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Targeted gene disruption to cure HIV

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Abstract

Purpose of review—Recent clinical research suggests that an HIV-infected patient with lymphoma who was transplanted with bone marrow homozygous for a disrupted mutant CCR5 allele has no remaining HIV replication and is effectively cured of HIV. Here we discuss approaches of disrupting host and viral genes involved in HIV replication and pathogenesis with the aim of curing patients with HIV.

Recent findings—Data from the ‘Berlin patient’ suggests that targeted gene disruption can lead to an HIV cure. This review discusses recent advances in the field of gene disruption towards the development of an anti-HIV therapy. We will introduce strategies to disrupt host and viral genes using precise disruptions, imprecise disruptions, or site-specific recombination. Furthermore, the production of engineered rare cutting endonucleases (zinc finger nucleases, TAL effector nucleases, and homing endonucleases) and recombinases that can recognize specific DNA target sequences and facilitate gene disruption will be discussed.

Summary—The discovery of a gene disruption approach that would cure or efficiently confine HIV infection could have broad implications for the treatment of millions of people infected with HIV. An efficient ‘one shot’ curative therapy would not only give infected patients hope of a drug- or treatment-free future, but could reduce the huge financial burden faced by many countries due to widespread administration of HAART.

Keywords

Homing endonuclease; zinc finger nuclease; TALEN; reservoir; gene targeting

Introduction

Despite extensive international research, an HIV cure remains elusive. Highly active antiretroviral therapy (HAART) has vastly improved the treatment and prognosis for HIV-

infected individuals, as HAART can effectively suppress viral replication and reduce viral loads. However, HAART cannot eliminate the virus and thus is not a cure. In addition, HAART requires a lifelong commitment that comes with a number of side effects. A main reason why HAART therapy cannot cure HIV is the ability of HIV to integrate its genome into the host genome, which enables viral persistence in reservoirs of latently infected cells. Consequently, individuals who develop drug resistance or withdraw from HAART rapidly develop high viral loads and eventually AIDS. Therefore, a curative therapy for HIV has remained a high priority in the HIV/AIDS field.

A cure for HIV must purge all HIV reservoirs or permanently prevent the spread of HIV to uninfected cells. Although this may seem daunting, several strategies are under development. An approach that eliminates all cells that are actively or latently infected with HIV would seem the most obvious. Unfortunately, this task is formidable, as HIV-infected cells are found throughout the body, often in discreet sanctuary sites. Although infected cells could be eliminated in many ways, a vaccination-based strategy, whereby circulating host CD8⁺ T cells are stimulated to target HIV-infected cells, currently provides the best hope for such a systemic approach (1-3). A strategy that prevents HIV spread by disrupting the cellular receptors that facilitate HIV entry in uninfected cells is also attractive. This approach should prevent further CD4 cell depletion, preventing the development of AIDS. Eventually, without new infection events, existing reservoirs of actively and latently infected cells could diminish or disappear. Even if full elimination of the reservoir took years, the absence of newly infected cells might result in a functional cure. Permanent inactivation of HIV genomes in infected cells is another route to an HIV cure. This could be accomplished by generating enzymes that selectively recognize and disrupt unique proviral sequences, and might allow inhibition of viral replication and spread without causing host cell toxicity.

While all these approaches could feasibly generate an HIV cure, this review will focus on the use of ‘designer’ enzymes to disrupt either HIV genomes or cellular receptors for HIV infection. Our aim is to introduce recent developments in this new field of anti-HIV research.

Genome disruption

In the context of this review, genome disruption is defined as the process by which a chosen genetic locus is altered in order to interrupt or change its normal function. Genes can be disrupted through introduction of precise ‘designer’ genomic deletions or additions, using gene-targeting techniques that rely upon homologous recombination (HR) between a targeted genomic sequence and an engineered DNA targeting vector (4-6). Alternatively, chosen loci can be cleaved at specific sequences and then repaired imprecisely by the host cell DNA repair machinery, thus introducing genomic indels (7). Disruption can also occur after DNA target sequences are excised by a site-specific recombinase (SSR). Recent advances in technologies to facilitate precise gene disruption, imprecise gene disruption, or site-specific recombination will be discussed.

Rare-cutting endonucleases

Gene disruption techniques that utilize precise gene targeting or imprecise repair both require a DNA double strand break (DSB) within the target region. For gene targeting the DSB can be introduced anywhere within the target region that will undergo HR, whereas disruption through imprecise repair requires DSB formation within a coding sequence in order to introduce a deletion or translational frame shift. For both techniques, the DSB must be introduced within a specific genomic region, ideally in a manner that does not indiscriminately introduce additional DSBs throughout the genome. To this end, several types of rare-cutting endonucleases that introduce DSBs with high specificity at unique DNA sequences have been developed.

Zinc finger nucleases—Zinc finger nucleases (ZFNs) are engineered proteins that can cleave dsDNA at specific sequences (reviewed in detail in (8)). Each ZFN comprises a pair of proteins that have a sequence of 3-6 Cys₂His₂ zinc finger domains, each bound to a single atom of zinc, coupled to the cleavage domain of the type IIS FokI restriction enzyme (Figure 1a). The N-terminal zinc finger domains bind to different 3 bp sections of DNA in a modular fashion, enabling rational selection of DNA-binding motifs. ZFN pairs bind to sequences on opposite strands of dsDNA 5-7 bp apart, and this enables the C-terminal FokI domains to dimerize and cleave DNA. ZFNs have been designed to target over 50 genomic loci in various organisms (8, 9). While instances of off-target cleavage due to ZFN subunit homodimerization have raised concerns over ZFN-mediated toxicity, newer ZFN scaffolds that allow ZFN cleavage only upon ZFN heterodimerization are much less toxic (10, 11).

TALENs—Like ZFNs, transcription activator-like effector nucleases (TALENs) are engineered proteins that can cleave specific dsDNA sequences. Unlike ZFNs, TALE proteins recognize DNA in a single base pair modular fashion (12, 13) and subsequently DNA binding occurs with less context specificity. This means that TALENs can be engineered against a wider number of DNA targets than ZFNs. TALENs contain an N-terminal engineered TALE binding domain derived from *Xanthomonas* and the FokI cleavage domain (Figure 1b), and like ZFNs must bind to target sequences as a pair to enable FokI dimerization and subsequent target site cleavage (14-16). TALENs have been designed to target many DNA sequences and have been used to disrupt a number of genes in both somatic and pluripotent cells (14-18), although the toxic effects due to off-target TALEN cleavage are yet to be explored.

Homing Endonucleases—Homing endonucleases (HEs) are highly specific DNA-cleaving enzymes encoded by certain selfish genetic elements. HEs are able to bind to long unique sequences (usually 16-30bp) with high fidelity and cleave them to form DSBs (Figure 1c). There are at least 5 families of HEs that bind and cleave DNA with distinct mechanisms (19). With some HE families containing over 200 members, there are potentially a large number of starting platforms for the development of novel site-specific nucleases (20)*, and a number of methods have now been developed to manipulate HE target site specificity (extensively reviewed in (19)). Designer HEs have now been used to target many genes for disruption/manipulation (21-27)**.

Exonuclease-driven mutagenesis

In mammalian cells, non-homologous end joining (NHEJ) is the predominant DNA repair pathway used to fix DSBs. However, NHEJ is error prone and can introduce mutations at repaired DSBs. Imprecise repair can therefore be advantageously disruptive, as it can be used to knockout a desired gene upon introduction of a DSB by a targeted rare cutting endonuclease. In the absence of a substrate for HR, rare cutting endonucleases introduce DSBs that are subsequently repaired, often precisely, by NHEJ. Cleavage of precisely repaired sites is iteratively repeated until an error is introduced that prevents subsequent endonuclease binding and cleavage. To accelerate the introduction of mutations at a site of rare-cutting endonuclease cleavage, exonucleases can be used to digest the single-stranded ends created at DSBs. Co-expression of end-processing exonucleases such as Trex2 and Artemis alongside rare cutting endonucleases increases the frequency of mutations at DSBs (28, 29)**, and can be used to maximize the efficiency of targeted mutagenesis.

Site-specific recombinases

The term SSR refers to a group of enzymes that can facilitate recombination in a highly precise fashion between specific sequences of DNA (reviewed in detail in (30)). The mechanism of SSR recombination has been studied best for Cre and Flp recombinases (Figure 1d), which can both facilitate genetic insertion or deletion through their respective *loxP* and *FRT* DNA recognition target sites (RTS). Genetic insertion occurs when a sequence flanked by two RTS is recombined into a single identical RTS within a desired genomic locus. Excision occurs when two identical RTS recombine to excise the flanked DNA and leave behind a single RTS. It is feasible that SSRs such as Cre and Flp could be used to disrupt DNA virus genomes including HIV, as the long terminal repeat (LTR) elements within the genome could potentially act as RTS-like substrates for recombinase-mediated excision of viral sequences. This makes SSRs promising candidate tools for the disruption of virus-infected cells.

Inhibiting HIV through host gene disruption

As an alternative to conventional HIV treatments, it may be possible to efficiently disrupt host genes that are essential to HIV infection (31). A number of recent studies have demonstrated the potential of this approach towards an effective HIV cure.

CCR5 and CXCR4—Cellular receptors for HIV represent excellent targets for novel therapeutic interventions. As the primary attachment receptor for HIV, CD4 would seem to be the first choice for targeted disruption, but CD4 plays an essential role in immune function, making its disruption impractical. However, the HIV co-receptors CCR5 and CXCR4 represent excellent targets for gene disruption, and a number of studies have targeted these molecules.

Although CCR5 deficiency can alter host responses to some pathogens (32), it is not essential for normal immune function. In fact, CCR5 mutations can reduce susceptibility to HIV infection (33), prompting studies targeting CCR5 for anti-HIV therapy. In a landmark study, an HIV-positive patient with leukemia received a bone marrow transplant from a donor homozygous for the CCR5 $\Delta 32$ mutation. This “Berlin patient” has been effectively

cured of HIV, showing no detectable HIV up to 45 months post transplant (34, 35)**. Although it is not absolutely clear whether the CCR5 $\Delta 32$ mutations from the donor are responsible for the cure, co-receptor disruption might be a viable route to therapeutic cure. Unfortunately, this is not an option for most HIV infected individuals, as the chances of finding a CCR5 $\Delta 32$ -homozygous and HLA-matched donor are extremely rare. Therefore, ex vivo disruption of CCR5 in hematopoietic cells from patients and subsequent repopulation would seem more realistic. Ideally CCR5 disruption would occur in hematopoietic stem cells (HSCs) in a manner that restricts disruption to lymphoid repopulating cells.

A number of studies have shown that CCR5 can be efficiently disrupted in hematopoietic cells or pluripotent stem cells. Yao and colleagues showed that ZFNs targeting CCR5 can be used to introduce the CCR5 $\Delta 32$ mutation into embryonic and induced pluripotent stem cells, and that these cells can differentiate into CD34⁺ HSCs and cells of hematopoietic lineage (36). Perez et al showed that ZFNs can efficiently disrupt the CCR5 locus in primary CD4⁺ T cells. When transplanted into immunodeficient mice, these cells are less susceptible to infection upon challenge with HIV (37, 38). Holt et al used ZFNs to introduce CCR5 disruptions to human CD34⁺ HSCs that could reconstitute the immune system of immunodeficient mice. Upon challenge with HIV, these mice had lower HIV levels and higher CD4⁺ T cell levels than mice engrafted with unmodified CD34⁺ HSCs (37, 38). More recently, Schleifman et al disrupted the CCR5 locus in human CD34⁺ HSCs using a CCR5-specific peptide nucleic acid (39). These CCR5-disrupted cells were able to successfully engraft immunodeficient mice for >4 months. Cumulatively, these studies suggest that disruption of CCR5 in patient stem cells followed by hematopoietic reconstitution could comprise an effective anti-HIV therapy.

Unlike CCR5, CXCR4 has an essential role in physiological processes including lymphoid and myeloid development (40). Nevertheless, CXCR4-knockout mice have normal T cell development (41), so disruption of CXCR4 in T cell-restricted hematopoietic precursors could be used to make individuals resistant to HIV infection. Two studies have demonstrated proof of concept for CXCR4 disruption in human CD4⁺ T cells using ZFNs (42, 43)**. CXCR4-deficient T cells were resistant to HIV infection, and also protected from X4-tropic HIV infection in a humanized mouse model. Unfortunately, protection was lost over time as R5-tropic HIV variants emerged in the mice. Although this data is promising, the targeting of specific T cells or T cell precursors for gene disruption remains challenging.

HIV as a target for viral genome disruption

With multiple essential genes and long single reading frame gag-pol transcript encoding multiple proteins, the HIV provirus has many excellent targets for gene disruption. Additionally, the majority of infected CD4⁺ T cells contain one provirus copy (44), and HIV infected cells are not easily super infected (45), suggesting that a gene disruption approach might only need to target one provirus in most infected cells. Furthermore, disruption of the provirus should result in HIV inactivation without killing the infected cell, which is desirable as it does not result in loss of cells potentially critical to host immunity.

Genome mutation—Viral genomes from HBV and HSV have been targeted for inhibitory disruption (24, 46), yet no studies have targeted HIV genomes. Nevertheless, a provirus-directed anti-HIV therapy remains an attractive approach towards an HIV cure. A recent study showed that the HE Y2-*AniI* could disrupt and inactivate gene expression from an integrated lentiviral reporter provirus with a recognition site for Y2-*AniI* inserted in the GFP ORF (47)**. Reporter GFP expression levels were significantly reduced in cells containing the integrated vector that had been treated with Y2-*AniI*, and frame shifts mutations were found at the Y2-*AniI* recognition site. Disruptions of the target site were observed in multiple cells types including T cells. This study provides proof of principle for HIV inhibition via provirus disruption, although Y2-*AniI* does not target HIV-specific sequences. Future efforts will need to focus on development of rare-cutting endonucleases that target essential HIV genes.

Genome excision—As an alternative to genome mutation, Flowers et al. first proposed that SSRs could be used to excise an integrated HIV provirus from the genome of infected cells (48). As proof of principle they used the Cre-lox system to reduce HIV replication in an in vitro model that introduced LoxP sites into the LTRs of the HIV (lox) laboratory strain of HIV. They found that Cre expression inhibited viral replication in HIV (lox)-infected cells and initiated genomic provirus excision, and this approach was subsequently extended by Lee et al (49, 50). This led to work from the groups of Buchholz and Hauber who were able to develop an evolved Cre recombinase that recognizes a loxP-like sequence found within the rare HIV isolate TZB0003. This ‘Tre recombinase’ can excise integrated HIV TZB0003 provirus after in vitro infection, and significantly reduce virus replication (51, 52)*. Taken together, these data suggest that genome excision could be used to inhibit or even eliminate HIV.

Therapeutic delivery

For an HIV gene disruption therapy to show efficacy a sufficient level of disruption will be required, which depends upon the approach being taken. To date the best studies in this regard are those using ex vivo disruption of CCR5 or CXCR4 in hematopoietic cells before transplantation and HIV challenge. In CD34+ cells, disruption of 17% of CCR5 alleles was sufficient to reduce levels of HIV in transplanted mice (37). In CD4+ T cells, disruption of 50% of CCR5 alleles was sufficient to reduce levels of HIV in transplanted mice (38), and disruption of 30-34% of CXCR4 alleles was sufficient to reduce levels of HIV in transplanted mice (42, 43). For direct targeting of provirus, the level of disruption will need to be substantially higher to have clinical benefit (7). Ultimately, the ability to efficiently induce target gene disruption in vivo, ideally with a single dose therapy, would allow these approaches to be available to populations in which hematopoietic cell transplantation is impractical.

Conclusion

Developments in the production of enzymes that can manipulate DNA at chosen target sequences have created possibilities for the cure of HIV. Although in its infancy, the production of an HIV-directed gene disruption therapy shows great potential given the

efficacy shown in a number of research studies. These approaches are now moving forward into the clinic with CCR5-specific ZFNs being used to disrupt CCR5 in autologous T cells transplanted into HIV-infected patients (clinicaltrials.gov NCT00842634 & NCT01044654). The rationale for such an approach has been shown to great effect in the ‘Berlin patient’ who received a genetically ‘disrupted’ allogeneic bone marrow transplant (35). Clinical studies using transplantation of gene-disrupted autologous cells may be more complicated and require a dual approach that targets both host and viral genes for disruption. By focusing future research efforts towards the design and delivery of enzymes targeting specific HIV or host genes, anti-HIV gene disruption therapeutics could one day become a reality.

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Key Points

1. Targets for anti-HIV gene disruption therapies can include genes from both the host and the pathogen.
2. Advances in the engineering of DNA endonucleases and recombinases have made it possible to target almost any gene for disruption/mutation.
3. The validity of anti-HIV gene disruption has been shown with engineered ZFNs that disrupt the HIV co-receptors CCR5 and CXCR4 in T cells and hematopoietic progenitor cells. Disruption of these co-receptors subsequently results in protection from HIV infection in animal models.

Figure 1.

DNA editing enzyme example structures and mechanisms of action. **a**, Composite ZFN structure showing 2 ZFN subunits (yellow & cyan) in proximity to a DNA target. Each subunit is composed of 3 zinc finger domains (pdb structure 1ZNF) and the catalytic domain of the restriction endonuclease *FokI* (pdb structure 2FOK). **b**, Composite TALEN structure showing 2 subunits (green & blue) bound to a DNA target site. Each subunit contains a DNA recognition TALE domain (pdb structure 3UGM) and the *FokI* endonuclease domain (pdb structure 2FOK). **c**, Crystal structure of the homing endonuclease Y2 I-Anil (purple) bound to its DNA target site (pdb structure 3EH8). **d**, Crystal structure of the synaptic complex formed between tetrameric Cre recombinase (orange) and 2 loxP sites during Cre-lox recombination (pdb structure 3MGV).