Development of lentiviral vectors for gene therapy for human diseases

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Retroviral vectors derived from murine retroviruses are being used in several clinical gene therapy trials. Recently, progress has been made in the development of vectors based on the lentivirus genus of retroviruses, which ironically includes a major human pathogen, human immunodeficiency virus (HIV). As these vector systems for clinical gene transfer are developed, it is important to understand the rationale behind their design and development. This article reviews the fundamental features of retrovirus replication and of the elements necessary for development of a retroviral vector system, and it discusses why vector systems based on HIV or other lentiviruses have the potential to become important tools in clinical gene therapy.

Introduction

Retroviral vector systems developed from murine oncogenic retroviruses have been extensively analyzed and used for preclinical and clinical gene transfer studies. Although much has been learned about the basic science of retrovirus replication and the issues surrounding the delivery of foreign genes into cells from these studies, the inability of these vectors to infect nondividing cells is 1 of the factors that limit their usefulness in clinical gene therapy applications. To circumvent this problem, vector systems based on the lentivirus genus of retroviruses, which includes human immunodeficiency virus (HIV), are being developed. Although these novel vectors show promise for future use in clinical studies, the complex biology and mechanisms of pathogenesis of the lentiviruses complicate their choice for clinical applications. The goal of this review is to discuss the advantages and disadvantages of these vectors so that rational decisions regarding their clinical use can be made and questions raised by patients and physicians interested in their application can be addressed adequately.

Basics of retrovirus biology

To discuss retroviral vector systems, a basic understanding of the retrovirus virion structure, genome, and replication cycle is necessary. Retroviruses are RNA viruses that replicate through a DNA intermediate. Retroviral particles consist of 2 identical single-stranded RNA molecules and virus replication enzymes contained within a viral protein core. This structure, in turn, is surrounded by the viral envelope, made up of host cell membrane and viral-encoded envelope glycoproteins. Basic features of a generic retroviral replication cycle are shown in Figure 1. Infection begins when a virion enters a susceptible target cell through the specific interactions between the envelope glycoprotein and the cellular receptor(s). Viral–cell membrane fusion and virion internalization (by direct fusion or endocytosis) result in release of the virus core into the cell cytoplasm. Viral RNA is reverse transcribed into double-stranded DNA and transported to the cell nucleus. The mechanism and timing of virion uncoating, reverse transcription, and nuclear transport are not entirely understood. Viral DNA is permanently integrated (hence referred to as the provirus) into chromosomal DNA, where it is replicated during the cell cycle, just as cellular genes are, and subsequently passed to daughter cells. This feature makes retroviral vectors useful for permanently introducing foreign genes into cells. Proviral DNA is transcribed into RNA and transported to the cytoplasm, where it can be translated into viral proteins. Viral precursor structural proteins and replication enzymes assemble with viral RNA to form new virion cores, which obtain the viral envelope glycoprotein as they bud from the cellular membrane. Further processing of the precursor core proteins result in the formation of mature, infectious progeny virus particles.

The earliest studied retroviruses were oncogenic retroviruses isolated from avian and murine hosts. These viruses share similar genome structures and are capable of causing neoplastic disease in their respective natural hosts. The genome structure for a prototypical oncoretrovirus, murine leukemia virus (MLV), is shown in Figure 2. All retroviruses encode gag, pol, and env genes. The protein products of these genes are unique to each type of retrovirus, but they do share basic common features. In infected cells, the proviral genome is transcribed into a single precursor mRNA from the viral promoter located in the 5' (upstream) long terminal repeat (LTR), with the 3' (downstream) LTR containing transcription termination and poly A signals. The gag gene encodes the viral core proteins, whereas the pol gene encodes the viral replication enzymes. These 2 genes are initially expressed as a Gag–Pol fusion polyprotein. The viral protease self-cleaves the Gag–Pol precursor and further processes the Pol protein to individual protease (for cleaving viral precursor proteins into their mature forms), reverse transcriptase (for replicating viral nucleic
Retroviral vector systems

Because retroviruses can permanently integrate into a chromosome of an infected cell and subsequently express viral genes in that cell and its progeny, oncoretrovirus derivatives have been used to introduce foreign genes of interest into cells, thereby setting the stage for the development of clinical gene therapy—the treatment of disease by the introduction of therapeutic genes into cells. In fact, MLV vectors are the most commonly used vector systems to introduce foreign genes into cells in clinical gene therapy trials (though MLV is a murine retrovirus, envelope glycoproteins from some MLV strains, such as the amphotropic group, can mediate virus entry into human cells).

Retroviral vectors are virus derivatives typically engineered to be replication defective. They are capable of infecting and integrating a foreign gene into a target cell but are unable to multiply and spread to other cells. Vectors are created by removing viral genes from a virus genome, leaving only the cis-acting sequences (regions recognized by viral proteins) necessary for a single round of replication. A diagram for a “typical” oncoretroviral vector is shown in Figure 3. As illustrated, all viral genes are removed and replaced with either a marker gene (for example, a gene that confers drug resistance) used to monitor the vector infection of cells or another foreign gene of interest. More complex vectors capable of expressing 2 or more foreign genes through additional heterologous, internal viral promoters or internal ribosome entry site sequences have been designed. Also illustrated in Figure 3 are cis-acting sequences that must be present on the vector for propagation to take place. These sites are used during various stages of virus replication, including reverse transcription of genomic RNA (LTR, primer binding site, polypurine tract), integration of the viral DNA into chromosomal DNA (att sites), transcription of the provirus (LTR), and packaging of viral RNA into progeny virions (encapsidation site).

Constructs containing these cis-acting sequences can be propagated in cultured cells and can therefore serve as vectors when gag, pol, and env gene products are provided in trans (expressed from other constructs). This trans-complementation can be accomplished in a number of ways. Vector and wild-type virus DNA plasmid constructs can be cotransfected (introduced by chemical or electrical means) into cells in culture. When vector and wild-type virus (also called “helper virus” because it assists in vector propagation) constructs are contained within a single cell, the gene products provided by the replication-competent virus result in the production of virus particles containing the vector RNA genome. These virions can then infect and introduce the vector genome into other cells. Although there are times when this strategy for vector production is useful, the fact that replicating wild-type virus is...
present prevents the study of a single viral replication cycle and obviously would be unacceptable for clinical use.

Techniques capable of propagating vectors without using wild-type virus have been developed. Cotransfection of a vector DNA construct and a plasmid expressing viral gene products (but not containing cis-acting sequences necessary for propagation) results in a single cycle of vector propagation. Because the construct(s) expressing viral proteins do not contain sequences necessary for replication, helper virus is not produced. After virions containing the vector genome are harvested from cotransfected cells and are used to infect fresh cells, the vector will not be propagated further because the infected cells will not be expressing viral proteins.

The third and probably best method for propagating retroviral vectors involves the use of packaging cell lines, which are cell lines that express viral proteins necessary for vector propagation. Such cell lines have improved greatly the efficiency with which virus containing vector genomes can be produced. Packaging cell lines are made by introducing viral genes into cells, where they are stably maintained so that viral structural proteins and replication enzymes are produced constitutively or on induction. After introduction of a vector genome into these cells, vector particles are produced (Figure 4). Again, helper virus should not be produced, and the vector is propagated only over a single replication cycle. Packaging cell lines are the preferred method to propagate a retroviral vector for 2 primary reasons. First, the process used to generate the virus is generally simpler and more efficient at producing a higher titer of vector virus than the cotransfection techniques. Second, the use of packaging cells reduces the possibility that wild-type virus will be regenerated inadvertently by recombination between helper and vector sequences during the transfection process; therefore, the virus is considered safer for clinical use. Nonetheless, packaging cell lines are regularly and rigorously tested for replication-competent retrovirus by using long-term culture to amplify any regenerated wild-type virus, thus increasing the sensitivity of detection.

**Lentiviral vector development**

The lentiviruses, when compared to oncogenic retroviruses, have a more complex genome and, consequently, a more complex replication cycle. HIV-1 has been the most studied lentivirus; its genome structure is illustrated in Figure 2. Although the basic genome organization is the same as for oncoretroviruses, HIV has additional accessory genes, some of which play crucial roles in the virus replication cycle. For example, the Tat and Rev proteins are essential for efficient viral gene expression. Tat activates the promoter in the HIV LTR so that viral RNA is produced efficiently. Rev interacts with a region of viral RNA known as the Rev-responsive element (rrr) and promotes the transport of viral RNA from the cell nucleus to cytoplasm.

More relevant to the significance of lentiviral vector development are viral components that enable HIV productively to infect nondividing and terminally differentiated cells, something that oncoretroviruses cannot do. Although oncoretroviruses can enter nondividing cells, the viral genome is excluded from the cell nucleus by the nuclear envelope, the breakdown of which occurs during the normal course of cell division; therefore, the replication cycle of an oncoretrovirus can only be completed in an actively dividing cell. Although the precise mechanism by which lentiviruses infect nondividing cells is unknown, for HIV it appears to be facilitated by several viral proteins: the integrase protein, the matrix protein from Gag, and the accessory protein Vpr. Both integrase and matrix protein contain signal sequences for nuclear localization. In contrast, Vpr appears to bind directly to the nuclear pore complex. Exactly how these elements direct the viral genome through the nuclear envelope and into the nucleus of nondividing cells and the timing of nuclear transport in relation to other aspects of viral cell entry, uncoating, and reverse transcription are not completely understood. However, these signals appear to operate through independent mechanisms because the removal of 1 or the other still allows infection of the nondividing cells.

Use of oncoretrovirus vectors could be limited in many potential clinical applications in which nondividing cells are the targets for gene therapy (examples include hematopoietic stem cells, which rarely divide, and terminally differentiated neurons; even tumor cells, another potential gene therapy target, do not all divide at any given moment). Because the lentiviruses can infect nondividing cells (though, in at least some cell types, progression through the cell cycle from G0 to at least G1 b is necessary for efficient, complete reverse transcription of the viral genome), there has been great interest in the development of lentiviral vectors for use in clinical gene transfer studies. Even so, the complex genome and replication cycle of lentiviruses, best exemplified by HIV-1, made the development of vectors and packaging cell lines more difficult than the development of analogous vector systems for oncogenic retroviruses. For example, whereas oncoretrovirus vectors could be propagated simply by providing analogous vector systems for oncogenic retroviruses. For example, whereas oncoretrovirus vectors could be propagated simply by providing analogous vector systems for oncogenic retroviruses. For example, whereas oncoretrovirus vectors could be propagated simply by providing analogous vector systems for oncogenic retroviruses. For example, whereas oncoretrovirus vectors could be propagated simply by providing analogous vector systems for oncogenic retroviruses.

In the section titled “HIV-1 vectors,” the reader is referred to the discussions of the retroviral packaging cell. A detailed description of the HIV-1 vector is found in Figure 4. In the section titled “HIV-1 packaging cell line,” the reader is referred to the discussions of the retroviral packaging cell. A detailed description of the HIV-1 vector is found in Figure 4.
generating stable packaging cell lines, possibly because of the toxicity of some HIV proteins.

Because of these difficulties, many of the first HIV vectors made were simply nearly intact viral genomes containing disruptions or deletions of the HIV env gene, with insertion of a marker gene expressed from an internal, heterologous viral promoter (for example, the simian virus 40 promoter driving expression of a drug-resistance gene) at this location. Because these constructs expressed all HIV genes except env, viral envelope was provided in trans for vector propagation. Use of the HIV envelope glycoprotein enabled vectors to be targeted to CD4-positive cells but limited the cell types that could be infected, and it consistently resulted in low vector titers. Other viral envelope proteins were found capable of substituting for the HIV envelope; use of the amphotropic MLV envelope glycoprotein or the vesicular stomatitis virus G-protein (VSV-G) broadens the types of cells that can be infected by the vector. The use of VSV-G also yields higher vector titer and results in greater stability of the vector virus particles.

A second type of vector more closely resembled the oncoretroviral vectors in that all viral genes were deleted, leaving only the necessary cis-acting sequences. Viral proteins were provided in trans. Marker genes were expressed either directly from the HIV LTR or from an internal promoter. Engineering gene expression from the HIV LTR (requiring Tat, normally found only in HIV-infected cells) enabled foreign gene expression to occur specifically in Tat-expressing cells, but use of an internal promoter increased the types of cells that could express the marker gene.

These vectors were useful for studying a single cycle of HIV replication and learning how to devise future vector systems but were still inferior to oncoretroviral vector systems. Because no packaging cell lines for HIV existed, vector propagation was dependent on cotransfection of vector and helper DNA plasmid constructs, resulting in lower titers and increased risk for generating recombinant replication-competent virus. This possibility is more of a concern with HIV than with oncoretroviruses because of overlapping cis-acting sequences that must occur on HIV vectors and helper constructs. For example, the rev sequence to which the Rev protein binds to promote RNA transport must be present on both constructs. In addition, the packaging signal for HIV extends into the gag gene; therefore, part of the gag gene must be present on the HIV vector to optimize vector propagation.22,24

Newer generations of HIV vectors, based on new knowledge of the HIV replication cycle, have been designed. Relatively high titers of VSV-G pseudotyped HIV-1 vectors have been produced. HIV-1 vectors expressing an internal marker gene were shown capable of infecting cells in culture that had been treated to arrest the cell cycle.25 Subsequent animal studies showed that HIV-1 vectors could infect terminally differentiated neural cells and that marker gene expression was stable for months.25-27 The HIV vectors, unlike oncoretroviral vectors, also have been shown to efficiently infect retinal, hepatic, and muscle cells.29 In addition, HIV-1 vectors have been shown to infect CD34+ NOD/SCID mouse repopulating cells; such infected cells were transplanted into mice and were shown to be capable of engrafting and differentiating into multiple hematopoietic cell lineages.30 A recent report notes the ability of such vector-infected cells to be serially transplanted in NOD/SCID mice, suggesting infection of the hematopoietic stem cells.31

Continued basic research has enabled HIV vector systems to be improved, resulting in safer and higher titered vectors. The HIV packaging signal has been further characterized.32 It also was found, in some cases, that Rev and RRE can be replaced by the cis-acting constitutive transport element from Mason–Pfizer monkey virus33 to minimize the overlap of sequences in vector and helper constructs. In addition, it has been shown that HIV-1 vectors can be propagated without the expression of any accessory genes.34 Stable expression of HIV proteins in cells, an important step in the development of HIV packaging cell lines, has been documented.

Although the HIV-1 vector systems have been the most extensively studied, other lentivirus vectors also have been developed. Development of human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus (SIV) vectors have faced technical problems similar to those encountered with HIV-1 vectors.37,38 HIV-2 is genetically more closely related to SIV than to HIV-1, is less pathogenic in humans, and can be studied in a primate animal model; therefore, HIV-2 vector systems may offer some advantages in clinical gene therapy applications. Furthermore, in certain situations, HIV-2 vectors may be preferable to HIV-1 vectors for delivering therapeutic genes that target HIV-1.39 In addition to HIV-2 and SIV vectors, chimeric vector systems composed of HIV-1 and HIV-2 components40 or of HIV-1 and SIV components41 have been described.

Lentivirus vectors from nonprimates, including felines and equines, are also being developed. Vector systems based on feline immunodeficiency virus (FIV) have been described.42,43 FIV causes acquired immunodeficiency syndrome in felines but is not known to infect humans or to cause human disease, though some viral strains can productively infect some human cells in culture.45 In the vector systems described, modification of the FIV LTR and use of a heterologous cytomegalovirus promoter were able to overcome a block to FIV replication in human cells and to enable the efficient expression of FIV vector constructs and viral proteins. The FIV vectors pseudotyped with the VSV-G envelope glycoprotein were capable of infecting a broad range of nondividing and terminally differentiated human cells. The titer of vector virus produced was in the range seen with HIV-1 vectors. In addition, an analogous vector system based on equine infectious anemia virus (EIAV) has been described.46

### Future applications and concerns

The continuing development of lentiviral vectors may be an important advance in moving clinical gene transfer closer to the goal of being a viable option for the routine treatment of a variety of diseases. Many disorders are potential targets for gene therapy using either lentiviral vectors or oncoretroviral vectors. These include genetic disorders, such as hemophilia, and neoplastic or infectious diseases. The ability of lentiviral vectors to infect nondividing or terminally differentiated cells may expand possible disease targets or facilitate the development of gene therapy treatments. For example, neurologic disorders may be more easily treatable with vectors that can deliver genes directly to neurons.

In addition, lentiviral vectors may increase the ease and efficiency of gene transfer to target cells. For many current clinical trials, gene transfer involves attempts to isolate and stimulate the division of hematopoietic stem cells, the ex vivo infection by vector-containing virus, and the return of treated cells to the patient. This is a time-consuming, inefficient, and expensive
process. Lentiviral vectors may decrease the need for much of the ex vivo cell manipulation and, though other obstacles remain that prevent efficient in vivo gene delivery, may bring the goal of direct in vivo retroviral vector infection of target cells closer to reality. A recent study demonstrated the ability to prevent or delay photoreceptor degeneration in a mouse model of retinitis pigmentosa by delivering the gene for the rod photoreceptor cyclic guanosine monophosphate phosphodiesterase-β subunit by HIV vector injections injected directly into the subretinal spaces of newborn mice.47 A report using an EIAV vector to correct, in lymphoid cells in culture, the defective gene present in Fanconi anemia group C was encouraging.48

Other concerns with current retroviral vector systems include the inability to target the vector to specific cells, insufficient vector titers, decreasing levels of foreign gene expression over time, the possibility of insertional mutagenesis by the vector as it integrates into the host genome, and the possibility of wild-type virus replication competent retrovirus) generation.49 Lentiviral vector systems share these concerns with oncoretroviral vector systems but also raise other issues. The use of HIV-derived vector systems clearly raises concern that wild-type HIV, a human pathogen, could be generated during vector production, even though no wild-type virus has been observed in the systems studied so far. Minimizing HIV sequences on the vector and on the helper sequences will help to prevent recombination from occurring, but the overlap of the HIV packaging signal in the vector and the gag gene in the packaging plasmid will not allow overlap to be completely avoided. To increase safety during clinical use, analysis of virus preparations using long-term culture and polymerase chain reaction assays should be used to prevent the possibility of RCR contamination. Regarding possible insertional mutagenesis by lentiviral vectors, it is encouraging that this phenomenon has not been observed, even in patients with HIV infection who have high viral loads. It must be kept in mind, however, that in patients infected with HIV, the cytopathic effect of wild-type HIV and the limited cell types that the virus naturally infects could mask the mutagenic potential of the virus.

A possible problem with the use of HIV vectors could occur if, after treatment with an HIV vector, a patient subsequently becomes infected with HIV. This situation might result in mobilization of the vector (because the wild-type HIV could then act as a helper virus) with subsequent spread to other tissues in the body or to other people. The design of vectors containing potential “suicide” genes which, if necessary, could be activated and thus destroy cells containing vector genomes (for example, expression of herpes simplex thymidine kinase, the toxicity of which could be activated by the administration of ganciclovir49) would help address this concern. Although HIV vector mobilization can be viewed as a drawback for gene therapy of non-HIV diseases, it can actually be an advantage for delivering anti-HIV genes in patients infected with HIV50 because the therapeutic genes would be spread to a greater number of appropriate target cells. Another issue with HIV vector use concerns the possibility that patients receiving this vector may mount an immune response to antigens in the virus particles, resulting in seroconversion. These patients then would test positive in an HIV antibody test, even though there had been no exposure to wild-type HIV. This could cause diagnostic confusion, psychological stress, and great inconvenience, though further testing could resolve the issue.

Use of non–HIV-1 lentiviral vectors avoids some, but not all, of the issues raised above. Clearly, the primate viruses HIV-2 and SIV are closely related to HIV-1, and some may feel there would be only limited advantages to their use in a clinical setting. The development of nonprimate vectors, such as those based on FIV and EIAV, is encouraging in that neither virus is known to cause human disease. Use of FIV or EIAV thus decreases the possibility that an infectious, wild-type virus known to be a human pathogen will be regenerated during vector production, and it eliminates the problem of possible HIV seroconversion. In addition, though there is no evidence to suggest that the use of HIV vectors might pose risks to patients receiving them or to the population at large, the use of nonprimate lentiviral vectors may help ease the psychological barriers to applying lentiviral vectors in clinical settings.

However, more information regarding the biology and the pathogenesis of primate and nonprimate lentiviruses in their natural hosts and further characterization of vector systems derived from these viruses are needed before any system can be deemed superior for clinical use. Furthermore, it is unknown whether any vector or packaging sequences could recombine with endogenous retroviral sequences present in cells or with other viruses49,51,52 to produce novel, dangerous pathogens. Potentially, such new pathogens may not only cause harm to the treated patient but could spread to other persons or even to other species, resulting in unpredictable consequences. The possibility of such an event, though remote, must be kept in mind when designing and developing vector systems, particularly when these systems use heterologous viral promoters and envelope glycoproteins, or, in some cases, chimeric viral particles. As recent events have shown,53 gene therapy is a young field with potentially unrecognized hazards that mandate a slow and thorough evaluation by those involved in the field.

Nevertheless, despite the challenges and questions that remain, lentiviral vectors do show promise for future clinical use. Thorough study and characterization of the molecular biology and pathogenesis of these viruses and of the vector systems developed from them are necessary but achievable steps in moving forward to clinical studies. Risks and benefits can be quantitated, as they can for any medical or surgical treatment in use, so the scientific and medical communities, along with patients, can make informed decisions as to how these technologies might be used.

References

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