USE OF GENE THERAPY TO CURE AIDS

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ABSTRACT

According to WHO survey in 2010 there are 34 million people who are infected with HIV and attained AIDS. Per year about 1.8 million people dies with AIDS and 2.7 million new people become infected with HIV. AIDS is a secondary immunodeficiency caused by HIV virus that belongs to retroviral family. During AIDS T-cell count in blood falls tremendously below 200 cells/l that results to be fatal in about 2-3 years. HIV can be transmitted from one person to another by unprotected homosexual or heterosexual sex, by transfusion of blood infected with HIV, by needle sharing between drug or steroid abusers, from mother to child during childbirth or during breast feeding and presence of sexually transmitted diseases (STDs) increase the threat of HIV infection. During AIDS many opportunistic infections and other immune deficiencies occur frequently that usually have negligible chances to affect a normal healthy person. It was found in Berlin patient of HIV, who was living on HAART, was transplanted with bone marrow from a person who was homozygous for CCR5Δ32bp deletion i.e. deletion of one base pair on 32 position in CCR5 gene after which patient became resistant to HIV infection. He discontinued the HAART therapy and lived like a normal person. Viral particles and viral reservoirs were vanished from his blood. This observation laid the basis for use of gene therapy against AIDS. By modifying stem cells taken from bone marrow in which using gene therapy genes for disrupted CCR5 were inserted in place of normal CCR5 gene and then the cells were re-implanted in bone marrow after which they started producing HIV resistant blood cells. Another observation was that modifying stem cells to produce interfering RNAs like sense RNA, antisense RNA, ribozymes that interfere in viral replication cycle or host cell function that is required for viral replication also provided good results to produce anti-HIV immune system. Like HAART in which combination of many drugs are used in similar way if many anti-HIV genes are used simultaneously to modify stem cells targeting different point in viral life cycle provide better results than results acquired by use of single anti-HIV gene to eradicate HIV infection. Although gene therapy provided good results against HIV infection but it is still in trial stage and have not been used on humans yet.

Keywords: HIV, AIDS, CD4+ TCells, HAART, Stem cells etc.

INTRODUCTION

1. HIV (Human Immunodeficiency Virus)

One of the most common secondary immunodeficiency is Acquired Immunodeficiency Syndrome, or AIDS, which results from infection with the human immunodeficiency virus (HIV). According to the World Health Organization (WHO), there were 34 million people living with AIDS, with 2.7 million new cases and 1.8 million deaths worldwide in the year 2010 (Organization, W.H. World health organization: global). HIV infected patients are symptomized by challenged immunity of the patient and various opportunistic infections readily occur to patients. Those microorganisms that healthy individuals can harbor with no ill consequences but can cause disease in those with impaired immune system [1]. In 1983 the virus that causes AIDS was isolated from the lymph node of an infected individual by Montagnier’s group in Paris and was called the human immunodeficiency virus or HIV for short. A second strain of HIV was identified in 1986; this was called HIV-2 and the first strain
was renamed HIV-1 [2].

Some characteristic features of HIV include following:

1) A long latent period
2) Tropism for hematopoietic and the nervous system
3) Several immunodeficiency
4) High mutation rate [3].

2. HIV- STRUCTURE AND GENETIC ORGANISATION

VIRAL ENVELOP

HIV virus is roughly spherical and approx 120 nm in diameter i.e. 1/10,000 of mm. Outermost coat of virus is known as Viral Envelop that generally consists of phospholipid bilayer that is derived from host cell membrane during the process of budding out from the host as shown in (figure 1). Envelop consists throughout the proteins of host in addition to 72 copies at average of Env protein embedded. Env protein appears as knob and consists of two types of glycoprotein:

1) gp120: forms cap of ENV with homo trimer
2) gp41: forms stem of Env with homo trimer.

These are transmembrane proteins and act as anchor for Env protein [5].

gp120 and gp41 are bound non-covalently enable HIV to enter CD4+T helper cells using CD4 a cell surface molecule on TH cell as viral receptor. These proteins are targeted by researchers to develop the HIV vaccine [6].

VIRAL CORE

The genome is surrounded by a nucleocapsid consisting of an inner layer of protein called p24 and an outer layer of matrix protein called p17. Core is in shape of bullet consist of approx 2,000 copies of viral protein p24.

Inside the core 2 HIV-RNA are present that are single stranded, identical and have associated enzymes: reverse transcriptase, integrase, protease, packed inside the core [7].

GENETIC ORGANISATION

Core consists of two identical single stranded RNA both RNA terminals possesses RNA sequence called Long Terminal Repeats (LTR) that act as switches to control production of new viruses and can be triggered by proteins either from HIV or the host cell. HIV genome consists of 9 genes out of which 3 genes are common to whole retroviral family these are env, gag and pol. Besides these 6 regulatory genes that are unique to HIV that guides the whole life cycle of HIV in various ways are tat, rev, nef, vpr, vif [8, 9].

<table>
<thead>
<tr>
<th>TABLE 1: The genomic organization of HIV [10]</th>
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<tr>
<td><strong>GENE</strong></td>
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<td>gag</td>
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<td>pol</td>
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<td>env</td>
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*Env gene produces protein called gp160 that is broken down by a viral enzyme to form gp120 and gp 41 the component of env protein.*
LIFE CYCLE OF HIV

a. INFECTION: The Env protein interacts with CD4 molecules on the surface of target T-cells. Conformational changes are induced in gp120 that permit interaction with chemokine receptor and the reaction fusing viral and cell membranes is triggered. This permits entry of viral genomes into the target T-cell.

b. VIRAL ENTRY IN CELL: Conformational changes occur on binding to receptor and coreceptor enabling gp41 mediate fusion of HIV with host cell membrane.

c. VIRAL RNA PRODUCTION: A cDNA copy of viral RNA is synthesized inside the host using the Reverse Transcriptase enzyme.

d. PROVIRUS PRODUCTION: cDNA enters the nucleus and integrates with host cell cDNA using viral integrase enzyme where it replicates with host. DNA numerous non-infectious proviruses are produced.

f. VIRAL RNA PRODUCTION AND TRANSLATION: Provirus transcription occurs with host DNA n viral RNA is produced which after splicing go to cytoplasm.

g. VIRAL PROTEIN PRODUCTION: Viral RNAs are translated in cytoplasm to characteristic regulatory proteins i.e. Tat, Rev, and structural proteins like gp160 MA, p17, CA, p24, NC, p7, p6, p1 and p2.

h. VIRAL PROTEINS MODIFICATION: Viral proteases cleave viral precursor proteins Like GP160 is cleaved into gp120 and gp41 that forms the viral receptors for T-cells.

i. PRODUCTION OF NEW VIRUSES: All the viral components i.e. viral proteins and viral RNA get assembled to form new viral particle.

j. VIRAL RELEASE FROM INFECTED TCELL: Assembled viral particles now buds out the host T-cell picking its lipid envelop with proteins of host cell membrane. Virus particles bud out of host cell.

k. NEW VIRUS PARTICLES FATE: After release from the host cell it may enter a latent phase and may get activated in response to any microbial infection or may infect other cell simultaneously.

ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS):

It is the terminal phase of HIV infection cycle. During AIDS the opportunistic infections become more frequent with decrease in CD4+ cell count to below 200µl⁻¹ that results in profound immunodeficiency indicates the final stage of HIV i.e. AIDS viral load (viaremia) is highest in this stage and level of CTL’s and antibodies are lowest and patient survives not longer than 2 years even with highest quality of sophisticated treatment and at last patient dies. AIDS is usually not caused just after the infection and till today it is not known how virus damages the immune system and if a person infected with HIV-1 will develop the AIDS. Great researches have been done but still a safe cure for HIV is not found.

The main priority for immunologists is to develop vaccine against AIDS. There are several effective strategies present to develop anti-viral drugs. Researchers target several points in HIV lifecycle that might be blocked by pharmaceutical agents. Two major challenges to produce vaccine for HIV are: (i) To find immunogens that can stimulate many cross-reacting neutralizing antibodies (ii) to find immunogens that can stimulate high levels of persisting CD8+ and CD4+ T cells. Both humoral and cellular immunity may be needed, but they require different types of immunogens; eventually vaccines could be mixed to achieve both.

It was thought that neutralizing antibodies would be stimulated by gp120 preparations, and they do against tissue culture adapted HIV strains but, all such vaccines failed to neutralize primary virus isolates. Now the aim is to design HIV envelope protein immunogens that will stimulate protective antibodies. Unfortunately it has many hurdles in between against this: (i) because of heavy glycosylation of the envelope it is non-immunogenic in nature. (ii) because it is conformationally variable, the chemokine receptor binding site, is not exposed unless CD4+ has bound; (iii) the CD4+ binding site is found interiorly and difficult to access by antibodies; (iv) In addition to carbohydrate gp120 surface is protected by hyper variable loops that vary by mutation at very high rate with no cost to virus
therefore escape neutralizing antibodies \[13\].

The anti-viral drugs are prepared with aim that these drugs must be specific for HIV-1 and must not interfere with the physiology of the normal cells. Many steps in the virus life cycle are potential targets for drugs, including: (i) entry; (ii) fusion; (iii) reverse transcription; (iv) integration; (v) transcription/ transactivation; (vi) assembly; and (vii) maturation \[14, 15\].

The first success in treatment of HIV-1 was with drugs interfering with the reverse transcription of viral RNA to cDNA; the drug was zidovudine, or AZT (azidothymidine). When AZT, a nucleoside analog, introduced into the growing cDNA chain of the retrovirus termination of the chain occurred. AZT is not effective in all patients, and long-term use of AZT has several adverse side effects and resistant viral mutants may also develop in treated patients. The administered AZT is used also by human DNA polymerase and incorporation of AZT into the DNA of host cells kills them. Precursors of red blood cells are also sensitive to AZT, therefore causes anemia in addition to other side effects. Another drug that can block reverse transcription is Nevirapine, which inhibits the action of the reverse transcriptase enzyme \[16\].

And the second success was inhibition of the cleavage of precursor proteins by protease to produce proteins that are assembled to produce new mature virion. Like other retroviruses HIV-1, synthesizes polyproteins each of which consists of several tandemly linked proteins. HIV-1 encodes two polyproteins, gag (55 kD) and gag–pol (160 kD), both are anchored to the plasma membrane via N-terminal myristoylation. These polyproteins are then cleaved by HIV-1 protease to their component proteins, but only after this enzyme has excised itself from gag–pol. This occurs after the virion has budded off from the host cell and results in reorganization of viral proteins to produce virion. The virion is thereby converted from its noninfectious immature form to its pathogenic mature form. If HIV-1 protease is inactivated, either mutagenically or by an inhibitor, the virion remains noninfectious. Hence HIV-1 protease is an opportune drug target \[17\].

A combination therapy, HAART (highly active anti-retroviral therapy) is that the latest procedure to cope with HIV infection. In HAART patient is treated with two nucleoside analogs and one protease inhibitor. This strategy helps to overcome the ability of the virus to rapidly drug resistant mutants. HAART decreases the viral load to undetectable level and health of patient improves. Though it is a bit successful in improving life of HIV infected people but it also has drawbacks like it includes strict time schedule and large no. of pills have to be taken in a every day. Additionally, it may lead to various side effects and some serious patient may not be benefitted by this treatment. Although HAART was a great success but some AIDS experts are not convinced as there is possibility of presence of latent CD4 T cells and macrophages may act as reservoir of infectious virus if provirus is activated by some means. In addition viruses present in brain may not be detected by anti-retroviral drugs as these can not penetrate these sites and these remain undetectable \[16, 18\].

**GENE THERAPY**

Gene therapy is a method that aims to cure an inherited disease by providing the patient with a correct copy of the defective gene so that the patient’s body can produce the correct enzyme or protein so as to eliminate the main cause of the disease. Gene therapy has now been extended to include attempts to cure any disease by introduction of a cloned gene into the patient. It differs from traditional drug-based approaches, in a way that it can treat problems by directly repairing the cause of genetic flaw i.e. it works at genetic level \[19\]. There are two basic approaches to gene therapy:

1. Germline therapy
2. Somatic cell therapy

**1. Germline therapy**

In this type of gene therapy, genetic alterations are done in germ cell (sperm or ova). Done a fertilized egg is provided with a copy of the normal gene and re-implanted into the mother. If the procedure succeeds, the gene will be present and expressed in all cells of the resulting individual. Germline therapy is done by microinjection of a somatic cell followed by nuclear transfer into an oocyte, and theoretically
could be used to treat any inherited disease. This can lead to genetic modifications in unborn and these modifications will then be passed on to the offspring [20].

2. Somatic cell therapy:

Somatic cell therapy involves modification of somatic cells. It is done in two ways:

1) Firstly, cells are removed from the organism, these are transfected using retroviral vectors, and then placed back in the body.

2) Cells are transfected in situ without removal.

The technique is very useful to treat inherited blood diseases (e.g. hemophilia and thalassemia), where genes are introduced into stem cells taken from the bone marrow, which give rise to all the specialized cell types in the blood [20].

Vectors used in gene therapy

Vectors used in gene therapy fall in two categories: (1) Viral vector (2) Non-viral vector. Because of the highly infectious and virulent nature of the viruses, these can deliver gene incorporated in viral genome using genetic engineering to any animal cell therefore genetically modified viruses carrying human DNA are used as vector for gene therapy.

Viral vectors: Three types of viruses are used in gene therapy:

a) Adenoviruses
b) Retroviruses
c) Adeno-associated viruses

Adenoviruses: These are used so as to avoid insertion of gene in wrong site. This vector does not get incorporated in host genome and transcribe the gene product freely in the host cell but re-administration of vector is required in growing cell line because the gene is not replicating with the host genome. Host range of adenovirus is very broad. It can evoke human immune response and induce inflammatory reaction but then also it is used to treat cancer of liver and ovaries [20].

Retroviral vector: Retroviruses were first to be used in gene therapy because of their great efficiency to infect any kind of cell. These contain RNA as their genetic material which is converted to DNA using enzyme encoded by viral RNA known as Reverse Transcriptase (RT) when viral RNA enters the host cell and as it occurs using another enzyme Integrase. DNA is incorporated into the host DNA and replicates with it [20]. Leukemia causing virus of mouse was used in one experiment which have no effect on humans. Disease causing genes were replaced by RNA copy of healthy genes and a small sequence ‘promoter’ was also inserted that acts as on/off switch for transcription of inserted gene in presence or absence of a specific drug. Though it is so efficient but still possesses some drawbacks like it usually infects actively dividing cells and it has no specific manner of insertion of gene in host genome. Therefore it may damage the host genome more than it was thought to repair it. Host may produce immune response against it. To avoid attack of retroviral vector to cell which was not required to be transformed researchers use ex-vivo gene therapy as described below:

1) Firstly healthy gene is inserted in vector.
2) Then cells which are to be transformed are isolated from patient.
3) Now the vector and isolated cells are mixed and cultured in laboratory.
4) Cells are monitored if they are working normally.
5) Modified cells fractioned out from mixture and are injected back to patient.

In this way, patient lacking same protein production starts producing it.

Adeno- associated virus: This virus is better vector than all because it can infect both dividing and non dividing cell and its host range is also very broad. Most importantly, it can insert gene specifically at a site in chromosome 19. This virus can be found in almost all humans as it is not pathogenic and do not evoke immune response against it. Still it has got drawbacks as it is very small and carries only two genes naturally therefore it can not carry large gene to transfer it to other cells. It is demonstrated that it can be used to repair genetic defect in animal and now it is used in initial studies to treat hemophilia in humans [20].
CHIMERAPLASTY

It is a non viral procedure of gene therapy used to fix defective gene directly by inserting new normal gene sequence in cell nucleus which binds at the defective gene at terminals in cell nucleus. The defective part in middle does not bind forming hump like structure in DNA which activates the DNA repair action of cell, DNA repair enzymes interchange the defective sequence with chimeraplast sequence and remove defective gene that is later degraded. Effectiveness of the chimeraplasty has been found to be 0.0002% effective in transforming yeast cells [20].

HUMAN APOBEC 3G - MEDIATED HYPERMUTATIONS

APOBEC3G or hA3G (human apolipoprotein B is a mRNA-editing enzyme catalytic polypeptide-like 3G). It belongs to a protein family including hA3D, hA3F, hA3H which possesses cytidine deaminase activity therefore acts as a potent host restriction factor of retroviral replication through, vif a HIV-1 accessory protein interacts with hA3G and protects the virus from its anti-viral activity [21,22]. In the presence of defective vif, hA3G/F/D/H induces mutation in the minus strand of the ssDNA by replacing dC-to-dU ,in response to this , dG-to-dA mutations in the plus strand of the cDNA occurs.

Viral replication is ceased by hyper mutations produced by hA3G which introduces stop codon in ORFs of retroviral gene mainly in the tryptopham residues (TGG-to-TGA/TAA/ TAG). Many sub-lethal hyper mutations have also been suggested to contribute to the HIV-1 genetic diversity [23] and greater genetic variations due to the low level of G-to-A mutation which allows HIV-1 evolution [24]. In proviral sequence cytidine deamination can generate drug-resistant progenies in vitro [25], pol sequences have been identified as a potential target sites for hA3G/F by computer prediction , but still the effect of hA3G in HIV-1 drug resistance in vivo is unknown and considered to be low [24].

HEMATOPOIETIC STEM CELL-BASED GENE THERAPY FOR HIV DISEASE

HIV gene therapy provides an alternative to HIV infected people living on HAART treatment which requires consuming loads of pill per day with tight timetable and in case of some carelessness sudden increment of plasma viral load occurs making patient fall sick seriously. HIV gene therapy targets to transduce hematopoietic stem cells in such a way that those stem cells start producing HIV resistant blood cells i.e. T-cells, B-cells, macrophages etc .Strategies and stages at which HIV virus is antagonized are shown in (figure 2) [38].

Figure 2: Applicable stages and strategies for gene therapy-based antagonism of HIV replication (a) Simplified diagram of hematopoiesis, highlighting myeloid and lymphoid lineages that are susceptible to HIV infection (red), or are refractory to infection (green). Yellow: HIV infection of hematopoietic progenitors remains controversial (b) Schematic of T-cell maturation, indicating applicable anti-HIV therapies at each stage of differentiation. Disruption or modification of some host genes may preferably be performed in more differentiated cells. (c) Three broad access points for inhibition of HIV infection and replication. HIV entry can be blocked by disrupting HIV co-receptor genes, and/or expression of fusion inhibitors. Genetic modification of TRIM5α, SAMHD1, TREX1 or APOBEC proteins may increase antagonism of viral uncoating and reverse transcription. Pharmacological inhibition of HIV integrase, or targeted disruption of integrated provirus, eliminate or prevent establishment of the latent viral reservoir [38].

(1) Disruption of co-receptors so as to avoid viral entry: Using gene therapy
hematopoietic stem cells can be modified in such a way that they start producing CD4+ T-cells with defective CCR5 (chemokine receptor 5) which is the HIV-1 co-receptor, this will avoid the interaction between T-cell and HIV-1 virus therefore T-cell will remain unaffected and will not die [26]. There is a well documentation for genetic disruption of CCR5 in human CD34+ hematopoietic stem cells and CD4+ T-helper cells. Perez and co-workers demonstrated robust disruption of CCR5 in CD4+ cells, engraftment in an NOG mouse model of HIV-1 infection and enrichment during HIV-1 challenge. These autologous, HIV-resistant CD4+ T cells are currently being tested in phase I clinical trials as a novel anti-viral therapeutic [27, 28]. Holt and co-workers performed analogous experiments in human cord blood-derived CD34+ stem cells. These cells engrafted and repopulated hematopoietic niches in all tissues tested in NSG mice, and were enriched following HIV-1 challenge [29].

Using this gene therapy, immune system will not be affected and person will show no symptoms of HIV infection and will be able to live a normal life. This was demonstrated clearly in HIV infected patient of Berlin to whom bone marrow from person who was homozygous for the CCR5-Δ32-bp allele is transplanted .At present patient is not using any kind of drug and is fully healthy. This shows how effective HIV gene therapy is and potential of HIV resistant cell to cure HIV infection [30, 31, 32].

Most of the concern is given to CCR5 but viruses can evolve and use another co-receptor CXCR4 (chemokine receptor 4) to enter in the CD4+ cell and (X4)-tropic HIV strain uses only this co-receptor to enter cell. Cells transduced with CCR5+ defective genes showed excellent protection against CCR5 (R5)-tropic [33], but not CXCR4 (X4)-tropic [34]. In humanized mouse model it has been already shown that disruption of CXCR4 reduces the HIV infection in similar way to CCR5 [35, 36]. In humans no stable CXCR5 null mutation occurs and CXCR4-null mice died during early embryonic development due to multiple defects in hematopoietic, vascular and neuronal development .T-cell specific CXCR4 knockout mice, however, are viable and lack appreciable T-cell defects [27], and Wilen and co-workers [35] have demonstrated that CCR5/CXCR4 doubly-disrupted T cells are viable in vitro. In nut shell, CCR5/CXCR4 double disruption is likely intractable in hematopoietic stem cells, but may represent a viable combinatorial therapy in CD4+ T cells [36].

Thus stem cell therapy offers a way that can pretend to act like transplant done in Berlin patient by engineering hematopoietic stem cells of patient to express anti-HIV genes to provide resistance to infection. Gene therapy provides us tool to reconstitute HIV resistant immune system and confers lifelong protection against HIV and main advantage is that it is a onetime treatment and no further consumption of drug or other treatment is required.

(2) HSCs transduced with triple-combination Anti-HIV Lentiviral Vector:

Today many anti-HIV genes are being designed to inhibit HIV replication. It is known to all that HIV have high rate of mutation therefore single anti-HIV gene may not provide satisfactory result therefore like HAART were combination of many drugs are used in same if we use many anti-HIV gene possibility of formation of escape mutant may end and viral load in patient may decrease to undetectable level or may vanish in small period of time [39]. Most common target sites of the anti-HIV gene include: attachment site, entry site, reverse transcription, and integration [33, 15, 40]. Anti-HIV gene has many benefits over the HAART treatment as it inhibits the formation of pro viruses and keep on replenishing the viral reservoirs that are biggest barrier between HIV infection cure [41, 42]. By combining many anti-HIV genes into a single vector, a efficient pre integration protection can be possessed against HIV infection. A strong pre integration protection from HIV-1 infection, in vitro, was established by Waler and co-workers using a triple combination anti-HIV lentiviral vector containing a human/ rhesus macaque TRIM5à isoform, a CCR5 short hairpin RNA (shRNA), and a TAR decoy [43]. CCR5 short hairpin RNA to antagonize viral entry, a rhesus macaque allele of TRIM5a to antagonize viral un coating, and a viral TAR decoy to down regulate reverse transcription of viral RNA. This vector not only prevented HIV integration in challenged cells but also blocked the generation of escape mutants.
The NRG mouse model (NOD-RAG1/IL2r) double mutant has the potential to evaluate multi-lineage human hematopoiesis from intra-hepatic injection of human CD34+ HSCs into newborn mice. After 3 months of transplantation, functional human T cells, B cells, and macrophages can be detected in lymphoid organs, including the spleen, thymus, and bone marrow. Humanized NRG mice infected with HIV display normal HIV disease symptoms, like, CD4+ cell count decrease and viral load increase in plasma [44]. This mouse model offers a unique preclinical in vivo system to evaluate anti-HIV gene therapy molecules in human cells at a level acceptable to regulatory agencies.

Recently, the preclinical safety and efficacy of a combination anti-HIV lentiviral vector has been evaluated, in vivo, in a humanized NRG mouse model. It was demonstrated that multi-lineage human hematopoiesis from anti-HIV lentiviral vector-transduced CD34+ HSCs in the peripheral blood and in various lymphoid organs, including the thymus, spleen, and bone marrow. After in vivo challenge with either an R5-tropic BaL-1 or an X4-tropic NL4-3 strain of HIV-1, maintenance of human CD4 cells and a selective survival advantage were observed in mice containing the anti-HIV vector-transduced cells. This combination anti-HIV lentiviral vector inhibiting HIV infection in a stem cell gene therapy has potential to be used on humans in future[45].

(3) HIV gene therapy using interfering RNA-based strategies

Recently, new antiviral approaches have been produced to treat HIV infection using RNA interference (RNAi) via gene therapy. It provided us a genetic tool that can be used in any viral or host cell function that is involved in HIV replication cycle [46].

RNAi can be induced by transfection of small interfering RNAs (siRNAs) or by short hairpin RNAs (shRNAs) that are intracellularly expressed from a gene cassette [47]. Viral RNAs or the mRNAs encoding cellular co-factors as target imposes some advantages as well as drawbacks. RNAi targeting host may cause cytotoxicity, but one also cannot prohibit adverse off-target effects of anti-HIV shRNAs. Selection of escape variants is one of the most prominent problem to target virus [48, 49, 50]. Targeting the most conserved and evolutionary static regions of the viral RNA genome may give way for anti-escape approach [51], the simultaneous use of multiple inhibitors in a combinatorial RNAi approach [52] or the use of RNAi reagents in combination with other RNA-based Inhibitors.

Various strategies using RNAi have been used to produce HIV resistant immune system. Like knockout of the chemokine co-receptor CCR5 that restricts the viral entry in CD4+ T cells by RNA interference [14, 34], hammerhead ribozymes [53] and DNA-editing zinc finger nucleases (ZFNs) [29] have been extensively studied. Alternatively, knockout strategies to introduce host restriction factors to block viral replication have also been reported. For example, the gp41-derived peptide (C46) blocks viral entry by preventing fusion [54]. Other restriction factors, such as TRIM5a from rhesus macaques [55], polynucleotide cytidine deaminase APOBEC 3G and 3F [56, 57] and BST2/tetherin [58] can also prevent HIV infection and expand the possible repertoire of molecular targets useful for gene therapy.

In a similar way viral mRNA can be targeted for degradation with RNAi during transcription therefore viral replication will be disturbed [59] and hammerhead ribozymes [60, 61] or sequestering the transcription activator Tat protein by TAR RNA decoy [62], or alternatively blocking Rev-mediated transport by expressing dominant negative Rev mutant (RevM10 [63, 64]), sequestering Rev protein by Rev binding element (RBE) RNA decoy [65] or degrading Rev mRNA by RNA interference [59].

Generally antisense RNAs [66], sense RNAs [67], and ribozymes [68, 69, 70] are used for RNA interference. To inhibit cellular or HIV RNA function antisense RNAs and ribozymes are designed, while sense RNAs is designed to disrupt HIV RNA/protein or RNA/RNA interactions. Live viruses may also be used to cause selective death of the HIV infected cells [71, 72]. A recombinant vesicular stomatitis virus (VSV) was engineered in which the gene encoding the viral glycoproteins was replaced with those encoding HIV-1 receptor (CD4) and co-receptor (CXCR4). This recombinant virus was shown to infect, propagate on, and kill the
HIV-infected cells [72].

**Interfering RNAs:** These are the RNAs which are intentionally designed so as to block HIV life cycle at one or other specific point by interfering with function of cellular (i.e. CCR5 or CXCR4) or HIV-1 RNA or proteins and effectiveness of the interfering RNA depends on site of interference and ability to avoid the formation of the escape mutants [73].

**Table 2.** Interfering RNA-based strategies in HIV gene therapy

**Antisense RNAs:**

Antisense RNAs can be designed to contain or sequences complementary to portions of cellular (i.e. CCR5 or CXCR4) or HIV-1 RNA. The RNA hybrids may then be cleaved by RNase1 [74], which would result in a permanent loss of the target RNA. Antisense RNAs spanning 800 nucleotides or more were shown to inhibit HIV replication more effectively [75, 76, 77, 78]. As antisense RNAs are not likely to be toxic to the cells, they may be expressed in a constitutive manner. Antisense RNAs could, upon hybridization with HIV RNA, disrupt Viral RNA splicing, translation, transactivation, nuclear export of all HIV mRNAs, RNA packaging, and/or reverse transcription of the progeny virus RNA. Lack of protein production would also result in inhibition of protein function. Inhibition of CCR5 mRNA translation would result in inhibition of viral entry and syncytium formation.

<table>
<thead>
<tr>
<th>Interfering RNAs used in HIV gene therapy</th>
<th>Target RNA/protein</th>
<th>Localization required for Activity</th>
<th>Interference site(s)</th>
<th>Fate of HIVinfected Genemodified cell</th>
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<tbody>
<tr>
<td>Antisense RNA</td>
<td>Cellular CCR5 mRNA</td>
<td>Nuclear/Cytoplasmic</td>
<td>Viral entry, syncytium formation with infected cells</td>
<td>Protected</td>
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<tr>
<td>HIV mRNAs</td>
<td></td>
<td>Nuclear/Cytoplasmic</td>
<td>RNA splicing, translation, transactivation, nuclear export</td>
<td>Protected</td>
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<tr>
<td>HIV progeny virus RNA</td>
<td></td>
<td>Cytoplasmic/Virion</td>
<td>RNA packaging, reverse transcription of progeny virus RNA</td>
<td>Subsequent rounds of Infection</td>
</tr>
<tr>
<td>Sense RNA U3-R-U5 RNA TAR/RRE RNA</td>
<td>Incoming virion RNA</td>
<td>Cytoplasmic</td>
<td>Virion RNA reverse transcription</td>
<td>Protected</td>
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<tr>
<td>HIV Tat/Rev proteins</td>
<td></td>
<td>Nuclear</td>
<td>Trans-activation/nuclear export of 4-5 kb and 9.3 kb HIV mRNAs</td>
<td>Protected</td>
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<td>Psi-e RNA</td>
<td>HIV Progeny virus RNA</td>
<td>Cytoplasmic/Virion</td>
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<td>Subsequent rounds of Infection</td>
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**Sense RNAs**

These are designed to contain specific sequences of HIV-1 RNA which are involved in specific viral RNA/RNA or RNA/protein interactions. These RNAs compete with the HIV RNA for binding to viral RNAs or proteins. These may be used to prevent trans-activation, nuclear export Packaging, or reverse transcription of the progeny virion RNA. Sense RNAs with specific sequences present in HIV TAR and RRE act as decoys. These RNAs binds to the corresponding Tat and Rev proteins to decrease the effective concentration of these proteins. And, as Tat/HIV-1 TAR and Rev/HIV-1 RRE interactions are required for transactivation and nuclear export, virus replication would be inhibited.

Sense RNAs possessing HIV-1 Psi signal may form dimers with HIV RNA, which would compete with HIV-1 RNA dimers for packaging into the virions. Furthermore, depending on the presence or absence of various cis-acting
elements required for HIV-1 RNA reverse transcription, the co-packaged sense RNA may either compete with HIV RNA for reverse transcription or prevent both sense and HIV RNA reverse transcription.

Several cellular factors have been characterized, which interact with HIV TAR and RRE. Thus, in addition to inhibiting Tat or Rev function, the decoy RNAs would also inhibit the normal function of these cellular factors and may cause toxicity. Therefore, TAR and RRE may have to be produced in a Tat-inducible manner. However, Tat inducible expression of a molecule that inhibits Tat function may not be ideal, as the amount of TAR produced in the cell may not reach excess concentration required to inhibit virus replication. To solve this problem, minimal TAR and RRE decoys (lacking the binding sites for cellular factors) are being developed that could be constitutively expressed without being cytotoxic. Sense RNAs containing HIV Psi-e (which includes RRE) may be produced in a Tat or Rev inducible manner.

Ribozymes

Hammerhead and hairpin ribozymes are small catalytic RNAs which can be designed to specifically pair with and cleave a specific target RNA in trans \[^68\]. The following criteria must be fulfilled for designing a specific hammerhead ribozyme \[^79, 80, 81\]. The cleavage site within the target RNA must contain an NUH (N, any nucleotide; H, C/U/A) \[^82\]. The ribozyme catalytic domain must contain 11 of the 13 conserved nucleotides \[^83\], and of the H adjacent to the cleavage site \[^79, 83\]. Cleavage by hammerhead ribozymes occurs 3' to the H and results in a 5' product with a 2', 3' cyclic phosphate and a 3' product with a 5' hydroxyl group. Hairpin ribozymes have been derived from the tobacco ring spot satellite virus RNA \[^81\]. The conserved nucleotides within the ribozyme catalytic domain are shown in figure below. The substrate specificity is conferred by providing the ribozyme with nucleotides complementary to the sequences flanking the NGUC adjacent to the cleavage site within the target RNA. Cleavage occurs 5' to the GUC sequence.

Ribozymes may be designed to specifically recognize and cleave a number of sites within a specific cellular RNA (i.e. CCR5 mRNA) or HIV RNA. The most important criteria in designing an HIV RNA-specific ribozyme is to chose a target site that is accessible and highly conserved. Ribozymes may be designed to cleave the incoming HIV virion RNA in the cytoplasm before reverse transcription occurs, the HIV transcripts in the nucleus or cytoplasm, and/or the virion RNA in the progeny virus. The incoming RNA in the cytoplasm or the primary HIV-1 transcripts within the nucleus may be targeted anywhere within the HIV-1 RNA. However, if the cleavage occurs post-splicing within the nucleus or in the cytoplasm, it may be preferable to target regions that are shared by all spliced and unspliced HIV mRNAs. These regions include the first 289 nucleotides within the 5' untranslated region (exon 1), 69 nucleotides near the center (exon 5), and the last 1259 nucleotides near the 3' end (exon 7) of HIV-1 RNA (figure 3). While CCR5 ribozymes would have to be expressed in a constitutive manner, anti HIV ribozymes may be expressed in a constitutive or constitutive and Tat-inducible manner (to allow overproduction in HIV-infected cells).

**Figure 3**: Secondary structure of a trans-cleaving hammerhead (top) and a hairpin (bottom) ribozyme. The catalytic domain is flanked by the 5' and 3' flanking complementary sequences, which are designed to be complementary to the H (hammerhead ribozymes) or NGUC (hairpin ribozyme) adjacent to the cleavage site (â). Target RNA sequences are shown in bold. Cleavage occurs 3' to the NUH (hammerhead ribozymes) or 5' to the GUC (hairpin ribozyme). N, any nucleotide; H, C/U/ A.
Various target sites to block viral life cycle using RNAi

There are many steps in viral life cycle that can be blocked using Interfering RNA-based strategies. These inferring RNA are designed to inactivate cellular RNA (e.g. CCR5 or CXCR4) or HIV RNA encoding factors required by HIV to replicate and expand infection in new cells or also be designed to inhibit the function of viral proteins (i.e. Tat and Rev).

Blocking of viral entry

Using antisense RNAs or ribozymes that inhibit co-receptor (i.e. CCR5, CXCR4) mRNA translation viral entry can be blocked. It also blocks the formation of syncytium in between gene modified HSCs and HIV infected.

Blocking at a pre-integration step:

Incoming HIV-1 virion RNA reverse transcription might be blocked using antisense RNA or ribozymes viral RNA reverse transcription can be restricted. Strategies can be designed to allow the interfering RNA can be designed that can access the incoming HIV virion RNA, before it is reverse transcribed as HIV RNA reverse transcription takes place in partially uncoated virions.

Blocking the post-integration step:

Interference with trans-activation of HIV gene expression:

For transactivation of HIV-1 gene expression Tat/TAR interaction is required. Against the TAR and/or the tat coding region Antisense RNAs or ribozymes could be designed to inhibit trans-activation or TAR decoy RNAs can be developed in use to block Tat protein function.

Interference with nuclear export of singly spliced and unspliced HIV mRNAs:

Interaction between Rev-RRE is required for nuclear export of singly-spliced and unspliced viral RNAs. Against the RRE sequence present in these viral RNAs antisense RNAs and ribozymes could be designed. To inhibit Rev function antisense RNAs or ribozymes may be designed against the rev coding region. RRE decoys can also be synthesised that can block Rev protein function.

Interference with HIV RNA translation:

A common sequence EXON 1 is present in all HIV mRNA against which antisense RNA or ribozymes can be designed to inhibit viral mRNA translation. Thus, further translation of all (2, 4-5, and 9.3 kb) HIV mRNAs would be inhibited.

Intervention at the level of infectious progeny virus production:

Antisense RNAs and ribozymes directed against the coding regions of several viral proteins including Pr55, Gag Pr160, Gag-Pol/Env, Vpu, and Vif that are required for the assembly, release, maturation, and infectivity of virus particles can be done to avoid formation of new viral particles or antisense RNA against factors that facilitate the viral packaging or co-packaging of ribozyme which degrades the viral RNA can be designed to inhibit the expansion of viral infection.

ROADBLOCKS IN GENE THERAPY TO CURE AIDS

siRNA/miRNA (small interfering/micro RNA) screenings, genome wide association studies (GWAS), and their meta-analyses were performed in different populations in order to understand the gross variability observed in genetic propensity towards HIV-1 [84,85,86]. However, the genetic prototype of natural viraemia controllers that empowers antiviral resistance remains largely enigmatic [86].

It was demonstrated in a report that although the recovered T cell population is resistant to CCR5-mediated HIV cell entry, these are not resistant to CXCR4-mediated cell entry by X4 tropic HIV. While this case study indicates the scope of gene therapy as a possible cure for HIV, it also raises issues of enhancing sensitivity of currently employed viral assays, risks from long lived non haematopoietic cell reservoirs, and restraints of X4 viruses. These are critical issues and hopefully answers to some of these would become available on long term follow up [87,86]. Variations in APOBEC3 genes might enhance resistance to vif and influence antiviral activity. A recent study has shown that an African variant of APOBEC3G H186R is associated with high
viral load and progression to AIDS \cite{88,89,86}. Similarly, a deletion of 29.5 kb from the 5 exon to 8 exon of \textit{APOBEC3B} leads to complete loss of \textit{APOBEC3B} and this has been reported to be associated with increased risk of HIV-1 infection and disease progression \cite{88,86}.

**FUTURE PERSPECTIVES**

Vaccines that can place appropriate immune effector responses at these early sites appear to provide meaningful protection. Although there remains much work to be done to optimize these approaches and translate this information to licensable vaccines, the HIV/AIDS vaccine field, for the first time, has a pathway to follow that is based on solid observations of efficacy and the foundation of an increasingly sophisticated understanding of lentiviral immunobiology.

Gene therapy approaches for the treatment of HIV/AIDS hold great promise for a functional cure of the disease. In the most recent study, patients with AIDS-related lymphoma undergoing autologous hematopoietic stem cell transplant were infused with gene-modified, HIV-resistant stem cells to evaluate the safety and feasibility of this approach. Although a low level of gene marking was observed, the feasibility of isolating, gene modifying and delivering a HSPC product was demonstrated. Additionally, this study demonstrated long term expression of anti-HIV RNA sequences (> 3 years) demonstrating the potential for a long-lasting antiviral effect. In order to obtain substantial numbers of gene-modified lymphocytes resistant to HIV infection, it is still necessary to use myeloablation for stem cell transplants or repeated infusions of modified differentiated T cells for T-cell therapy. There is added difficulty of efficiently transducing stem cells to provide a sufficient population of gene-modified stem cells that can give rise to HIV-resistant progeny in the long term.

Additionally, the large-scale production of viral vectors required for current gene therapy protocols can be costly. Thus, there is a need for better stem cell isolation and expansion protocols that do not result in loss of pluripotency. Results from early stage clinical trials have shown that gene-modified stem cells can engraft and reconstitute the hematopoietic niches. This is promising in combination with reports about naturally cycling repopulating cells. It is possible that in the future the approach of permanent HIV gene therapy will change based on these findings.

For example, it is conceivable that future protocols will comprise a continuous treatment with only few but highly pure repopulating cells that have been genetically modified prior to infusion. Repeated infusions of small numbers of cells could eventually lead to filling marrow niches with gene-modified progenitors. It may also be feasible to reconstruct the ‘Berlin patient’ results using ZFNs to disrupt the CCR5 gene in hematopoietic stem cells, followed by mini-transplants. Improvements in combinatorial approaches involving anti-HIV small RNAs and proteins with low immunogenic profiles and identification of promoters better suited for continuous gene expression are expected to enhance anti-HIV resistance of individual cells. With the advances in our knowledge of HIV-1 biology, novel cellular targets can be identified, thereby expanding the repertoire of potential targets for HIV-1 gene therapies while reducing the likelihood of viral escape. As these obstacles are overcome, expect to see wider applications of gene therapy for the treatment and perhaps ‘curing’ of HIV-1 infection.

**REFERENCES**

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