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Possible applications for replicating HIV 1 vectors

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Abstract

Since its discovery some 25 years ago, much has been learned about HIV type 1 and the molecular details of its replication cycle. This insight has been used to develop lentiviral vector systems that have advantages over conventional retroviral vector systems. For safety reasons, the lentiviral vector systems are replication incompetent and the risk of generating a replication competent virus has been minimized. Nevertheless, there may be certain applications for replication competent HIV based vector systems, and we will review our activities in this particular field. This includes the generation of a conditionally replicating HIV 1 variant as a safe live attenuated virus vaccine, the construction of mini HIV variants as cancer selective viruses for virotherapy against leukemia, and the use of a conditionally live anti HIV gene therapy vector. Although safety concerns will undoubtedly remain for the use of replication competent HIV based vector systems, some of the results in cell culture systems are very promising and warrant further testing in appropriate animal models.

Keywords

HIV; RNAi; vaccine; vector; virotherapy

Conditionally live HIV 1 as AIDS vaccine

Live-attenuated virus vaccines have proven to be very successful at inducing protective immunity against pathogenic viruses such as those causing smallpox, polio and measles. Research on the development of a live-attenuated HIV/AIDS vaccine has predominantly been performed in the experimental model system in which macaques are infected with pathogenic simian immunodeficiency virus (SIV). In most of these studies, several accessory functions have been deleted from the viral genome, either individually or in combination (reviewed in [1–4]). The majority of monkeys vaccinated with such deletion mutants of SIV can efficiently control replication of pathogenic challenge virus strains. However, the attenuated virus could revert to virulence and cause disease over time in vaccinated animals [5–9]. Similarly, some of the long-term nonprogressors of the Sydney Blood Bank Cohort infected with an HIV-1 variant in which *nef* and long terminal repeat (LTR) sequences were deleted eventually showed progression to AIDS [10], and an HIV-1 $\Delta 3$ variant with deletions in the *vpr*, *nef* and LTR sequences regained substantial replication capacity in long-term cell culture infections by acquiring compensatory changes in the viral genome [11]. These results highlight the genetic

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instability and evolutionary capacity of attenuated SIV/HIV strains, which poses a serious safety risk for any future experimentation with live-attenuated HIV vaccines in humans.

The major safety problem with live-attenuated HIV/SIV vaccines is caused by the persistence of the attenuated virus in combination with the ongoing low-level replication. Due to the error-prone replication machinery of the virus, this may eventually lead to the appearance of fitter and more pathogenic virus variants. To improve safety, the vaccine strain can be further attenuated through additional deletions or mutations in accessory genes or regulatory elements, which will reduce the pathogenicity of the virus. However, this will also reduce the vaccine efficacy of the virus [12,13]. As an alternative strategy to prevent evolution toward a pathogenic variant, replication of the vaccine virus should be limited to the extent that is needed to provide full protection. For instance, virus replication can be stopped upon vaccination by administration of antiviral drugs [14]. Whereas this is a good strategy for *in vitro* studies, application in humans seems problematic because long-term virus inhibition will require continuous drug administration, and the virus may develop drug resistance. Alternatively, a virus that can execute only a single round of replication can be used as vaccine [15–18]. However, due to the limited replication, such a single-cycle virus vaccine may be less potent for the induction of protective immunity.

We and others previously presented an approach that uses a conditionally live HIV-1 virus [19–23]. HIV gene expression and replication are naturally controlled by the viral Tat protein that binds to the 5' *trans*-acting responsive (TAR) region in the nascent RNA transcript to enhance transcription [24]. For the construction of a conditionally live HIV variant, this Tat–TAR regulatory mechanism was inactivated by mutation and functionally replaced by the doxycycline (*dox*)-inducible gene expression system (Tet-On system) [25]. This *E. coli*-derived gene expression system is controlled by the rtTA protein. Binding of *dox* to the rtTA protein triggers a conformational switch that allows binding to *tet* operator (*tetO*) elements and activation of transcription from the downstream-positioned promoter. To replace the Tat–TAR axis with the Tet-On system, we modified the HIV genome by: inactivation of Tat and TAR through mutation, introduction of *tetO* elements in the U3 promoter region and introduction of the *rtTA* gene at the *nef* gene locus (HIV-rtTA in Figure 1). Transcription of the resulting HIV-rtTA variant is thereby no longer activated by binding of Tat to TAR, but instead by binding of the *dox*–rtTA complex to the *tetO*-LTR promoter. As a result, the HIV-rtTA variant replicates exclusively when *dox* is administered. Upon vaccination with this virus, replication can be temporarily activated by transient *dox* administration to the extent needed for induction of the immune system. The initial HIV-rtTA construct has been improved significantly by virus evolution [26–32], and we have shown efficient and *dox*-dependent virus replication not only *in vitro* in T-cell lines, but also *ex vivo* in human lymphoid tissue [33].

In addition, we constructed an HIV-1 variant that depends not only on *dox* for gene expression, but also on the T20 peptide for cell entry [34]. T20 (Fuzeon®) is a 36-mer peptide that mimics part of the HR2 domain of the envelope gp41 protein (Env-gp41), which is intrinsically involved in the fusion of the viral and cellular membranes [35]. T20 inhibits viral entry because it prevents the formation of the 6-helix bundle structure in Env-gp41 during fusion. We were able to isolate T20-resistant HIV-1 variants, but also a T20-dependent virus from a patient on T20 therapy [36]. This latter variant carries two mutations in Env-gp41 that cause the premature formation of the 6-helix bundle, which hampers cell entry. Further analysis revealed that the T20 peptide can rescue this hyperfusogenic Env protein by preventing the premature conformational switch, thus restoring virus infectivity and replication [37]. Introduction of the Env-gp41 mutations in HIV-rtTA resulted in a virus that replicates exclusively in the presence of both *dox* and T20 [34]. Hence, replication of this vaccine strain can be restricted to the level and time window required for induction of protective immunity by transient administration of

dox and T20. Subsequent withdrawal of these inducers efficiently blocks viral replication and prevents evolution.

Mini HIV as vector for leukemia virotherapy

The use of live viruses as a strategy to cure cancer has been investigated for more than a century [38]. This virotherapy aims to remove the cancer from the patient by the selective replication of oncolytic viruses in malignant cells, which should result in the specific destruction of these cells [39,40]. At the start of therapy, the presence of a large number of cancerous target cells will allow a fast-spreading viral infection, which will result in a rapid decrease in the number of target cells and a concurrent vanishing of the therapeutic virus. Over the past decade, several therapeutic viruses have been tested in humans and, although significant advances have been made, safety and efficacy issues remain [41,42]. For example, the virus should replicate exclusively in the malignant cells, yet be minimally toxic to their normal counterparts, and the virus must spread to all target cells before the infection is controlled by the immune system. Most described virotherapy strategies have focused on solid tumors using a large variety of viruses, either natural isolates or genetically modified variants [42].

We explored the possibility to use viruses based on HIV-1 strains that use CD4 and CXCR4 for cell entry as a therapeutic virus against malignancies such as T-lymphoblastic leukemia/lymphoma, natural killer-cell leukemia and some myeloid leukemias [43]. Our approach is based on the observation that several accessory viral proteins are not needed for HIV-1 replication in transformed T-cell lines, yet are important for virus replication in primary cells. A minimized derivative of HIV-1 with five gene deletions (Δvif , vpR , vpU , nef and $U3$; HIV- $\Delta 5$ in Figure 1) was demonstrated to replicate in several leukemic T-cell lines and to induce cell death. This minimized HIV variant does not replicate in normal peripheral blood mononuclear cells, not even when the peripheral blood mononuclear cells were co-cultured with the susceptible SupT1 T-cell line that continuously produced new infectious virus particles. The mini-HIV variant is thus able to efficiently remove leukemic cells from a mixed culture with untransformed cells.

We combined the mini-HIV approach with the dox-controllable HIV-rtTA approach [19]. This mini-HIV-rtTA variant [44] replicates efficiently in leukemic T-cell lines and, as expected, virus replication is strictly dependent on dox addition. In a therapeutic setting, the minimized virus can be used to target the leukemic cells in the presence of dox. This will result in a self-limiting viral infection since the target cells are killed by the virus. Withdrawal of dox provides an additional safety feature to block ongoing replication after the leukemic cells are removed. The mini-HIV virotherapy approach may benefit from the incorporation of a therapeutic RNAi cassette, for example expressing an siRNA against an oncogene. Elimination of all cancer cells will likely require the combined action of the therapeutic virus and the cellular immune system.

Replicating HIV vector that inhibits wild type HIV 1 through RNAi

dsRNA can induce sequence-specific gene silencing via a process known as RNAi [45,46]. The effector molecules of this evolutionarily conserved mechanism are siRNAs of approximately 22 nt [47–49]. Transfection of siRNAs into cells has proven to be a powerful tool to transiently suppress gene expression [48]. Stable expression of shRNAs, which are processed into effective siRNAs by the ribonuclease Dicer, can result in long-term gene suppression [50,51]. We and others have shown that shRNA-mediated RNAi against HIV-1 potently inhibits virus replication and makes cells resistant to HIV-1 [52–58]. Thus, RNAi shows potential as a means to achieve intracellular immunization against HIV-1.

The development of an RNAi-based gene therapy against HIV requires the stable transduction of T cells or hematopoietic blood stem cells with a vector expressing anti-HIV shRNAs. At

present, the HIV-1 based lentiviral vector system is preferred for this objective. This procedure requires that cells are isolated for each patient, followed by *ex vivo* transduction and reintroduction of the transduced cells in the infected individual. The complexity and cost of this gene therapy procedure forms a major bottleneck to perform this procedure with large populations of infected individuals. As an alternative, less elaborate method, we recently explored the potential of the HIV-rtTA variant as a replicating vector for the efficient delivery of anti-HIV shRNAs [59]. We introduced an RNA polymerase III-driven shRNA cassette into the HIV-rtTA genome (HIV-rtTA-shRNA^{nef} in Figure 1). The shRNA targets the viral *nef* sequence, which is present in wild-type HIV-1 but not in the HIV-rtTA vector where the *nef* gene has been replaced by the *rtTA* gene. A spreading infection of this therapeutic HIV-rtTA-shRNA^{nef} variant in all HIV-susceptible cells can be controlled by transient dox treatment. Subsequent dox withdrawal generates cells containing a silent integrated provirus with a constitutively active shRNA^{Nef} expression cassette. As a result, cells are harnessed with shRNAs that efficiently inhibit replication of wild-type HIV-1. This strategy seems particularly suitable for patients infected with a multidrug-resistant virus that can no longer be treated with the current antivirals.

The HIV-rtTA-shRNA^{nef} variant may allow interesting combinations of vaccination and RNAi-inhibition strategies. When used as prophylactic vaccine, the RNAi cargo of this virus will protect all infected cells against a future exposure to HIV-1, thus boosting vaccine protection. When used as a therapeutic virus, the vaccine effect may boost the RNAi-mediated virus inhibition. To prevent unwanted off-target effects of the anti-HIV shRNA, the vector can be improved by imposing conditional shRNA expression, for example with a promoter that is activated only upon HIV-1 infection by the viral Tat protein [60]. Furthermore, to preclude silencing of shRNA expression by flanking chromosomal sequences at the site of integration, the shRNA cassette can be surrounded by insulator elements [61,62].

Future perspective

Testing the safety & efficiency of replicating vectors in animal models

Obvious safety concerns and regulatory issues remain for the development of replicating vectors based on the human pathogen HIV-1. One of the major concerns is that even attenuated HIV-1 variants will cause a chronic infection. This fact, combined with the high mutation and recombination rate of HIV-1, may result in the generation over time of variants with altered replication characteristics. However, the dox-controlled HIV-rtTA variant will cause a latent infection upon dox withdrawal, with silent integrated proviruses that will less likely contribute to ongoing virus evolution. Another concern is that the vector may integrate near the 5'-end of a proto-oncogene. In this position, the viral LTR promoter or enhancer elements may activate proto-oncogene expression, which could result in cell proliferation and ultimately cause cancer. In fact, insertional oncogenesis has been observed in X-linked severe combined immune deficiency and X-linked chronic granulomatous disease patients who received gene therapy with a γ -retroviral vector [63–65]. However, whereas γ -retroviruses favor integration near transcription start sites, HIV-1 favors integration within actively transcribed genes [66,67]. The idea that lentiviral vectors are safe for use in gene therapy is supported by the results of the first clinical trials. Levine *et al.* introduced a lentiviral vector that expresses an antisense HIV-1 *env* gene in the CD4⁺ T cells of five HIV-infected individuals who failed on at least two antiviral regimens [68]. Cartier *et al.* used a lentiviral vector to genetically correct the CD34⁺ hematopoietic stem cells of two X-linked adrenoleukodystrophy patients [69]. In both studies, there was no indication for abnormal expansion of cells due to vector-mediated insertional activation of proto-oncogenes in the approximately 4 and 2 years of follow-up, respectively [68–70]. The risk of insertional oncogenesis can be further reduced by minimizing the transcriptional activity of the LTR promoter. For this reason, so-called ‘self-inactivating’ LTRs, in which strong transcription-promoting elements have been removed, are present in

the third-generation lentiviral vectors [71]. The tetO-LTR promoter in our HIV-rtTA vectors is inactive upon dox withdrawal, which will similarly reduce the risk of activation of adjacent genes.

We have extensively studied the evolutionary possibilities of HIV-rtTA in long-term cultures and never observed a reversion to the Tat-TAR mechanism of transcription control. The introduced mutations in Tat and TAR, and the components of the Tet-On system, which are essential for virus replication, were stably maintained in the viral genome. In fact, the virus acquired mutations in both the *rtTA* gene and the tetO elements that significantly improved replication [26–28,30]. The mutations in rtTA increased the transcriptional activity and dox sensitivity of this protein. Interestingly, the tetO mutations were found to reduce the background activity of the viral promoter in the absence of dox, which is important to prevent vector spread after dox withdrawal. Furthermore, the absence of background activity will prevent constitutive expression of the viral proteins, including rtTA. Otherwise, this could induce immune responses and cytotoxic effects that eventually result in the loss of transduced cells in protocols with the replicating RNAi vector. We have also observed specific mutations in rtTA that reduced dox control [29], and developed a novel rtTA variant that blocks this undesired evolutionary route [29,31]. As a next step, the genetic stability and immunogenicity of the HIV-rtTA variant will be tested in mice with a humanized immune system [72]. Furthermore, we recently constructed a similar dox-dependent SIV variant that is currently used to study the efficacy and safety of a conditionally live virus vaccine against AIDS in macaques [73]. In addition, this SIV variant may be an attractive tool to study the correlates of immune protection upon vaccination, because the level and duration of replication can be controlled by dox administration.

For the mini-HIV approach, it could be argued that repair of gene deletions would be impossible, but one cannot exclude alternative viral strategies that improve viral fitness or replication capacity. Such an indirect escape strategy has been reported for an HIV-1 vaccine candidate with three gene deletions [11]. Gradual improvement of viral fitness has also been reported for persons infected with a *nef*-deleted virus variant, coinciding with AIDS disease progression in some of these patients [74]. The mini-HIV-rtTA variant replicates exclusively in leukemic cells and in the presence of dox. Withdrawal of dox after the leukemic cells are removed introduces an additional block to continued replication and evolution of the virus. We plan to set up a T-cell acute lymphoblastic leukemia model in severe combined immunodeficiency mice to test the capacity of these therapeutic viruses to selectively remove leukemic cells *in vivo*.

RNAi is a highly sequence-specific process in which the siRNAs have to be complementary to the target sequence. As a consequence, HIV-1 can escape from RNAi inhibition through nucleotide substitutions or deletions in the target sequence [52,53,55]. The use of a combinatorial shRNA therapy, in which multiple conserved viral RNA sequences are simultaneously targeted by multiple shRNAs, may block the emergence of RNAi-resistant variants [75–78]. When multiple shRNA-expression cassettes are inserted in the HIV-rtTA genome, it may be necessary to use different polymerase III promoters to avoid recombination at the repeated sequences [77]. Alternatively, one could use extended shRNAs or miRNA clusters that encode multiple siRNAs [79–81]. In general, the anti-HIV siRNAs should be carefully chosen such that they do not target the HIV-based replicating vector [82]. When such complementarity is present, it may be necessary to modify the target sequence in the vector without interfering with its replication capacity.

HIV-1 escape from RNAi-mediated inhibition can also be prevented by targeting cellular functions that are essential for viral replication. For example, the HIV-rtTA variant can be used to harness cells with shRNAs that target the CCR5 coreceptor for HIV-1 entry. Several

observations indicate that knockdown of CCR5 may indeed provide an effective therapy for HIV-infected individuals. First, it has been shown that siRNA- or shRNA-induced targeting of CCR5 does inhibit HIV-1 replication in cell culture [83–90]. Second, individuals who are homozygous for the CCR5 Δ 32 allele that prevents CCR5 cell surface expression are resistant to HIV-1-infection, but otherwise apparently normal [91–94]. Third, it was recently described that HIV-1 replication was efficiently suppressed in an HIV-1 infected patient who had undergone allogeneic stem cell transplantation with complete replacement of his stem cells with CCR5 Δ 32/ Δ 32 CD34⁺ peripheral stem cells [95]. Targeting of the cellular CCR5 protein does not interfere with our approach, as CCR5 will be knocked-down after the cells have been transduced with the HIV-rtTA-shRNA^{CCR5} vector. The humanized immune system mouse model again seems to be the appropriate system to test this HIV-based replicating vector strategy against HIV-1 [58].

Executive summary

Conditionally replicating HIV-1 variant as a safe live-attenuated virus vaccine

- Live-attenuated simian immunodeficiency virus variants have shown promise as AIDS vaccine in animal studies, but attenuated HIV-1 variants are considered unsafe for use in humans.
- The attenuated vaccine strain will cause a chronic infection and can evolve into a pathogenic variant.
- Replication of a conditionally live virus can be restricted to the time needed to induce the immune system.
- The ability to switch virus replication completely off after vaccination prevents evolution of the vaccine strain and, thus, increases safety.

Conditionally replicating mini-HIV variants as virotherapy against leukemia

- Virotherapy aims to destroy cancer cells by the use of virus variants that replicate selectively in malignant cells.
- Mini-HIV strains that lack accessory viral functions replicate in leukemic T cells but not in normal T cells.
- Mini-HIV variants specifically kill malignant cells and demonstrate potential as therapeutic virus against leukemia.
- Conditional replication of the therapeutic mini-HIV variant provides an additional safety feature as replication can be stopped after the leukemic cells are removed.

Conditionally replicating anti-HIV gene therapy vector

- Cells can be made resistant to HIV-1 by RNAi induced by antiviral shRNA molecules.
- A gene therapy approach using lentiviral vectors that express the shRNAs can provide durable protection. This therapy requires complex and costly procedures, which may present a bottleneck for treatment of large numbers of HIV-infected individuals.
- A replicating HIV vector that spreads the RNAi antivirals to all HIV-susceptible target cells will provide a more efficient and less elaborate delivery method. For safety reasons, such a vector should be conditionally replication competent.

- This strategy seems particularly suitable for patients infected with a multidrug-resistant virus that fail on current therapy regimens.

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Figure 1. Design of replicating HIV-based vectors

Schematic of the HIV-1 proviral genome and replication-competent derivatives. In the doxycycline-inducible HIV-rtTA variant, the Tat–TAR regulatory mechanism was inactivated through mutation of Tat and TAR (*tat^m* and *TAR^m*). The Tet-On regulatory mechanism was incorporated through the insertion of *tetO* elements in the U3 promoter region, and the *rtTA* gene at the site of the *nef* gene. In the HIV-Δ5 mini-HIV variant, deletions were introduced in the *vif*, *vpr*, *vpu*, *nef* and U3 sequences of HIV-1. In the therapeutic HIV-rtTA-shRNA^{nef} variant, a polymerase III promoter-shRNA^{nef} expression cassette is inserted into the 3' LTR. Upon virus replication, the cassette is inherited in both LTRs. The shRNAs are processed by Dicer into siRNAs, which target the *nef* sequence in wild-type HIV-1 RNA.

LTR: Long terminal repeat; TAR: *trans*-active responsive region; *tetO*: *tet* operator; Pol III: RNA polymerase III promoter.