Ex vivo gene therapy for HIV-1 treatment

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Until recently, progress in ex vivo gene therapy (GT) for human immunodeficiency virus-1 (HIV-1) treatment has been incremental. Long-term HIV-1 remission in a patient who received a heterologous stem cell transplant for acquired immunodeficiency syndrome-related lymphoma from a CCR5−/− donor, even after discontinuation of conventional therapy, has energized the field. We review the status of current approaches as well as future directions in the areas of therapeutic targets, combinatorial strategies, vector design, introduction of therapeutics into stem cells and enrichment/expansion of gene-modified cells. Finally, we discuss recent advances towards clinical application of HIV-1 GT.

INTRODUCTION

Although highly active anti-retroviral therapy (HAART) regimens have improved considerably, cost and side effects are significant, and some patients ultimately fail. Subtle declines in immune function can occur over time even with conventional treatment owing to the difficulty in eradicating latent reservoirs, particularly as new potential compartments continue to be identified (1–3). Gene therapy (GT) has the potential to not only control viral replication and prevent CD4+ T cell depletion, but also provide a pool of immune cells with sustained intrinsic protection. With this in mind, we discuss some of the recent exciting developments in ex vivo therapy for human immunodeficiency virus (HIV) treatment, focusing primarily on those GT approaches targeting hematopoietic stem cells (HSCs). Typically obtained by enriching either human bone marrow or umbilical cord blood for the early marker CD34+, their genetic modification and engraftment can potentially reconstitute the entire hematopoietic system with cells resistant to HIV-1 infection and/or replication. We focus on recent studies that inform GT design and implementation in the treatment of HIV-1.

MODELING DESIGN PARAMETERS

Computational modeling has been used extensively in an attempt to better understand HIV-1 replication dynamics and evolution. The predictions of these models could be validated against the large body of clinical data and subsequently adjusted. Consequently, accurate in silico models exist to describe outgrowth of HIV-1 resistance in response to HAART therapies and can be informative in evaluating new conventional drugs.

Based on this success, interest in computational modeling of GT regimens is increasing, particularly as HIV-1 GT moves closer to the clinic. In silico models of disease dynamics and response to GT are increasingly sophisticated and pertinent. Evaluation of these models is hampered by the lack of patient data; nonetheless, computational studies raise important issues that can inform GT design. For instance, mathematical modeling divides anti-HIV therapeutics into three classes (4): Class I inhibits HIV-1 entry and replication before virus integration; Class II inhibits viral regulatory and structural protein expression; and Class III inhibits viral assembly and release. Class I antivirals are by far the best inhibitors, consistent with recent in vivo comparisons (5).

Two recent in silico computational models of HIV-1 replication and development of resistance to GT regimens form a useful backdrop for discussion. Both models include the use of different classes of anti-HIV inhibitors and incorporate features designed to better simulate in vivo GT regimens. These include the following parameters: starting with a population of engrafting cells containing a mixture of GT-modified (GTM) and unmodified (UM) cells, rather than 100% GTM; assuming that the viral population at the time of engraftment consists of mixed quasi-species with differing sensitivities to the introduced gene therapeutic(s) as opposed to a single wild-type strain; and assuming that viral replication continues during engraftment and expansion.

The first analysis (6) compares the dynamics of emerging viral resistance in gene-modified CD4+ T cells versus HAART treatment using pre-integration inhibitors starting with realistic GTM/UM ratios. The model describes a homeostatic mechanism for maintaining the total CD4+ T cell pool, with regeneration of both the GTM and UM cells by...
self-proliferation and minor contribution from the bone marrow. The viral population is also heterogeneous, consisting of both GT-sensitive wild-type virus and evolving resistant strains. Viral resistance increases as mutations accumulate; concomitantly, replicative fitness decreases. The results suggest that HIV-1 evolution differs fundamentally in response to GT and HAART treatment. In the GT model, HIV-1 continues to replicate in the UM population during engraftment and as the GTM population expands slowly, viral load declines gradually. In contrast, HAART blocks viral replication nearly immediately upon administration in nearly all cells of the target population.

These results implicate two aspects of the GT design critical to viral control. The first is the inhibition factor or the ability of the gene therapeutic to attenuate the rate of viral infectivity in GTM cells. Potent inhibition is an important factor in controlling viral resistance, which acts by weakening early mutants before they can replicate sufficiently to evolve the additional mutations required to compete with wild-type virus replication and successfully escape. The authors also modeled the effect of increasing the rate of proliferation of GTM relative to UM cells, causing a more rapid rise in the GTM to UM ratio.

The importance of potent inhibition also supports the use of a combinatorial approach. The typical combinatorial approach expresses multiple anti-virals in the same cell, usually from the same vector. The authors evaluated a different combinatorial scenario, comparing the onset of resistant viral outgrowth in a homogeneous population where a single gene therapeutic is used, as opposed to a heterogeneous mixture of two populations, each containing a single gene therapeutic targeting a different viral functionality. This is known as the split combinatorial or ‘divide and conquer’ approach. Viral replication is suppressed more efficiently in the second case—all other things being equal; but a single therapeutic with a greater genetic barrier to evolving escape can perform as well as the split combinatorial approach with two weaker gene therapeutics under some conditions.

This highlights another important factor to emerge from the Arkin laboratory analysis: replication fitness cost associated with the mutation of a viral target site of a gene therapeutic, which functions by inhibiting viral replication in the UM population. In practice, this supports the importance of choosing conserved viral targets, as conservation implies that escape mutations will impose a heavy fitness cost. However, relatively small reductions in fitness cost resulted in benefits comparable to much larger increases in therapeutic potency, implying that individual components of combinatorial approaches should be designed to have synergistic inhibitory effects for maximum therapeutic benefit. The authors point out that the split combinatorial approach is not limited to populations expressing only a single therapeutic, but multiplexed therapeutics as well. The use of independent multiplexed therapies would not only increase the fitness cost to any escaped virus, but also potentially bridge the gap when the predicted effective number of therapeutics required to prevent resistance significantly exceeds the safe number of therapeutics for single cells. This may be very important in maximizing the effectiveness of RNA interference (RNAi) strategies while avoiding toxicity (see the following discussion).

In the second example, Applegate et al. (7) modeled the emergence of HIV-1 resistance in CD4+ T cells of a three-dimensional matrix simulating lymphoid tissue derived from standard engraftment of gene-modified CD34+ cells. Direct cell–cell transmission and latency were included in the model of survival and replacement of infected cells. Specifically, this model tested the number of anti-viral short hairpin RNAs (shRNAs) required for effective suppression using two, four or six validated shRNA target sites (unspecified) that exerted inhibition post- and/or pre-integration. Other variables included loss of efficacy of the shRNA owing to mutation in the target, the effect of mutations on viral fitness and the GTM/UM ratio in the engrafting population. Briefly, their model shows that better inhibition is achieved (i) when the starting population of CD34+ cells contains 20%, as opposed to 1% of GTM cells; (ii) as the numbers of anti-HIV shRNAs increase; and (iii) when both Class I and II viral targets are used as opposed to only Class II targets. Their results are consistent with the low-level inhibition by a single Class II shRNA compared with a Class I entry inhibitor (5,8).

In addition, Applegate et al. modeled the effects of reduction in viral fitness due to mutations in the target site. In their model, two shRNAs were relatively poor at viral control, while four or six were highly protective. Reduced viral fitness confers no extra benefit when four or six shRNAs are used, but has a pronounced beneficial effect with two shRNAs.

As the authors themselves note, the exact output of both the models depends upon the initial assumptions. For instance, Applegate et al. note that while more shRNAs are better, their data do not show an exact number required for good control of HIV-1. Although the two modeling scenarios differ significantly, the results are important for the trends they illustrate and show some striking parallels. More gene therapeutics are better, consistent with the need for potent viral inhibition. GT benefits from higher starting proportions of GTM cells by limiting viral replication, modeled as increased proliferation in the study of Applegate et al. and higher starting transduction efficiency in the study of Aviran et al. However, some continuing wild-type HIV-1 replication in the UM compartment can be beneficial, by competing with viral escape mutants, highlighting the complexity of viral population dynamics. Practically, however, the potential advantages of some low-level wild-type replication in suppressing viral escape would have to be weighed against the likelihood of increased levels of spontaneous mutation in the wild-type population as well as memory T-cell death and immune system compromise. Concurrent HAART therapy can reduce the costs associated with viral replication in the unmodified cells, but problematically also eliminates the selective pressure on modified cells. In any case, HAART therapy will likely continue in GT protocols in the foreseeable future for ethical reasons. We will discuss methodologies under development to circumvent this paradox. In the meantime, these results await validation in animal models of HIV-1 infection, which have recently expanded to include the cat (9) and the rabbit (10).
HIV-1 PRE-ENTRY AND PRE-INTEGRATION INHIBITORS

Both of the above models considered only viral targets, but host cell factors are potential targets for both conventional and GT (summarized in Table 1). Fueled by recent success targeting CCR5 (see below), screens to identify host cell components involved in HIV-1 replication in various cell types (11) and investigations into the mechanisms of known host cell factors continue. CCR5 is the major co-receptor used by HIV-1 to initiate infection of T cells and macrophages as well as other cell types. Individuals who are heterozygous or homozygous for CCR5Δ32 mutation in CCR5 are slower progressors or are resistant to HIV-1 infection, respectively (12). Approximately 3–5% of individuals of Western European descent carry a CCR5Δ32 allele. The homozygous CCR5Δ32 genotype has no pronounced deleterious effects, making CCR5 an attractive cellular target for GT of acquired immunodeficiency syndrome (AIDS). The potential of this approach has received a boost from the results of a single study, in which an HIV-1-infected individual treated for lymphoma by an allogeneic bone marrow transplant (BMT) with a CCR5 homozygous null (CCR5Δ32/Δ32) HLA-matched donor was able to discontinue conventional HAART treatment (13). He has recently reached the 3-year mark with no detectable trace of HIV-1 (14) and is tentatively being considered the first possible ‘cure’ for HIV-1.

While enormously encouraging, this approach is not likely to be generally applicable. Allogeneic BMTs require full bone marrow ablation and immune suppression treatment and thus are not justified solely for HIV-1 treatment at this time, except in the context of treating a separate medical condition. Even in the event that should change, the pool of CCR5Δ32/Δ32 HLA-matched donors is limiting. However, autologous regimens are potentially less toxic, as they may not require full bone marrow ablation or subsequent immune suppression for engraftment. In this setting, the goal would be to disable the CCR5 gene in enough cells to confer benefit and reintroduce these cells back into the patient; consequently, a number of investigators have pursued CCR5 knockout strategies alone or in combination (15–20). Holt et al. (21) recently described an engineered zinc finger nuclease (ZFN) to generate CCR5 homozygous disruptions in an estimated 5–7% of treated umbilical cord CD34+ cells. Both ZFN-treated and untreated CD34+ cells were capable of secondary serial engraftment in the NOD/SCID/IL2rgnull mouse model and showed similar developmental profiles in multilineage assays. These results indicate that at least some of the ZFN-modified cells were stem cells that could subsequently undergo normal expansion and development. CD4+ T cells in peripheral blood, spleen and intestines became depleted over time in HIV-1-challenged animals engrafted with UM but not in ZFN-modified CD34+ cells; moreover, the proportion of CCR5Δ32 cells increased in the later population over time and viremia was greatly reduced. The majority (59/60) of ZFN cleavage site sequences derived from intestinal mucosal cells were edited and polyclonal in origin only in animals engrafted with ZFN-modified cells, consistent with modification of multiple CD34+ precursors. Taken together, these results suggest that ZFN-induced CCR5 disruption in CD34+ cells protects the derivative CD4+ T lymphocytes from HIV-1 infection, resulting in a selective advantage for CCR5Δ32 cells, thus providing support for the CCR5 knockout GT strategy.

Studies of the underlying mechanisms of host restriction factors are expanding the repertoire of possible molecular GT approaches. The TRIM (tripartite motif)α protein of old world monkeys such as rhesus (rh) macaques prevents transduction by HIV-1, while the human orthologue is permissive [reviewed in (22)]. TRIM5αrhr disrupts cytoplasmic uncoating of viral capsid in the cytoplasm prior to viral integration (23–25) possibly by forming a hexagonal lattice that binds to the HIV-1 capsid hexagonal lattice (24). Only one or two amino acid differences between the human and rhesus TRIM5α proteins are responsible for restriction in the TRIM5α domain that interacts with the HIV-1 capsid (22,26,27). In addition, TRIM5αrhr can restrict HIV-1 at a late phase of viral replication (28). Studies of Chinese seronegative intravenous drug users suggest a possible natural protective effect of some polymorphisms in this protein (29) lending support for the use of chimeric human–rhesus TRIM5α variants (TRIM5αrhr-hu) (19) or engineered human variants (27,30) that minimize antigenicity as gene therapeutics. While viral escape is possible (31), expressing modified TRIM5α as part of the combinatorial approach shows promise (19).

Some members of the polynucleotide cytidine deaminase APOBEC3 (A3) family of proteins, particularly A3F and A3G, are known HIV-1 host cell restriction factors. A3G is packaged in the virion and blocks reverse transcription by deaminating minus-strand viral cDNA during reverse transcription prior to integration; it may inhibit HIV-1 via other mechanisms as well. HIV-1 vif (viral infectivity factor) directs A3G ubiquitination and degradation by linkage to E3 ligase complex, thus preventing A3G incorporation into the virion [reviewed in (32–34)]. Research continues into the biology of APOBEC3 proteins in HIV-1 replication in various relevant cell types [see references in above reviews, also (35–40)]. Class III gene therapeutic strategies aim to exploit this interaction by restoring APOBEC encapsidation, which can be accomplished in a number of ways. Underscoring the need for careful design and evaluation of GT strategies based on the APOBEC-vif axis are reports that A3G can cause sublethal, as well as lethal, levels of mutation that can contribute to HIV-1 diversification, evolution of resistance and potential escape (41,42). Another strategy utilizes Chim3, a dominant-negative vif mutant derivative that acts as a Class I inhibitor. Chim3 does not block the integration by inducing A3G degradation; rather, it blocks the accumulation of retrotranscripts and decreases HIV-1-induced cell cycle perturbation in HIV-1-infected CD4+ T cells (43). The molecular biology of the HIV-1 vpu/host BST-2 (tetherin) axis is the subject of intense interest and may yield a new generation of HIV-1 inhibitors (44–59).

INCREASING LEVELS OF GENE-MODIFIED CELLS

Both the molecular modeling scenarios already discussed as well as early preclinical trials (discussed below) emphasize
Combinatorial approaches (5,8,15,19,66,85)

Vectors FV (8)

Selection of GTM cells MGMTP140K transgene expression (8,66)

General GT modality Specific modality References

Cellular CCR5 co-receptor I RBZ-mediated mRNA knockdown (15)
I shRNA-mediated mRNA knockdown (8,16–20)
I ZFN gene knockout (21)
I CCR5+ allogeneic transplant (13,14)
I TRIM5α/h-hu chimera (19)
I Variant human TRIM5α (30,31)

Cellular/viral APOBEC3G/vif axis I Chim3 (vif-dominant negative) (43)
Viral TAR protein I TAR decoy (15,19)
II shRNA-mediated mRNA knockdown (5,8,15,66)
II shRNA-mediated mRNA knockdown (8)
II Long antisense RNA-mediated mRNA knockdown (5)
I Membrane-anchored C46 peptide fusion inhibitor (5,8,66,
I shRNA-mediated mRNA knockdown (85)

Vectors FV (8)
Retroviral (31)
Lentiviral (5,15,16,18–20,30,43,66,85)

Combinatorial approaches

the desirability of increasing the proportion of gene-modified cells in patients. One approach is to increase the proportion of gene-modified cells prior to implantation, which has proved to be challenging particularly with lentiviral vectors. Research continues on methods to increase lentiviral transduction efficiency (60,61) [reviewed in (62,63)]. Another approach would be to expand the transduced population of cells (with or without selection) ex vivo, but here the challenge is to maintain the pluripotency or ‘stemness’ of transduced cells in culture. The knowledge of the molecular mechanisms for inducing pluripotent stem cells may be applicable in this context.

Post-transplantation, continuing viral replication will theoretically select for GTM cells but molecular modeling shows the advantages of increasing the proportion of GTM cells independent of viral selection. One approach takes advantage of chemotherapy used to treat lymphoma in AIDS patients to select for GTM cells. The P140K mutant of O6-methylguanine-DNA methyltransferase (MGMT) confers resistance to chemotherapeutic reagents such as lysomustine (64), temozolomide and bis-chloronitrosourea (BCNU). MGMTP140K, unlike the endogenous wild-type gene, is also insensitive to O6-benzylguanine (O6BG). Therefore, after autologous transplantation, cells expressing MGMTP140K (along with any anti-HIV genes) are selected by a combination O6BG and either BCNU or temozolomide treatment. This approach was successfully used in a clinically relevant pigtailed macaque model of HIV/simian immunodeficiency virus (SIV) infection and potentially circumvents the problems associated with permitting HIV-1 replication to select gene-modified cells (8,65,66).

VECTORS

GT vectors are continually being developed and improved [reviewed recently in (67–70)]. Lentiviral vectors are currently still preferred because of their ability to transduce non-dividing cells, albeit less efficiently than dividing cells, and the lentiviral integration site spectrum is less problematic than that of murine leukemia virus (MLV). Other GT vectors include Sleeping Beauty (SB) [(71); and reviewed in (72–74)] which has been approved for use in a clinical trial. Integration of the SB transposon carrying the therapeutic gene into the host cell requires transient expression of the SB transposase, typically from a co-transfected plasmid that theoretically should be lost after cell division. Nonetheless, there are reports of SB transposase gene integration that raises safety issues if the transposase is active in the host cell. This problem is potentially rectified by using transposase mRNA rather than the gene-encoding plasmid to express the SB transposase (75). Tol2 and piggyBAC are two other candidate transposable element vector systems for use in GT [compared in (76,77)].

Foamy viral (FV) vectors have a number of advantages for GT. The parent virus is non-pathogenic and not endemic to humans. Vectors are not targeted by anti-HIV sequences (unlike some lentiviral vectors or packaging constructs), have a large transgene capacity and broad tropism. The packaged vector is also resistant to degradation in human serum. Although requiring cell division for integration, transduction intermediates are relatively stable in the target cell as reverse transcription is completed in the virion, which may underlie their ability to transduce quiescent HSCs. FV vectors integrate less frequently within genes or near transcription start sites than lentiviral or MLV vectors, respectively, and efficiently transduce murine, canine and human HSCs [(78) and reviewed in (65)].

Site-specific vector integration is one of the goals for GT. While an area of active research for some time (79), new directions have recently emerged, fueled again by a better understanding of the molecular mechanism of HIV-1
integration. The cellular protein lens-epithelial-derived growth factor (LEDGF) acts as a bridge, tethering the HIV-1 pre-integration complex to the host cell chromatin at the future site of integration. Specifically, the C-terminal domain of LEDGF binds the C-terminal domain of HIV-1 integrase (INT), while the N-terminal chromatin-binding domain (CBD) of LEDGF binds chromatin (80). Substituting the LEDGF CBD with heterologous CBDs can alter the HIV-1 integration spectrum (80,81). Efficiency is potentially reduced by cellular LEDGF competing with chimeric LEDGF for INT binding, but this can be circumvented if HIV-1 INT provided during viral packaging can be altered to retain binding to the heterologous CBD but not to endogenous LEDGF (82).

Other approaches include making retroviral-ZFN protein hybrids to alter vector integration site specificity. Lim et al. (83) screened for permissive insertion sites for ZFN domains throughout the MLV gag-pol region. While engineered virus did not insert into genomic sites originally targeted by the ZFN, integration shifted away from transcription start sites, and some fusions showed distinct integration site preferences. Presumably, the approach can be improved and similarly applied to lentiviral vectors. Development of ZFN hybrids of SB and piggyBAC transposons is also underway (84).

COMBINATORIAL APPROACHES

Several combinatorial RNAi strategies are being investigated to meet the challenge of HIV-1 escape, as single mutations in a target site can severely impair RNAi knockdown. Schopman et al. expressed three shRNAs from different promoters in a single lentiviral vector against the same target: one against the wild-type target and two additional shRNAs against the anticipated dominant escape mutants, referred to as second-generation shRNAs (85). The feasibility of this approach was based on a previous evolution study demonstrating limited escape routes from the highly conserved HIV-1 int targets. The triple shRNA effectively inhibited HIV-1 replication and outgrowth of the two expected major escape mutants. New escape mutants did emerge, possibly of reduced fitness as they were present as minor populations in the original study and caused amino acid substitutions. In addition, competition between the shRNAs reduced the initial level of virus inhibition, potentially facilitating escape.

These results highlight two important design issues for therapeutic shRNAs. The first is the saturation of cellular RISC, manifesting as competition between different shRNAs and/or cellular toxicity; this is largely a consequence of the high transcription rates from RNA polymerase III (Pol III) promoters, such as U6, HI and 7SK typically used to express shRNAs. The second is that combinatorial shRNA strategies directed against different conserved viral targets may be more effective in controlling viral replication and escape. One approach mitigating both these factors uses an endogenous polycistronic microRNA scaffold to express multiple RNAi effector units from a single RNA polmerase II (Pol II) transcript (86). Many Pol II promoters have lower expression levels than the standard Pol III promoters and the potential to confer cell-type specificity or inducible control. Modified tRNAs may also be used to express lower levels of individual shRNAs. These options have the additional advantage of reserving the standard Pol III promoters for expressing other RNA therapeutics, such as aptamers that benefit from
high expression levels. These alternatives allow one to circumvent the repetitive use of the same promoter, which can lead to cassette deletion (8,87).

Mixed anti-HIV combinations, employing different types of RNA therapeutics, can also mediate effective HIV-1 inhibition and may be important in controlling replication in cells with limited Dicer (88). These typically employ triple combinations including at least one Class I inhibitor. A foamy virus vector expressing two shRNAs targeting HIV-1 tat and rev, the C46 envelope fusion inhibitor as well as the MGMTP140K gene for selection inhibits viral replication by over 4 logs in vivo. CD34+-transduced cells efficiently engrafted NOD/SCID IL2Rγnull mice and expanded upon selection for MGMTP140K resistance. As FV vectors also inhibit SHIV expressing two shRNAs targeting HIV-1 and may be important in controlling replication in cells with RNA therapeutics, can also mediate effective HIV-1 inhibition in the absence of significant viremia, an intrinsic selective advantage may be required. The results of the studies of Hutter et al. and DiGiusto et al. suggest that these hurdles can be overcome.

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