmann, 1992; Lin et al., 2003; Marcello and Giaretta, 1998; Muesing et al., 1987; Rosen et al., 1985) [reviewed in Karn, 1999], as high as 100-fold in the lymphoid cell line HUT-78 stably expressing an LTR-TK construct after infection with a high titer of HIV-1 (Caruso and Klatzmann, 1992). Marcello and Giaretta (1998) observed that TK expression from a suicide vector in the fibroblast cell line COS-1 increased 3.3-, 5-, and 7-fold when co-transfected with a vector coding for HIV-1 Rev, Tat, or both, respectively. Since the toxicity of the TK-GCV suicide system is dual dose-dependent, the low level of TK expression resulting from basal transcription will likely phosphorylate a level of GCV that is non-toxic to cells. Miyake et al. (2001) used a LTR-driven TK suicide vector in various cell lines as a prospective prophylactic for productive HIV infection and observed no difference in GCV-mediated growth inhibition in the absence of HIV-1 infection between cells transduced with a LTR-TK construct versus an empty LTR construct. These results support the idea that in the absence of viral transactivators of the LTR, any basal transcription from the LTR produces only a negligible level of TK. Therefore, the concentration of GCV that is toxic to cells in which the LTR has been transactivated by Tat will likely be too low to have a toxic effect on cells expressing only a basal level of the suicide gene.

The vector model described here could be modified to decrease potential basal activity and to increase cell specificity. One method to achieve this would be by mutating specific transcription factor binding sites in the LTR. Specific alterations to the NF-κB binding sites in the LTR promoter reduce basal activity while retaining a high degree of transactivation in the presence of Tat (Brady et al., 1994). The two NF-κB binding motifs have been shown to be crucial for Tat-independent expression from the LTR in many cells types, including unstimulated lymphoid cells (Perkins et al., 1993). Recent work in our laboratory has confirmed these observations, demonstrating that alterations in the NF-κB binding sites in the HIV-1 LTR greatly reduce basal transcription from reporter constructs in the absence of Tat (Raymond et al., in press).

The addition of a Rev response element (RRE) and splice donor and acceptor sites flanking the TK gene could increase the sensitivity of the vector to the presence of the HIV protein Rev. Cellular restrictions hinder the transport of incompletely spliced mRNAs from the nucleus. Early in the course of HIV-1 replication prior to the expression of the viral protein Rev, only fully spliced viral mRNAs (one of which codes for Rev) are exported to the cytoplasm. Once Rev is produced it enters the nucleus and binds the RRE on unspliced and singly-spliced viral mRNAs, allowing their export. Therefore, the addition of the RRE in front of *tk* could increase the transport of TK mRNA to the cytoplasm in cells where HIV-1 proteins are being produced. Chenciner et al. (1995) demonstrated increased activity of a LTR reporter construct flanked by a RRE and splice donor and acceptor sites when co-transfected with a plasmid encoding Rev. This alteration would increase the expression of TK in HIV-infected cells producing Rev, thus lowering the dose of GCV necessary to induce apoptosis and reducing the likelihood of side effects in uninfected cells.

The vector design could also be modified to alter StpC expression. By replacing the CMV promoter driving the expression of StpC with the Lck promoter, we could limit expression of StpC to T cells (Browning et al., 1997; Wildin et al., 1991), the primary cellular reservoir for latent HIV-1. The use of cell type-specific promoters could also target the vector to other cell subsets that may serve as minor reservoirs of HIV-1, such as macrophages and NK cells.

Since StpC has been characterized as an oncoprotein its safety must be considered. It has been reported that the concerted expression of both StpC and the HVS protein Tip are necessary to induce transformation of T cells (Duboise et al., 1998). Although StpC has been shown to have high transformation potential in rat fibroblasts (Jung et al., 1991), multiple attempts to transform T cells by StpC expression have failed. Infection of marmosets in vivo and primary marmoset T cells in vitro with mutant forms of *H. saimiri* subgroup C strain 488 containing deletions in Tip resulted in no lymphomas or immortalization, respectively; however, all animals infected with wild-type virus developed lymphomas and 23 of 24 marmoset T cell cultures were immortalized (Duboise et al., 1998). Likewise, StpC-transgenic animals develop epithelial but not lymphoid tumors (Murphy et al., 1994). Human primary T cells transduced with the pCSPP vector resulted in stable StpC expression, but were not transformed (Hasham and Tsygankov, 2004). Therefore, the independent expression StpC in T cells should not result in transformation.

Although the vector described here did not achieve complete eradication of latently infected cells, even the modest killing observed could be utilized as an eradication strategy, since GCV treatment in vivo could be continued for a relatively long period of time compared to the in vitro studies presented here in which GCV treatment was carried out for only 7–10 days. Even if induction with a suicide vector only reduced the latently infected cell population by 20% with each treatment, the turnover rate of the latent reservoir could be greatly affected. In addition, the use of other inducers in combination with the suicide vector may have a synergistic effect, and could possibly be used at lower concentrations to minimize potential adverse side effects. Future studies will need to address the efficacy of the suicide vector when used in combination with other inducers.

These results show that vectors encoding *H. saimiri* StpC can be used to activate latent HIV-1, and the addition of HSV TK confers sensitivity to GCV in transduced cells. This prototype vector serves as a proof of concept of the utility of novel gene-based induction agents acting in collaboration with suicide genes as a new method for targeting cellular reservoirs of latent HIV-1.

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