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Gene Therapy for HIV Infection by 'Intracellular Immunization' with Antiviral Genes

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Abstract

Gene therapy for HIV infection either aims to eliminate HIV-infected cells or to suppress virus replication by expression of antiviral genes in the major target cells for HIV. Here, the latter approach, which has been termed 'intracellular immunization', is described. The elements that are crucial for this strategy to be effective are optimized gene transfer tools and protocols, the careful choice of a target cell for genetic modification, and a highly effective combination of antiviral genes. Only if all these components are integrated into an optimized gene therapeutic process is there a chance for gene therapy to be effective clinically in HIV infection.

Key words

Gene therapy. Antiviral genes. HIV dynamics.

Introduction

Antiviral drug therapy (HAART) has considerably improved the survival of HIV-infected patients. However, limitations of HAART, such as drug-resistant HIV variants and toxicity have become more and more evident. The development of additional therapies for HIV infection, such as gene- and immunotherapy, therefore appear warranted.

A large number of different gene therapeutic strategies have been proposed (reviewed in[9]). These can be grouped into two basic approaches. The first aims to eliminate HIV-infected cells. Examples are the induction of cell death by HIV-activated caspases, targeting toxins to HIV-infected cells, introducing a universal T cell receptor for HIV-infected cells into cytotoxic T cells (CD4zeta), or augmenting the patients' immune response by genetic vaccination protocols[20,23,44,67]. The second basic approach involves the introduction of antiviral genes into target cells of HIV-1 or their progenitor cells. Lymphocytes or hematopoietic stem cells are collected, genetically engineered *ex vivo* with an antiviral gene and then infused back into the patient. This approach has been termed 'intracellular immunization', although the name may be misleading as no immune reaction is actually required for this principle to be effective.

For both therapeutic approaches extensive basic research and preclinical studies have been performed. In addition, more than 20 clinical trials have been approved by the NIH[1]. Yet, only few trials have been completed and the results published. None of these could show a clear benefit of a gene therapeutic strategy for the HIV-infected patient. Nevertheless, it is by far too early to conclude that gene therapy for HIV infection will never be effective. In contrast to a therapy with drugs, gene therapy for HIV infection involves a complex therapeutic process in which all steps must be optimized. Here, the major components required for a successful intracellular vaccination for HIV infection are discussed with a focus on the predicted impact of different antiviral genes on virus and cell dynamics in the HIV-infected patient.

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Tools for gene transfer

Inefficiency of gene transfer has been one of the major obstacles on the way to successful gene therapy for many diseases. Initially, several groups used physicochemical gene transfer methods, but currently viral vectors are most widely used. Protocols for retroviral vectors have been extensively optimized to allow high levels of transduction especially for lymphocytes but also for hematopoietic stem cells. One crucial improvement has been the use of the envelope protein of gibbon ape leukemia virus (GALV) to pseudotype retroviral vectors. In addition, the transduction on fibronectin leads to colocalization of vector and cells and thereby augments transduction efficiency considerably[10,11,51].

A promising alternative is the use of lentiviral vectors (LV) derived from HIV which have several advantages over the murine leukemia virus (MLV)-derived retroviral vectors generally used. In contrast to MLV vectors, LVs transduce non-dividing lymphocytes, which reduces the time for *ex vivo* culture for gene transfer. Cis elements of LVs can act as RNA decoys and thereby additionally inhibit HIV replication. Furthermore, LVs can be mobilized and transferred to new T cells by replicating HIV thus increasing the number of gene-modified cells. Although the possible contamination of vector stocks with a replication-competent recombinant lentivirus is no more a major problem with the newest generation of safety-modified packaging systems, lentiviral vectors still have major drawbacks. Stable producer cells for lentiviral vectors are not available, which makes large-scale production of vector stocks for clinical application difficult. Some anti-HIV genes also interfere with the production of vector with the HIV-derived lentiviral packaging systems[42]. In addition, the GALV Env, which mediates high level transduction of lymphocytes and stem cells, is incorporated inefficiently into lentiviral particles[8]. In conclusion, as gene transfer techniques are being further improved, future clinical trials are expected to show higher levels of gene transfer and thus more likely a clinical benefit for the HIV-infected patient.

Genetic modification of lymphocytes versus hematopoietic stem cells

There are several obvious reasons why many groups have targeted anti-HIV genes to T lymphocytes. T helper cells are the major reservoir for HIV[69]. Protocols for efficient gene transfer into primary peripheral blood lymphocytes have been established[10]. Moreover, lymphocytes in the adult apparently regenerate mainly from pre-existing mature T cells[70]. It is therefore not unexpected that in clinical trials gene-modified T cells were found to survive for many years, especially if the transgene confers a selective advantage[43,47].

Hematopoietic stem cells have also been used as targets for genetic modification with anti-HIV genes since they have two major advantages. They are long-lived and theoretically allow a life-long production of gene-modified progeny. Moreover, although T lymphocytes are the major reservoir of HIV infection, virus replication can also be detected in monocytes and macrophages[26,68,69]. Stem cell gene transfer would lead to genetic modification of all these cellular reservoirs for HIV. However, the principal problem of a stem cell-based gene therapy for AIDS is that antiviral genes do not confer a major selective advantage to stem cells and therefore conditioning of the patient before stem cell transplantation is expected to be required to achieve significant engraftment. Therefore, many trials aim to show clinical benefit of an antiviral gene (proof of principle) with gene-modified lymphocytes before considering the more invasive stem cell therapies.

Antiviral genes

The gene product that mediates the antiviral effect is either a protein or an RNA. Both have advantages and disadvantages. Proteins can be immunogenic, while RNAs are not. On the other hand, some proteins can be engineered to act on non-transduced cells (bystander effect, such as secreted sCD4, neutralizing antibodies, cytokines), which is a major advantage in comparison to the antiviral RNAs.

Several types of antiviral proteins are known. Dominant-negative mutants of viral proteins can inhibit the function of the wild-type viral proteins. Examples are transdominant Rev and Tat mutants[39,48]. Correspondingly, expression of mutated cellular proteins, such as soluble CD4 or mutEIF-5A[5], can interfere with essential functions of the wild-type cellular proteins within the viral life cycle. Ectopic expression of certain cytokines has been shown to inhibit HIV replication. Examples are interferon α/β and interleukin[13,16,32,56,75]. Finally, antibodies that bind and inactivate viral proteins can be expressed within the cell as single-chain fragments (SFvs: anti-Rev, anti-IN, anti-RT) or secreted as a neutralizing antibody into the supernatant[35,55,61].

Three major groups of antiviral RNAs have been used for intracellular immunization against HIV: (i) Antisense-RNAs have been described for many regions of the HIV RNA genome. (ii) Expression of small RNA fragments containing RNA elements crucial for HIV replication can lead to competitive inhibition of the function of this element in the viral genome. Examples for these so-called RNA decoys are the RRE-, TAR- and primer binding site (PBS) decoys. (iii) Ribozymes that target and cut specific sequences within HIV mRNA and the RNA genome have been shown to effectively inhibit HIV replication at several levels.

A proposed classification of antiviral genes

The effect of an antiviral drug on virus and T cell dynamics in the patient is expected to depend directly on the degree of inhibition of HIV replication. Moreover, the overall decrease of viral load in the patient is largely independent of the step at which the viral replication cycle is inhibited[14]. This is ex-

pected to be different for antiviral genes. Since the total number of target cells for HIV-1 in the patient is over 10^{11}, genetic modification *ex vivo* of all these cells will not be feasible in near future, neither by a T cell nor by a stem cell-based approach. Application of cells containing an antiviral gene can thus only lead to an overall reduction of viral load and a clear clinical benefit, if the genetically protected cells have a selective advantage over the non-modified cells and accumulate with time to a significant portion. Virus and T cell dynamics will thus be complicated by the fact that two groups of T cells are present in the patient, a genetically protected and a susceptible population.

In a complex system, such as the virus-infected patient, it is difficult to predict the overall outcome of the modification of one element in the system, such as the transfer of genetically protected cells. However, certain effects of the different antiviral genes in the patient seem probable. Inhibitory genes have been described for all major steps of HIV replication (Fig. 1). Here, we argue that the outcome will depend crucially on which steps of the viral life cycle are inhibited in gene-modified cells. A novel classification of antiviral genes into three groups is proposed based on the predicted effect on virus dynamics (Table I).

In the first class, we have grouped those genes that inhibit early steps of virus replication, including the integration of provirus, and thereby prevent virus infection of the cell. With respect to virus and T cell dynamics (adapted from[49]), as represented in figure 2, class I genes cause a reduction of the rate constant (κ) for the generation of infected T cells (T*) from non-infected cells (T). Examples are the membrane-anchored peptides as described below that inhibit entry of HIV and intracellular single-chain antibodies to reverse transcriptase (RT) and integrase (IN). The second group of genes do not interfere substantially with the early phase of infection, but inhibit expression of viral proteins and thereby reduce the amount of virus released from infected cells (N in Fig. 2) and the viral cytopathic effect (reduction of death rate, δ in Fig. 2). Only genes in this group have been used so far in clinical trials as they have the strongest antiviral activity in cell culture. Examples are tdRev (RevM10) and RRE decoys[4,72]. Genes in the third class reduce the number of infectious virions released from the cell. However, these class III antiviral gene products neither protect the

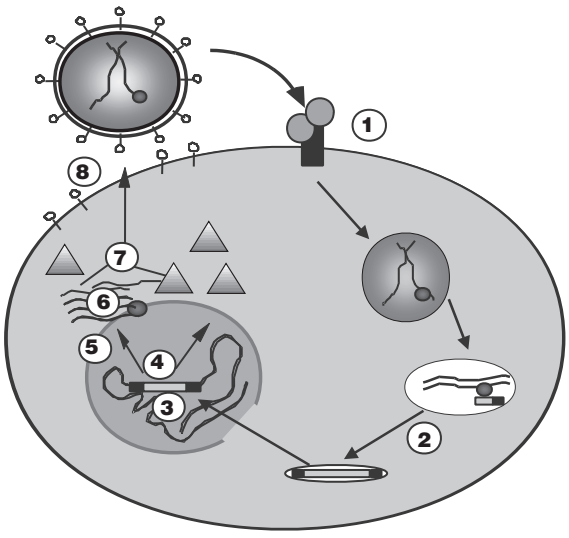

Figure 1. *Action of different antiviral genes. Secreted neutralizing antibody, secreted sCD4 and membrane-anchored peptides can inhibit entry of HIV (1). Intracellular SFvs to reverse transcriptase and integrase inhibit reverse transcription and integration, respectively (2, 3). TAR decoys and transdominant (td) Tat mutants inhibit expression of viral proteins (4). RRE decoys and tdRev inhibit export of unspliced viral RNA (5). Ribozymes cut viral RNA and antisense RNAs most likely decrease the stability of viral RNAs (6). Td Gag proteins interfere with the assembly of infectious virions (7), while Gag-nuclease fusion proteins lead to the degradation of viral genomic RNA in the virions (8).*

Table I. *Classification of antiviral genes according to the effect on virus and T cell dynamics*

Group	I	II	III
Replication step inhibited	Before integration	Protein synthesis	Infectious particle production
Parameter primarily affected*	$\kappa \searrow$	$N \searrow, \delta \searrow$	$N \searrow$
Selective advantage	yes	yes	no
'Pseudolatency'	no	yes	no

*As in figure 2.

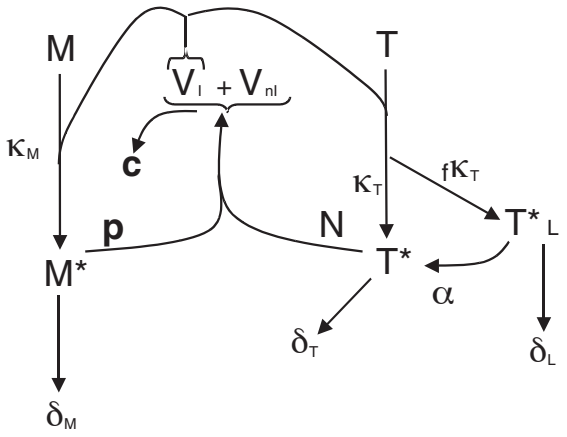

Figure 2. *Schematic representation of virus and T cell dynamics. T, uninfected T cells; κT, rate constant for infection of T; VI, infectious free virus; VNI, non-infectious virus; c, clearance rate of VI and VNI; T*, infected T cells; N, burst size of V produced from T; δT, death rate of T*; T*L, latently infected T cells; δL, death rate of T*L; fκT, rate constant for generation of T*L; α, rate at which T*L are activated to T; M, non-infected macrophages; κM, rate constant for the infection of macrophages; M*, infected M; δM, death rate of M; p, rate of virus production by M* (adapted from[14].)*

cell from a viral cytopathic effect nor from recognition by the immune system, as virus protein production is not reduced (N is reduced while the death rate of infected cells, δ, is not influenced). Examples are td Gag proteins and Gag-nuclease fusion proteins[49,59,65].

Predicted effect of antiviral genes on T cell and virus dynamics

These three classes of antiviral genes are anticipated to differ considerably in their effect on virus and T cell dynamics (Table I). Genes in the first group are clearly expected to confer a selective advantage to the cell and lead to the accumulation of gene-modified, non-infected cells. Genes of the second group also confer a selective advantage. However, gene-modified cells that are selected and accumulate in the patient will harbor suppressed HIV-provirus. These cells resemble latently infected cells and are quasi in a 'pseudolatent' state. The effect on total virus production of such an accumulation of 'pseudolatently' infected cells is difficult to predict, but will not necessarily involve a substantial reduction of viral load. Class III genes do not confer a selective advantage. Their possible benefit in gene therapy of HIV infection could therefore only be in combination with group I or II antiviral genes that allow accumulation of genetically protected cells. These considerations are clearly in favor of the inclusion of group I antiviral genes in intracellular immunization strategies.

Class I antiviral genes

Only few class I genes have been described (Table II). Virus entry can be inhibited by a neutralizing human monoclonal antibody (2F5) or by soluble (s)CD4, both secreted by cells, into which the corresponding genes have been introduced[55]. Reverse transcription and integration can be inhibited by expression of intracellular single-chain Fv (SFv) antibody fragments. These antiviral genes, however, have low antiviral activity[35,61]. Entry of M-tropic viruses can be inhibited by downregulation of the CCR5 receptor. This has been achieved by expression of an anti-CCR-5 ribozyme as well as SFv antibodies to CCR5 that are retained in the ER (intrabody)[18,62].

Recently, we have described a membrane-anchored peptide expressed from a retroviral vector that inhibits entry of a broad range of HIV isolates with high efficiency[24]. The peptide used is derived from the C-terminal heptad repeat of HIV gp41 envelope glycoprotein (T20) and, as a free peptide, is known to inhibit HIV-1 fusion and entry at nanomolar concentrations[17]. The proposed mechanism of action is shown in figure 3. However, the free peptide is not orally bioavailable, has an extremely short half-life of less than 2 hours, and large-scale production is expensive still. The aim was to overcome these drawbacks by direct expression of the inhibitory peptide in the target cell of HIV-1.

A retroviral vector was designed to express a membrane-anchored form of T20 as a fusion protein (M87)[24]. This protein has an N-terminal signal domain derived from the human low affinity nerve growth factor receptor (LNGFR) followed by the antiviral peptide sequence (T20), a flexible linker derived from the murine IgG heavy chain and a C-terminal membrane anchor (MSD) derived from the truncated LNGFR (Fig. 4).

The T helper cell line PM-1 was transduced with M87. Cell surface expression of T20 was detected by flow cytometry and replication of HIV-1 was inhibited more than 100-fold in the M87-transduced bulk cultures (Fig. 5). Expression of T20 as well as inhibition of HIV replication was also shown for infected peripheral blood T lymphocytes. Replication of several primary HIV isolates from different clades (B, D, E, O) and even of HIV-2 was reduced in T cells expressing membrane-anchored T20 (M87).

The model that M87 acts at the level of virus entry was tested by single-round infections with different

Table II. *Summary of published anti-HIV genes*

Class	Gene	Citations	Potentially immunogenic	Potential bystander Effect
I	Secreted soluble CD4	45,46	no	yes
	Secreted neutralizing antibody	55	yes	yes
	Anti-CCR5 ribozyme	3,18	no	no
	CCR5-intrabody	62	yes	no
	Membrane-anchored entry inhibitors	24	yes	yes
	SFv to RT	61	yes	no
	SFv to IN	6,29,35	yes	no
II	IL16	75	no	yes
	Antisense RNA	12,41	no	no
	TdRev	39,40,50,73	yes	no
	TdTat	19,48	yes	yes
	MutEIF-5A	5	(yes)	no
	TAR decoy	37,63	no	no
	RRE decoy	2,33	no	no
	Antisense RNA	27,33,42,66	no	no
	Anti-Rev SFv	16,25	yes	no
	Anti-Tat SFv	52	yes	no
	Ribozymes	21,22,30,36,57,71,74	no	no
III	tdGag	65	yes	no
	Mutated $tRNA^{Lys}$	38	no	no
	Psi-sense/antisense RNA	15	no	no
	PBS decoy	28	no	no
	Gag-nuclease fusion protein	59	yes	no
	CD4 chimera	54	(yes)	?
Non-classified	Interferon α/β	13,32,56	no	yes

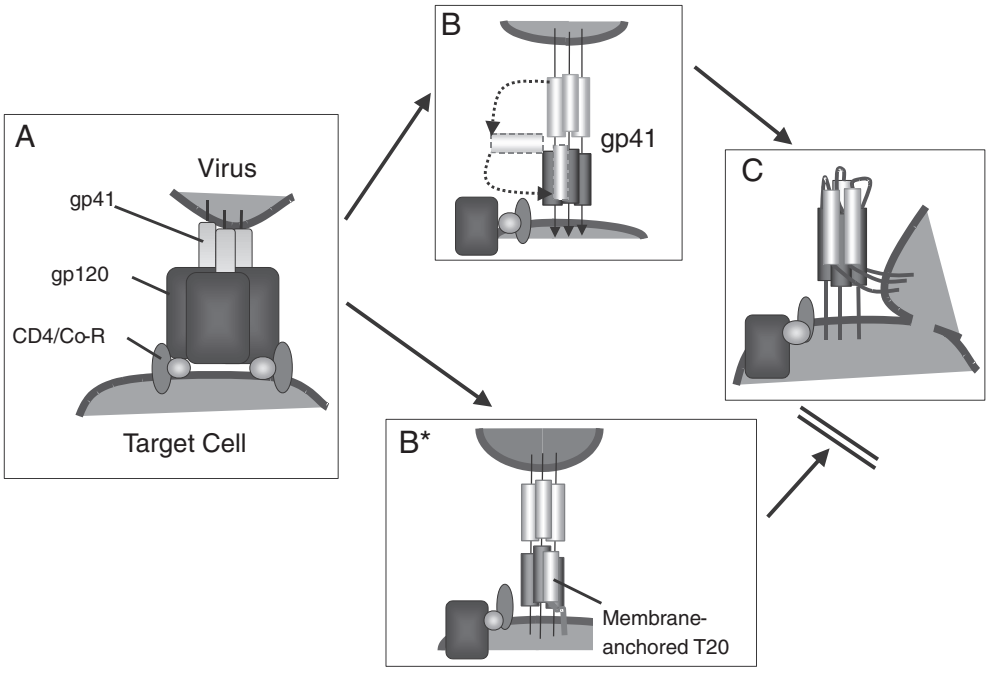

Figure 3. *Inhibition of entry by M87. HIV envelope glycoprotein trimer is composed of the surface protein gp120SU, which is anchored to the virus membrane by gp41TM. Virus entry is initiated by binding of gp120 to the CD4 receptor and a chemokine receptor, both associated with a conformational change of gp120 (A). Fusion of the viral and cellular membrane is then mediated by gp41 (B->C). gp41 has two alpha helical heptad repeat regions, here represented by cylinders. Three N-terminal helices form a central coiled-coil and the C-terminal helices pack into the three grooves thereby formed (C). The membrane-anchored peptide, which is derived from the C-terminal heptad repeat, binds to the N-terminal coiled-coil of HIV gp41 and thereby locks the gp41 into a fusion-incompetent state (B*).*

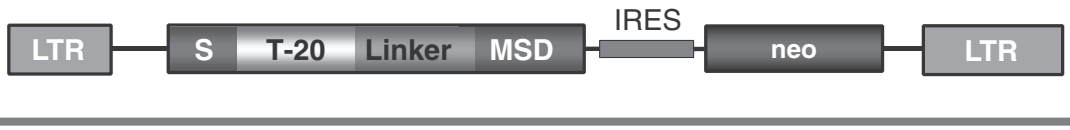

Figure 4. *The M87 retroviral vector encoding membrane-anchored T20. Membrane-anchored T20 is a fusion protein composed of an N-terminal signal peptide derived from LNGFR that directs transport into the ER lumen, followed by the T20 peptide, a flexible linker derived from the IgG hinge and a C-terminal membrane-spanning domain again derived from LNGFR.*

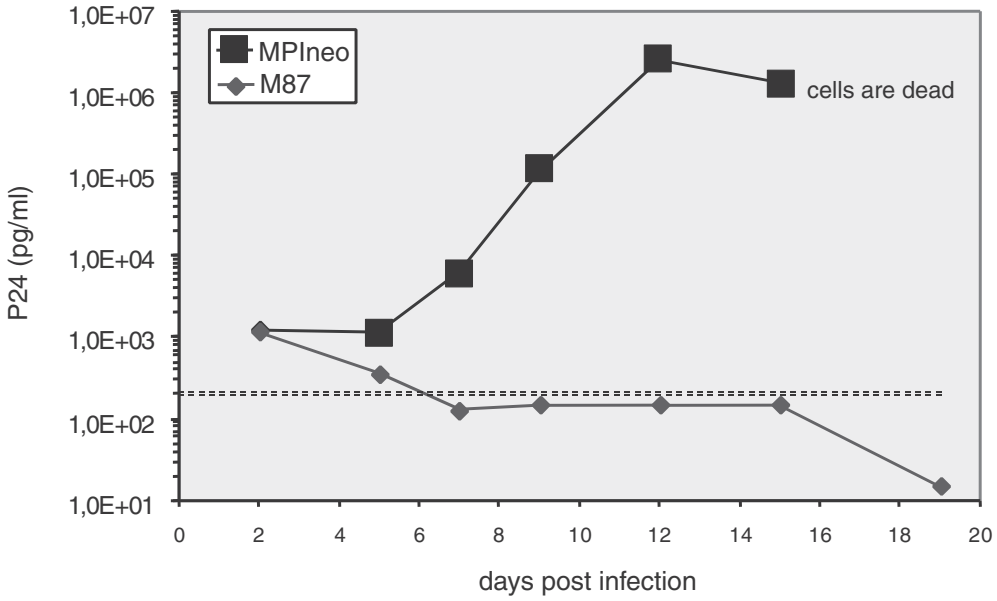

Figure 5. *M87 inhibits replication of HIV-1. PM-1 cells were transduced with M87 expressing membrane-anchored T20 and in parallel with a control vector containing the neo selection marker only. G418-selected bulk cultures were infected with an moi of 0.05 NL4-3 and p24 production was monitored. The cut off of the p24 ELISA is shown (dotted line).*

replication-incompetent viruses. Single-round infection with replication-incompetent HIV-1 was inhibited by M87 independent of co-receptor usage. In addition, the transduction efficiency of a murine leukemia virus-derived vector pseudotyped with a truncated HIV env was also reduced for cells expressing M87. In contrast, infection with a vesicular stomatitis virus G protein-pseudotyped HIV lentiviral vector was not inhibited. These results clearly show that virus replication was blocked at the level of HIV Env-mediated entry.

No adverse effects on cell growth or expression of surface antigens were seen. Currently, detailed preclinical toxicity and efficacy studies are performed to evaluate the feasibility of a clinical application of M87. Although a combination of antiviral genes will most likely be required for patients to benefit, membrane-anchored fusion inhibitors are a good candidate for a group I gene component in an effective gene therapeutic strategy for AIDS.

Class II antiviral genes

Most anti-HIV genes described to date are class II genes (Table II). In this class, expression of viral proteins is suppressed which leads to a prolonged survival of infected cells and a reduction of virus production. Transcription of viral RNA can be inhibited by TAR decoys or transdominant Tat protein[7,19]. Both interfere with the interaction of the viral Tat protein with the viral TAR RNA element, which is required for efficient transactivation of HIV transcription.

Nuclear export of unspliced genomic HIV RNA, which is translated into viral structural proteins and packaged into viral progeny, is crucial for efficient virus replication and is highly regulated. Export of this RNA is dependent on the binding of the viral Rev protein to an RNA element in the HIV RNA, the RRE sequence. Rev is produced from spliced RNA early after infection. As Rev accumulates, it mediates a switch from the expression of spliced RNA to genomic RNA by its interaction with the RRE sequence within the viral genome. Transdominant Rev (tdRev such as RevM10) as well as an RNA decoy derived from the RRE element can both inhibit this interaction of Rev and RRE and thereby prevent efficient export of HIV genomic RNA[34,39,73]. Clinical trials have been performed for both tdRev and for RRE decoys. A selective advantage for lymphocytes transduced with tdRev was found in all three trials

performed[53,64,72]. However, *in vivo* selection of protected cells was not sufficient to convey an overall clinical benefit. An RRE decoy was retrovirally transduced into hematopoietic progenitors in a further trial involving HIV-infected children. In this study, the number of gene-modified cells was too low to draw any conclusions[31].

Ribozymes can specifically cut HIV RNA. Although we have grouped ribozymes into the class II, ribozymes most likely also act at the pre-integration level of the viral life cycle by reacting with the infecting RNA genome[57,74]. A major drawback of ribozymes, however, is that single nucleotide substitutions can lead to resistance and the emergence of virus escape mutants. Two small clinical studies, one with CD4+ lymphocytes and one with CD34+ hematopoietic progenitors were performed. Marking in both studies was low but there was some indication for a possible selective advantage conferred by the ribozyme[9].

Antisense RNAs can be expressed as a long antisense molecules of 1.000 bp and more from an RNA Pol II-dependent promoter or as a short antisense sequence of less than 100 bp within a tRNA or snRNA[42]. Antisense RNAs to HIV most likely inhibit viral gene expression at several levels[21,60,71]. Proposed mechanisms of action are interference with RNA processing, degradation of viral RNA by cellular enzymes, or hindrance of translation. In addition, some antisense RNAs are packaged into viral particles and thereby reduce virus infectivity. One major advantage, especially of the long antisense RNAs, is that mutations within the viral target RNA leading to as much as 14% sequence divergence are well tolerated. Resistant mutants are therefore less likely to arise for antisense RNA than for ribozymes[42].

Class III antiviral genes

Class III genes allow production of viral proteins and thereby do not protect the cell from the cytopathic effect of HIV. However, they inhibit the production of infectious particles either by interfering with the assembly of virions or by decreasing the infectivity of produced particles. Several examples of class III genes have been described. Expression of a CD4 chimera with the ER retention signal of the CD3epsilon chain interferes with the transport of HIV Env protein, which interacts with CD4 in the ER, to the cell membrane. This leads to a reduction of the number and infectivity of released particles[54]. Transdominant Gag proteins have been described that inhibit packaging of HIV[65]. Gag-nuclease fusion proteins are packaged into virions and then decrease the infectivity of released particles by digestion of the virus genome[58]. A mutated $tRNA^{Lys-3}$ that binds to the TAR region of the HIV genome instead of to the primer binding site was expressed in HIV-infected cells. This mutant tRNA is packaged into virions and considerably reduces virus infectivity [38]. Finally, antisense RNA to the primer binding site and primer binding site RNA decoys decrease the infectivity of virions released from infected cells[15,28]. None of these class III genes have been tested in clinical trials so far.

Conclusions and outlook

Success of gene therapy for HIV infection will depend on three crucial elements. Gene transfer vehicles and protocols must be used that allow a high level of gene transfer without loss of function of the gene-modified cells. The cell type that will provide a long lasting pool of target cells protected from HIV infection must be defined. Finally, antiviral genes must be carefully evaluated and selected with respect to those parameters that will be crucial for success. These parameters are: inhibitory activity, potential toxicity and immunogenicity, a bystander effect of the gene product and, as discussed here in detail, the step in the virus life cycle that is inhibited. A particular challenge will be to define a combination of genes that act synergistically to allow a high level of inhibition of HIV with a minimal chance of resistance to the antiviral gene product.

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