Retroviral vectors and gene therapy: An update

S K Maurya*, Sushant Srivastava and R K Joshi
Department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry
Narendra Deva University of Agriculture and Technology, Kumarganj, Faizabad 224 229, India

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Gene therapy aims at treatment of diseases by transfer of genetic material into specific cells of a patient. So the transduction of appropriate target cell is critical. Retroviruses infect nearly every cell in the target population and become integrated in host cell genome for a stable expression. All retroviruses have only three structural genes gag, pol and env, except for lentiviruses where two other genes tat, rev and four accessory genes are required (vif, vpu, nif, vpr). There are two components of retroviral vector system—i) Packaging cell lines, which provide the products of gag, pol & env genes but are unable to package itself as they lack the ψ sequence, and ii) retroviral vectors where gag, pol and env are deleted but ψ sequence along with LTR is present. Several other types of vectors are also described.

Keywords: Gene therapy, retroviruses, vector

Introduction

Development of recombinant DNA technology provided a strong ray of hope for improving the practice of medicine. Most of the advances affecting the clinical management of patients have involved either the development of new molecular techniques for the diagnosis of specified inherited and acquired diseases, or the development of new therapeutic products made possible by the ability to engineer the over expression of specific genes. However, the idea underlying gene therapy—that diseases might be treated by transfer of genetic material into specific cells of a patient, rather than by conventional drugs—has yet to make its mark in medicine. One of the challenges of gene therapy is the efficient delivery of genes to target cells. Although the nucleic acids containing the genes can be generated in the laboratory with relative ease, the delivery of these materials into a specific set of cells in the body is far from simple. Various methods have been developed to deliver the genes into cells. Some of these methods use physical approaches to delivery, such as, direct DNA injection, encapsulation of DNA into liposomes, and gene gun technology1-3. Other methods take advantage of viruses, a class of intracellular parasites, to deliver the genes into the target cells. In general, viruses are more efficient at delivering genes to target cells than physical methods. The transduction of appropriate target cells represents the critical first step in gene therapy; consequently, the development of methods of gene transfer suitable for different forms of therapy has been a major focus of research. The single common feature of these methods is the efficient delivery of genes into the cells.

Viruses from several different families have been modified to generate viral vectors for gene delivery. These viruses include retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses, picornaviruses, and alphaviruses4-7. Retroviruses were the first viruses to be modified for gene delivery, and retroviral vectors are used in the majority of all gene therapy clinical trials8. In the case of retroviral vectors and adeno-associated virus vectors, the transferred DNA sequences are stably integrated into the chromosomal DNA of the target cell. These vectors have been considered most often for ex vivo gene therapy, which involves removal of the relevant target cells from the body, transduction of the cells in vitro and subsequent reintroduction of the modified cells into the patients.

Retroviruses: Genome and Its Life Cycle

Retroviruses are found throughout the animal kingdom, including almost all species, and were first identified in chickens as cell free oncogenic factors. The retroviral virion is a spherical particle of about 80-100 nm in diam. They are single stranded RNA viruses. Retroviruses are RNA viruses that replicate
through an integrated DNA intermediate. The virions contain reverse transcriptase enzyme, integrase, and two identical subunits of RNA (diploid) held together in dimer linkage in a capsid surrounded by a host lipid bilayer (envelope) into which the viral env gene product is embedded. Upon infection, RNA is converted into DNA by reverse transcriptase, initially in cytoplasm. Subsequently, DNA and integrase along with associated proteins, the pre-integration complex, is translocated into nucleus where linear viral DNA gets into the host chromosomes. Most interesting and unique to retroviruses is the generation of long terminal repeats (LTRs) in DNA, which serves as sites for covalent association with host DNA, a step generally referred to as integration and is carried out by viral integrase in conjunction with host cell proteins. The integrated genome commonly referred to as provirus, with flanking LTRs serves as template for synthesis of viral RNA by the host RNA polymerase II. LTRs also contain powerful transcription initiation and termination/polyadenylation signals, 5’ LTR serving as the site of initiation and 3’ LTR serving as the site of termination/polyadenylation.

All retroviruses carry a minimum of three structural genes gag, pol and env, which are essential for replication and encapsidation of viral particles. In simple viruses like avian leukemia virus (ALC) and murine leukemia virus (MLV), the three genes, gag, pol and env, are sufficient. A protease encoded as a part of gag or pol is essential for processing of gag or pol polyprotein. In complex human retroviruses, as exemplified by human immunodeficiency virus (HIV), several additional proteins are required both for efficient expression of viral genes and for pathogenesis. These include tat, rev, nef, vpr, vif and vpu. Tat is necessary for transactivation of viral and cellular genes and rev functions in regulation of RNA splicing and in RNA export to cytoplasm. Nef appears to be involved in signal transduction and progression to AIDS. Vif and vpu are required for assembly of infectious virus particles and release by budding from the membrane, respectively. Vpr is involved in early stages of infection.

In addition, retroviruses must contain in their genome packaging signals for encapsidation of viral RNA. This packaging sequence which is called psi is localized at 5’ end usually between 5’ LTR and gag gene. Viral RNA can efficiently packaged and replicated provided the genome contains LTR and psi sequence. All structural genes can be replaced with foreign genes of interest and the products gag, pol and env can be supplied in trans, which forms the basis of retroviral vectors used in gene therapy. It is the ease of manipulation of retroviral genome, because of the unique arrangement of their genome, that makes retroviruses so attractive for vectors of gene therapy.

During pathogenesis, the virus first infects the target cells. The env proteins bind to the cellular receptor and the particle enters the host cell, probably via a process of receptor-mediated endocytosis. Following shedding of the outer envelope, the viral RNA is copied into DNA by the process of reverse transcription. This takes place within the RNA/protein complex and is catalyzed by the reverse transcriptase enzyme of the pol region.

The double stranded linear DNA molecule produced by reverse transcription is made circular and inserted in random sites in the cells’ genome using a pol product, integrase protein. This protein recognizes sequences at the ends of the LTRs to direct linear integration of the DNA provirus in such a way that the provirus is always joined to host DNA, 2 bp from the ends of the LTRs. Host cell enzyme, such as RNA polymerase II, are now used by the integrated virus to express the gag, pol and env proteins from spliced RNA transcripts. A splice-donor signal (AGGT) at nucleotides 203-206 downstream of the 5’ LTR is used in conjunction with splice-acceptor (sa) sites in the genome, principally a major 3’ splice site at about 6 kbp in to the MoMLV genome, upstream of the env sequences.

The final step in pathogenesis is the packaging of viral components. The full-length transcripts are packaged in to viral particles at the membrane of the cell, following recruitment of the proteins synthesized earlier in the cycles. Packaging of the genomic transcripts in to the virion particles requires an RNA signal known as the psi sequence that lies 3’ of the 5’ LTR and, therefore, downstream of the splice-donor site. This ensures that any spliced messages from the viral genome will not include the psi site and so will not be packaged. Hence, only full-length genomic transcripts become packaged into virions. Further studies of psi sequence have shown that high titer packaging of genomes is possible by inclusion of small region of the gag gene from bases 215 to 1039 in the MLV. This revised psi packaging sequence is included in most of high titer retroviral vectors.
A viral protease encoded in the pol region cleaves the envelope polyproteins into smaller components, and virus particles “bud off” from the cell surfaces. Many of the retroviruses are shown to be replication-defective that produce oncogenicity after substitution of a part of their normal viral gene complement with an oncogene sequence. Second category of retroviruses are replication competent retrovirus that also cause malignant disease and a range of other pathogenic states in a wide range of species. However, many retroviruses cause life long infections and appear to be relatively benign in their normal host species. In case of mice, there are retroviruses that are very closely related to oncogenic retroviruses but are not oncogenic themselves. There are some retroviruses like spuma virus or foamy virus that are not linked to any pathogenic state. Similarly, there is also a range of endogenous retrovirus sequences that are not associated with specific pathologies.

**Strategies for Retroviral Vector Construction**

To achieve the aims of carriage and expression of non-viral genes, the construction strategy must encompass two considerations:

- The vector must be able to behave as a viral genome to allow it to pass as a virus from the producer cell line. Hence, its DNA must contain the regions of the wild type retroviral genome required in cis for incorporation in a retroviral particle.
- The vector must contain regulatory signals that lead to the optimal expression of the cloned gene once the vector is integrated in the target cell as a provirus. These may or may not be provided by viral DNA sequences.

Almost all the genomic sequence can be deleted to make a vector; however, there are some essential sequences and these are:-

1. The ψ sequence that ensures the packaging of the vector DNA into virions. This sequence corresponds to nucleotides 215-565 in the MoMLV sequence. More recent vector constructs contain the extended ψ sequence, which extends up to nucleotide 1039, incorporates the start of the gag gene (ψ+), but with the AUG start codon of the viral gene mutated.
2. The tRNA binding site that is necessary to prime reverse transcription of the RNA from the vector (in the virion) into the target cell (-PBS).
3. The sequences in the LTRs that permit the ‘jumping’ of the reverse transcriptase between RNA strands during DNA synthesis.
4. Specific sequences near the ends of the LTRs that are necessary for the integration of the vector DNA in to the host cell chromosome in the ordered and reproducible manner characteristic of retroviruses.
5. The sequences adjoining the 3’ LTR that serves as the priming site for synthesis of the plus strand of DNA molecule (+PBS).

**Construction of Vector**

Retroviral vectors may be either replication-competent or replication-defective. The viral gag, pol and env genes encode diffusible proteins, which act in trans and, therefore, can be deleted from the vector and replaced with exogenous genes. The missing functions can be supplied by another virus, which acts as a helper. Helper cells are designed to support the propagation of retroviral vectors. The viral proteins in the helper cells are expressed from helper constructs that are transfected into mammalian cells. In helper cell lines that were initially developed, all of the viral genes were expressed from one helper construct. Examples of these helper cells are C3A2 and ψ-28-29.

Virions containing replication-defective vectors are generated in packaging cell lines30 carrying helper provirus. Thus, a retroviral vector system can be divided in two components—i) the packaging cell lines, and ii) the retroviral vector.

i) Packaging Cell Lines

The design of retrovirus packaging cell lines has been evolved to address the problem of spontaneous helper virus production encountered with early designs31. Early packaging cells contained replication-competent retroviral genomes, from which the packaging signals of the virus have been deleted. These deleted viruses produced all of the retroviral proteins, but genomic RNA was poorly encapsidated into virions and the virus spread very slowly. However, a single recombination event between this deleted retrovirus and a retroviral vector introduced in to the packaging cells could result in the production of wild-type virus. Further modifications of the viral genome contained in packaging cell lines were done where both packaging signals and 3’ LTR were deleted. These require two recombination events to produce wild type virus. More recently, helper free MoMLV derived packaging lines have been...
developed. These constructions employ a strategy in which the \textit{gag} and \textit{pol} genes are expressed on a single plasmid and the \textit{env} gene is expressed on a second plasmid, essentially eliminating the production of helper virus via recombination.

ii) Retroviral Vectors

The usefulness of retroviral vectors is enhanced by the inclusion of a dominant selected marker, such as, the gene for neomycin resistance, \textit{neo}. Selection of cells with the drug G418 allows for the isolation of \textit{neo}-resistant colonies which contain the retroviral vector. The efficiency of gene transfer is dependent upon the titer of the vector. The titer of a virus producing cell line is assayed by infecting target cells with vector containing culture supernatant from the producer cell line and selected for \textit{neo} colonies. Viral titer is calculated as colony-forming units per milliliter of culture supernatant used for infection (cfu/mL). Virus titer can be influenced by vector size, in which vectors too small or too large are not packaged efficiently in to virions. This also can be influenced by \textit{cis} elements. For example, MoMLV vectors containing packaging signals plus a 5' portion of \textit{gag} sequences produce up to 200-fold higher titers than corresponding vectors with only the packaging signal.

Recently, a simple approach has been developed that dramatically expands the range of genetic elements transferable by retroviral vectors. The system employs \textit{in vitro} generated RNA to nucleate retroviral virions, completely circumventing packaging cell genomic transcription and translation.

The utility of a vector depends not only on the number of cells infected, but also on the level of expression of the transduced gene. A variety of vector designs have been developed.

\textit{Double Expression (DE) Vectors}—They carry two genes, a selectable marker gene and the cloned gene of interest, both of which are driven by the 5' LTR promoter. This design depends on proper splicing to occur because one of the genes can only be expressed as a spliced mRNA. However, expression of both functions from the LTR has often been shown to give disappointing levels of co-expression, and low titers from producer lines. This may be, in part, because the retroviral \textit{(gag, pol)} message and the \textit{env} message are generated at different levels during expression of the provirus. LTR shut-off also limits the usefulness of this design.

\textit{Vectors with Internal Promoters (VIP)}—They express the selectable marker using the 5' LTR promoter and the cDNA of interest from a promoter cloned 3' to the marker gene. The internal promoter is generally a SV\textsubscript{40} early promoter. Since no splicing is required to express any mRNA species, the vector also contains the \textit{sd} mutation. However, in vectors with two transcription units, in which one is expressed using the LTR based promoter and the other is driven from an internal promoter, problem can arise both in virus titers and in the relative level of expression of the two functions. This arises from an effect known as "promoter selection". It has been noted that an LTR can exert a negative effect on other promoter downstream of it.

A VIP type vector N2 contains the packaging signal sequence plus 418 bp (base pairs) of \textit{gag} sequence fused to \textit{neo} and produces 10-50 fold higher titers than similar vectors with out the \textit{gag} sequences.

\textit{Self Inactivating Vectors (SIN)}—The retroviral DNA will integrate into the genome of the target cell largely at random, but in a predictable, structurally and functionally intact fashion—a fact that is an attraction of the system. However, if a retrovirus integrates with in a critical cellular gene, it may disrupt the coding sequence; alternatively integration near such a cellular gene may lead to the phenomenon of insertional mutagenesis. In these instances, the retroviral regulatory sequences can influence expression of the nearby cellular gene. This can lead to the inappropriate (over) expression of the cellular sequences, primarily as a result of the influence of the strong retroviral-enhanced elements in the both the 5' and 3' LTRs. Several enhancer deleted vectors with a deletion in the U3 region of the 3' LTR have been reported. The original such a vector contained a 299 bp deletion that removed the 72 bp repeats (with promoter and enhancer activity).

A self-inactivating vector was developed where U3 region of the 5' LTR in vector constructs was replaced with cytomegalovirus promoter, resulting in \textit{Tat}-independent transcription but still maintaining high level of expression. A self inactivating vector was constructed by deleting 133 bp in U3 region of 3' LTR, including the \texttt{TATA} box and binding sites for transcription factors Sp1 and \texttt{NF-kB}.

A SIN lentivirus was constructed by deleting 400 bp in 3' LTR, which included \texttt{TATA} box also. These vectors are able to transduce neurons and T cells.
Double Copy Vectors (DC)—They also attempt to bypass transcriptional interference from LTR. In this design, the transduced cDNA and a promoter are cloned in to U3 of vector 3’ LTR. As in SIN vectors, the modification is copied in to both 5’ and 3’ of the provirus. The copy in the 5’ LTR is upstream of the retroviral transcriptional units and thus avoids any transcriptional interference from the LTR. In addition, the 5’ gene transcript runs through the promoter/enhancer sequence of the 5’ LTR U3 before reaching the poly A signals in the 5’ LTR segment. This may cause interference of the 5’ LTR driven viral transcript and may, therefore, indirectly relieve transcriptional interference of the downstream gene, potentially allowing for even higher gene expression. The Double Copy Reverse Design—It prevents any interference between the 5’ gene transcript and the LTR driven transcript. Highly successful modification design of the DC design include the use of RNA Pol III driven tRNA promoters for the efficient expression of small RNA transcript and the combination of two genes between the LTRs and one gene in the double copy position to express three different cDNA in a single vector.

Avian Retrovirus Vectors

It is highly beneficial to develop transgenic poultry free of leucosis or to increase genetic traits. Avian retrovirus vectors are best suited for this purpose. Several groups constructed replication competent avian retrovirus vectors for the transfer and expression of gene cassettes in avian cells. Using this vector, they have successfully inserted retroviral genes into the chicken germ line. In order to expand host range of avian retroviruses, chimeric vectors containing env gene of amphotropic MLV and gag and pol genes of Rous sarcoma virus have been constructed and shown to transfer genes into cultured mammalian cells. Avian leucosis virus has been used to transduce exogenous genes into early somatic stem cell or into germ cells. Microinjection of vector directly under blastoderm was shown to be expressing in the semen of hatched male birds. Primordial germ cells (PGCs) isolated from gonads could be transfected by a retrovirus carrying β-galactosidase gene.

Murine Retrovirus Vectors

Idea of using retroviruses to transfer genes into cultured cells, animals and humans for correcting various genetic defects dates back to early 1980s. Since then several improvements have been made in the design of safe vectors, which found their way into clinical trials. A replication-defective murine leukemia virus (MLV) vector is by far the most frequently used retrovirus vector for gene transfer and several clinical trials are ongoing. MLV are more stable, have faster growth kinetics but the level of expression seems to be cell line dependent.

Lentivirus Vectors

Human immunodeficiency virus (HIV) has been incriminated as the etiologic agent for acquired immunodeficiency syndrome (AIDS). It infects a gamut of different cells which bears CD4+ receptor and co-receptors, such as CXCR-4 or CCR-5. The co-receptors, termed chemokine receptors, are present on a number of cells including macrophages (CCR-5), T-lymphocytes (CXCR-4), dendritic and neuronal cells. Gene transfer to central nervous system targeting a number of neurological diseases required introduction of genes into terminally differentiated neurons, which are refractory to infection by MLV based vectors. Since lentiviruses infect dividing and non-dividing cells, vectors based on these retroviruses are being used for gene therapy.

In order to construct a lentivirus vector, it is essential to supply in trans not only the structural proteins but also the regulatory proteins. Packaging cell lines based on HIV-1 has been developed and their betterment is going on. In a packaging cell line a small portion of env gene was deleted and the downstream or 3’ LTR was replaced by heterologous poly A signal to express viral proteins. As necessary to eliminate packaging of this viral RNA, a 37 bp sequence, which constitutes packaging signal, was deleted. A selectable marker can be used to introduce this DNA in to HeLa cells. Then the packaging vector can be transfected and viruses can be produced with a titer of about 10^6 units/mL.

Clinical Trials and Obstacles

A large number of studies have successfully demonstrated introduction of genes into tissue culture cells, animals and humans. The National Institute of Health (NIH), USA has approved more than 100 gene therapy protocols since 1989. Severe combined immunodeficiency (SCID), caused by the deficiency of adenosine deaminase (ADA), is the first genetic disorder approved for gene therapy. Using the self inactivating retroviral vectors, it has been shown that ADA gene could be successfully transferred into
cultured mammalian cells and mice, and into bone marrow and peripheral blood lymphocytes of patients with SCID. A related MLV vector LASN, in which ADA gene is driven to expression by MLV LTR and a bacterial neoR gene under control of SV40 promoter, has been used to successfully transfer and express ADA enzyme in neonates who were diagnosed as having ADA deficiency. Umbilical cord blood was collected at birth from these three infants, transfected ex vivo with MLV-ADA and then the cells were transplanted back into the patients by infused intravenously into each neonate on d 4 of life. All three children are making the enzyme even after 3 yr, although at a very low level54. Now attempts are being made to increase the frequency of gene containing cells55,56. These results show a tremendous promise for gene therapy for other genetic disorders as well as for HIV-infection in newborns.

A partial success has been reported in treating malignant brain tumours by intra-tumoural implantation of retroviral vector-producing cells57. Herpes simplex virus thymidine kinase (HSV-tk), which can efficiently phosphorylate ganciclovir (GCV), has been cloned in MLV immediately downstream of 5′ LTR, followed by SV40 early promoter driving neoR gene used as a selected marker. The vector is packaged in PA 317 cells and the virus producing cells were directly implanted into 15 patients by computed tomography (CT)-guided stenotaxic injection in linear tracts. Treatment of patients with GCV has resulted in reduced tumor size in 5 of the smaller tumors. These techniques, however, need to be improved.

Apart from the strategies for elimination of cancer cells by suicide gene therapy as above, reversion of cancer cells to normal cells by delivery of a functional tumour suppressor gene58 and modification of cancer cells to elicit stronger immune responses59 are also being used.

Retroviral vector-based gene therapies have also been used in clinical trials to treat HIV-1 infection. Generally, these treatments have involved modification of the syngeneic lymphocytes ex vivo using retroviral vectors and are designed to suppress the expression of viral genes. These strategies include use of antisense RNA, mutant trans-dominant regulatory proteins or ribozymes that are targeted to cleave viral RNA. Using MLV-based vector for delivery, hairpin ribozyme that cleave conserved sites in HIV-1 LTR and pol have shown to confer protection from HIV-1 infection of T cell lines, primary T cells and macrophages-like progeny of CD34+ hemopoietic progenitor cells60-62. Another approach is to modify autologous fibroblasts to express a part of the HIV Env so that a host immune response can be elicited63.

Another potential application of gene therapy is to prevent severe graft-versus-host disease that often results from allogeneic bone marrow transplantation. In a gene therapy clinical trial, the bone marrow donors’ lymphocytes were first transduced with a retroviral vector encoding the HSV-tk gene. HSV-tk is not toxic by itself; however, HSV-tk can phosphorylate a nontoxic prodrug named ganciclovir (GCV) to activate the toxicity of the drug. The HSV-tk-expressing cells were then used for bone marrow transplantation. Patients that developed graft-versus-host disease were treated with GCV to eradicate the donor cells that were mounting an immune response against the host. The results of this clinical trial showed that this approach is effective in controlling graft-versus-host disease64.

Apart from these successes in case of retroviral mediated gene therapy, replication-competent viruses65 and insertional mutagenesis66,67 are the two main risk factors. The two known instances where insertional mutagenesis/oncogene activation has resulted from the administration of a replication-defective retroviral vector has suggested that, the design of the vector aside, there are additional risk factors that influence the probability of an adverse event, the most obvious of these being the specific transgene expressed from the vector66,69, which in both cases is a gene capable of influencing cell growth (although in neither case can it be considered a classical oncogene). In terms of the influence of vector design, it is interesting to note that in both of these instances the same vector, pMFG, was used70,71. This vector is derived from MoMLV, a strongly oncogenic murine retrovirus, and notably uses the viral LTR to drive expression of the transgene. Indeed, it is generally believed that, in general, the risk of insertional mutagenesis, while poorly defined, is probably substantially lower than seen in the X-SCID trial72, where there appear to be a number of specific secondary risk factors.

**Conclusion**

Replication competency comprises the most important determinant of the safety of retroviral vectors. The technologies available for the production of vector virions would appear to be able to preclude
the production of replication-competent virus by recombination of the constituent parts of the vector system, i.e., vector and helper plasmids, with a very high degree of certainty. However, production of replication-competent virus from the cell lines used for virus production remains a theoretical possibility and more work needs to be done on generic assays for replication-competent retroviruses.

As both oncogenic (MoMLV derived) and lentiviral (HIV-1 derived) vectors have been shown to preferentially integrate into transcribed sequences, it would appear logical that the likelihood of proviral integration near cellular genes involved in the positive regulation of cell growth would be increased in actively growing cell populations. This suggests that the use of transduction protocols that target non-cycling cells, or cells that are subjected to the minimum of stimulatory signals, would be greatly advantageous in terms of minimizing the risk of malignant events after the stimulatory signals are removed.\(^7\)

It can be said that retroviral mediated gene transfer remains an extremely attractive option for gene therapy when the stable and permanent genetic modification of the target cell is optimal. However, a greater care must be taken to ensure the utilization of resources, so that proper and stable expression could be achieved and adverse events are to be minimized.

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