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Visions & Reflections

Retrovirus molecular conjugates

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Abstract. Retrovirus-derived vectors are currently the preferred vectors used for human gene therapy protocols. Serious safety concerns persist, however, which are specifically related to the formation of a replication-competent virus, and no synthesis method currently employed precludes its formation with certainty. For many cell types, a low transduction efficiency results in insufficient therapeutic benefit. We describe the development of a molecular conjugate system, which permits transient chemical modification of a retrovirus with polylysine. This modification not only introduces additional safety features over standard unmodified retrovirus vectors, but also provides enhanced transduction efficiency.

Key words. Retrovirus; polylysine; molecular conjugate vector.

Gene therapy

Gene therapy is defined as a therapeutic strategy in which nucleic acids are utilized to treat disease or other medical conditions. Delivery of the genetic material to the target cells is accomplished with specialized vehicles, or vectors, which are either synthetic, natural, virus derived, or a combination of these vehicles. DNA, after an intermediary step into RNA, is translated into proteins, the active form of the therapeutic molecule. Although transient gene expression is sufficient for some medical applications (e.g., vaccine protocols, suicide gene therapy), durable or lifelong transgene expression is necessary for other disorders such as inherited deficiency syndromes, or the introduction of drug resistance genes into hematopoietic stem cells. Here, permanent gene replacement and physiological gene expression is the goal [1–3]. In most instances, this requires integration of the therapeutic gene into the genome of target cells. The most commonly used vectors for this purpose belong to the retrovirus family [4–6].

Retroviruses

Retroviruses consist of a family of viruses, several types of which have been developed for gene therapy approaches: (i) oncoretroviruses, consisting mainly of the mammalian and avian C-type retroviruses, (ii) lentiviruses (such as HIV and other immunodeficiency viruses), and (iii) spumaviruses. The most commonly used retrovirus vectors for both research and human clinical trials are murine Moloney-based oncoretrovirus vectors. Based on the spectrum of susceptible host cells, they are categorized into ecotropic (rodent cell tropism only) and amphotropic (rodent and human cell tropism) types. Retroviruses are lipid-enveloped particles comprising a homodimer of linear, positive-sense, single-stranded RNA genomes of 7–13 kb. Up to 8 kb of exogenous DNA can be inserted and expressed in place of the viral genes

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[7]. The viral envelope glycoprotein dictates the host range of retroviral particles through its interaction with receptors on target cells. Following entry into target cells, the RNA genome is retrotranscribed into linear double-stranded DNA and integrated into the host genome [8, 9]. Retroviruses can establish chronic infections, which are usually well tolerated by the host but may also cause latent diseases ranging from malignancy to immunodeficiency [8]. Although no known human diseases are associated with the most commonly used murine Moloney-based oncoretroviridae, one must follow absolute safety requirements to minimize the risk of introducing diseases into human patients. Nearly a decade of human trial experience has been gained with murine Moloney oncoretrovirus vectors. Most applications had been ex vivo, with a major thrust to stably transduce human hematopoietic stem cells in culture. Although in vivo clinical approaches for retrovirus gene therapy are uncommon, one well-known concept is being pursued for the treatment of malignant glioblastomas of the brain in humans. A retrovirus producer cell line is directly infused into the anatomic region of the cancer. The producer cells generate locally large quantities of recombinant retrovirus. The therapeutic gene is the herpes simplex thymidine suicide gene (HSV-tk) [10, 11]. The virus selectively transduces rapidly proliferating tumor tissue, but fails to transduce normal neuronal tissue. Transduced cells are amenable to selective killing with the drug ganciclovir [11, 12]. Nevertheless, despite prolonged and extensive experience, long-term side effects of retroviral gene therapy are still uncertain and, thus far, experimental treatments have been almost exclusively administered to patients with limited life expectancies of less than 2 years.

One safety concern is related to random integration, which could potentially inactivate tumor suppressor genes or activate oncogenes, with subsequent tumor formation. The most serious safety risk, however, is considered the potential formation of a replication-competent retrovirus in the host. Current safety features for retroviral-based vectors are primarily designed to avoid viral replication in the host, and retrovirus vectors are technically rendered replication incompetent. Viral vector sequences are expressed by heterologous transcriptional signals from two separate constructs lacking most viral cis-acting sequences which are stably incorporated in packaging cell lines [13]. Despite a split construct design, which improves the biosafety of the vector by increasing the number of recombination events required to reconstitute a replication-competent genome, generation of a replication- competent virus still occurs (RCR) [14, 15]. Studies in non-human primates link RCR formation to a significant risk for the development of lymphoma, thus underlining the importance of this safety concern [16, 17]. The propagation of RCR could be prevented if its progeny were blocked from entering cells within the host. However, the dilemma is that recombinant vectors are required to infect human cells to fulfill their therapeutic purpose, preferably at high efficiency. On the other hand, if a vector that lacks tropism to human cells (such as the ecotropic retrovirus) could be transiently modified to enable a one-time transduction of human cells, this could greatly enhance safety features. In contrast to genetic modifications, where formation of RCR results in propagation of wild-type virions, physical or chemical modifications are transient and will not be passed onto replicating virions. Formation of RCR, then, could not result in further expansion of its progeny in humans because an ecotropic virus cannot propagate in humans due to its tropism restriction.

Molecular conjugate vectors

Cation-based molecular conjugate vectors (MCVs) were one of the first gene delivery vehicles developed for gene therapy. Electrostatic forces condense cationic (e.g., polylysine) and anionic (DNA) components to form toroid-shaped structures with a diameter of 80–100 nm [18, 19].

In addition to the ability of the vector to enter cells via cation-specific mechanisms, the vector can also be redirected to enter cells via specific receptors through incorporation of the corresponding ligands into the vector complex [20–25]. To further enhance gene expression, replication-deficient adenovirus particles were introduced into the vector to escape endosomalysis, which increased gene expression by 2000-fold [26, 27]. Although gene transfer efficiency and transgene expression are higher with MCVs than with most other gene transfer systems in most cell lines and primary cells, the introduced DNA, delivered with conventional MCVs, does not integrate, and, diluted with each cell division, results in transient transgene expression [28]. In contrast, formation of polylysine-based recombinant adenovirus molecular conglomerates (recMCVs) has dramatically improved gene expression characteristics over the unmodified recombinant adenovirus, with integration events and significantly prolonged transgene expression. Maximum gene expression was increased by 50-fold in adenovirus conglomerate-transduced human cell lines. While, as expected, the unconjugated adenovirus resulted only in transient gene expression for 30 days or less, stable transduction was achieved within cells that were transduced with equivalent multiplicities of infection (MOIs) delivered as adenovirus conglomerate vectors. The transduction efficiency was reported at a frequency of 35–50%. In stably transduced cultures, gene expression remained high at baseline expression levels of 90% or greater for over 6 months [29, 30]. These results indicated the potential for applying this general concept of polylysine conjugation

successfully to other viral vector systems to enhance their transduction efficiency. As a next step, we explored and developed this concept for retrovirus vectors.

Retrovirus molecular conjugates

We have explored the concept of transient modification by chemically linking ecotropic retrovirus to poly-L-lysine to form recombinant retrovirus molecular conjugates. This modification redirects or enables vector uptake via the polylysine component, which binds to heparan receptors and also adheres to cell membranes charge dependently, thus permitting efficient cellular transduction of both cell lines and primary human cells with ecotropic retrovirus [30–32]. Most importantly, however, this new vector can provide additional safety features over amphotropic retrovirus vectors currently used in human trials. Even the formation of a replication-competent ecotropic virus would be, theoretically, a self-limited problem, as progeny of a transiently modified ecotropic virus could not propagate further in humans due to the tropism restriction. The transduction efficiency in a panel of different human cell lines and primary human bone marrow stroma cells with ecotropic retrovirus MCVs was shown to be identical to amphotropic retrovirus MCVs. Moreover, the transduction efficiency with retrovirus MCVs was significantly enhanced (up to ten-fold) over equivalent MOIs of a conventional, unconjugated retrovirus [33]. The transduction efficiency in human primary bone marrow stroma cells was also enhanced up to fivefold over a VSV pseudotyped retrovirus (MOI of 1) [unpublished observation]. Based on these observations, we hypothesize that this vector could specifically enhance the transduction efficiency with amphotropic retrovirus of human tissue with low expression of amphotropic receptors such as hematopoietic cells.

Vector uptake is largely determined by the presence of virus receptors and the cell surface receptor density, which is a primary condition for subsequent transduction [34–36]. The formation of a polylysine-based retrovirus MCV, therefore, proved to be one strategy that can overcome ecotropic/amphotropic receptor-dependent vector uptake and enhanced cellular transduction efficiency through circumventing the natural pathway of cell entry by the virus (fig. 1). Although polylysine conjugates enable or enhance retrovirus uptake, additional mechanisms may enhance cellular transduction. Conjugates consist of multiple virus particles bound to polylysine, thus providing a higher likelihood of delivering one intact virus to the nucleus compared to individual retrovirus particles. Although immediate vector uptake constitutes a critical step, other vector and cell-specific properties determine the success of gene transfer. These include trafficking

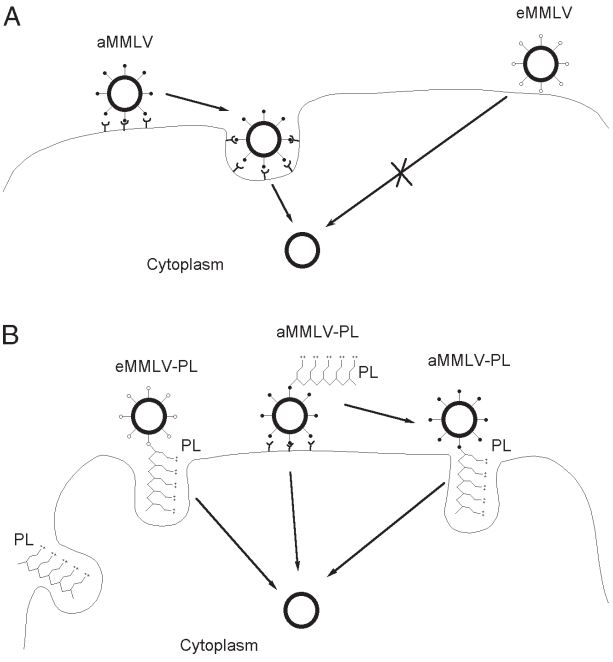

Figure 1. Retrovirus molecular conjugates. The envelope of amphotropic murine Moloney leukemia retrovirus (aMMLV) contains an epitope, which binds to specific receptors on the cell surface of human cells, thus enabling virus uptake into the cytosol. There is no corresponding receptor for ecotropic murine Moloney leukemia retrovirus (eMMLV) on human cells and, therefore, intracellular virus uptake does not occur (*A*). Polylysine (PL) binds to and enters human cells via heparan receptors and non-specifically via electrostatic charges. Conjugation of eMMLV to PL (eMMLV-PL) permits uptake of eMMLV into human cells. Conjugation of aMMLV to PL (aMMLV-PL) provides one additional mechanism for introducing aMMLV into cells, a modification previously shown to enhance virus uptake (*B*).

within the cytosol and resistance to cellular defense mechanisms, the ability to penetrate the nuclear membrane, and the ability to integrate into the host genome. Formation of retrovirus MCV may confer a survival benefit to a bound retrovirus by protecting it from nuclease digestion within the polylysine conglomerate. For example, in studies where DNA bound to polylysine, it was found to have significantly enhanced resistance to intracellular degradation over free DNA [37]. We hypothesized that the improved transduction efficiency could result from a combined effect of both polylysine-mediated enhanced uptake and also improved retrovirus survival from cytosolic degradation. This conclusion of enhanced uptake and resistance to degradation is further supported by the fact that the copy number integrated into the host genome is still one per cell, and did not change over cells that were stably transduced with an unconjugated retrovirus [33]. This potentially protective effect could possibly be further enhanced by the additional introduction of endosomalytic components. Lastly, the polylysine-based retrovirus system may enable specific receptor retarget-

Table 1. Comparison of retrovirus and retrovirus molecular conjugates. The properties of amphotropic murine Moloney leukemia retrovirus (aMMLV), its retrovirus molecular conjugate (aMMLV-PL), ecotropic Murine Moloney leukemia retrovirus (eMMLV) and its conjugate ecotropic Murine Moloney leukemia retrovirus (eMMLV-PL) are compared.

Property	aMMLV	aMMVL-PL	eMMVL	eMMLV-PL
Host range	rodent and human	rodent and human	rodent	rodent and human
Transduction efficiency	very efficient for all rodent cells; highly variable for human target tissue (poor in hematopoietic cells)	significantly enhanced in all cell lines over aMMLV only	efficient for all rodent cells including hematopoietic rodent cells	efficient in many human cells including primary bone marrow; transduction effeciency is comparable to aMMLV-PL
Safety concerns for human use	(i) occurrence of replication – competent virus (ii) insertional mutagenesis	risk is similar to unconjugated aMMLV	not applicable	(i) safety risk for replication – competent virus is eliminated (ii) insertional mutagenesis risk likely to be similar to aMMLV
Vector manufacture	well established	additional conjugation step	well established	additional conjugation step
Quality control	well established	more complex	well established	more complex
Toxicity	only at high doses	increased at high doses	only at high doses	increased at high doses
Experience in humans	multiple trials completed	none	not applicable	none

ing by the formation of receptor-targeted retrovirus MCVs [21–25, 29].

Numerous gene transfer studies in animal models relevant to human disease, including a limited number of clinical trials, have been published using polylysine-based MCVs, although so far none has been reported with a retrovirus MCV [38] [20, 39–41]. Preliminary unpublished studies from our laboratory using a retrovirus MCV indicate that persistent transgene expression in human hematopoietic stem cells can be accomplished with increased transduction efficiency in the NOD-SCID xenotransplant model compared to an equivalent unconjugated retrovirus. The most intriguing and promising reasons to pursue further development of this technology for ultimate clinical utilization are the improved safety, by using an ecotropic retrovirus for the transduction of human tissue, and the enhanced transduction efficiency with this vector. In summary, formation of retrovirus MCVs enhances retrovirus uptake and overcomes natural tropism barriers for an ecotropic retrovirus. These properties could provide additional safety features for clinical applications. The advantages of a retrovirus MCV are directly compared with a recombinant virus in table 1.

Although clinical gene therapy trials with polylysine-based conjugate vectors have been reported from Europe [38], conjugate vectors are not commonly used for human clinical trials in the USA because of concerns of greater lot-to-lot variation compared to purely recombinant viral vectors. However, conjugate technology is widely used with protein-based therapies (e.g., polyethylene glycol modifications of recombinant cytokines where similar concerns preceded their incorporation into clinical medicine).

If future in vivo studies demonstrate and consolidate the known advantages of viral molecular conjugate systems, potential quality control concerns will likely be quickly and adequately addressed by pharmaceutical developers, allowing the concept of moving a retrovirus MCV into human gene therapy trials. Therefore, development of this technology in the near future has to be focused on demonstrating advantages of this novel system in clinically relevant animal models. Moreover, technology needs to be developed preferably by industrial partners, which will permit the production of clinical-scale retrovirus molecular conjugate vectors in compliance with requirements of the regulatory authorities for individual countries.

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