Retrovirus-Mediated Gene Transfer (RMGT)

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Gene transfer is a technique previously used in the laboratory for studying the function of a specific gene of interest. From the initial research work, the idea of transferring genes has been extended into the realm of therapeutics, where it is known as gene therapy. At its inception, gene therapy was mainly aimed at the correction of heritable genetic lesions by replacement of the aberrant gene, such as adenosine deaminase (ADA),[1-3] in severe combined immunodeficiency (SCID). Recently, gene transfer technology has been applied to the treatment of a wide variety of diseases, for example AIDS and cancer.[4-7] Gene transfer techniques can be divided into two groups, non-viral and virus-based techniques. Non-viral gene transfer techniques or so-called "physical mechanisms" were developed before the virus-based methods.[8] Some concepts relevant to these techniques will be reviewed briefly but more attention will be paid to retrovirus-mediated gene transfer (RMGT).

Non-viral techniques[8]

1. Calcium phosphate transfection

Calcium phosphate transfection was the first non-viral method of gene transfer developed. This technique is based on precipitates of plasmid DNA and calcium ions. The precipitates are thought to enter cells by endocytosis. It is simple and inexpensive but the efficiency of transfer is low. Cells can be either stably or transiently transfected by this technique.

2. Microinjection

Microinjection is a method which directly delivers small amounts of plasmid DNA into target cell nuclei. This technique is thought to disturb cells minutely since cells are closely monitored under a microscope during manipulation.

3. Electroporation

Electroporation is a method applied to mixing target cells in suspension with DNA. The DNA enters the cells through holes in the cell membrane formed when an electrical pulse is passed through the cell suspension. This method may not be suitable for mammalian cells since many cells do not survive the high voltage electrical pulse that is required.

4. Liposomes

Liposomes form complexes with DNA or entrap DNA and deliver it into cells by endocytosis. Lipofectin transfection is an example of gene transfer based on liposomes.

5. Ballistic DNA injection

This technique is also known as particle bombardment, microprojectile gene transfer or gene-gun. Basically, a plasmid carrying the gene of interest is coated onto microbeads (1-3 micron sized gold or tungsten particles) and accelerated by a motive force to penetrate cell membranes. Approaching the cell membrane, microbeads will be stopped by a retaining screen and only the plasmid is able to pass through to the target cell surface.

6. Naked plasmid DNA injection

This technique was developed from the observation of transgene expression in rodent and primate muscle. Following intramuscular injection of plasmid DNA, the transgene was found to be expressed for longer than 19 months. No conclusive information has been provided about how the plasmid DNA is taken up. As this method is very simple, it has been tested on other tissues. Unfortunately, the utility of this method seems to be restricted to skin, thymus and striated muscle.

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Virus-based gene transfer[9]

These gene transfer techniques are based on modified viral vectors. As retroviruses were the first type of virus available for gene transfer technology, they have been widely studied. Initially there were some concerns about safety but subsequently the development of "helper-free packaging cell lines" made retrovirus-mediated gene transfer safer and more convenient[10-14]. Conversely, extensive study of retroviral vectors has led to an appreciation of its limitations. For example, retroviral integration needs cycling target cells.[15] This is not possible for some target cells, such as quiescent primitive haemopoietic stem cells unless they are induced to divide, in which case they may lose their primitive phenotype and function.[16-18]

The later generation of viral vectors, which are adenovirus, herpes simplex virus (HSV) and adeno-associated virus (AAV), were designed to overcome the restrictions of retroviral vectors. Some characteristics of viral vectors used in gene transfer are summarised to indicate their comparative advantages and disadvantages in Table 1.

Table 1. Characteristics of common vectors used in gene transfer technology

	Retrovirus	Adenovirus	AAV	HSV
Insert size	up to 8 kb	up to 8 kb	< 4 kb	> 20 kb
Titre	10^7 - 10^{10}	10^{11}	10^9	10^8
Integration	Yes, random	No	Yes, random?	No
Target DNA expression	Variable	Transient	Transient	Transient
Target cells	Cycling cells	Quiescent and cycling cells		

Retrovirus-based systems for gene transfer[10-12,14,19-21]

A retroviral vector used in gene delivery technology is constructed in order to obtain effective expression of the DNA under investigation and also to maintain some useful characteristics of the retrovirus, such as its ability for DNA integration into the host genome. Retroviral vectors infect cells by binding of viral envelope glycoprotein to receptor molecules expressed on the surface of the target cells. Retroviral receptors are classified by their host range. Ecotropic virus is able to infect only murine cells since it needs receptors unique to mice. Amphotropic viruses need different receptors which are found in both murine and non-murine species.

Ordinarily, a retrovirus requires three main genes acting in *cis* in order to form an infective viral partical. These are **gag**, **pol** and **env.** The **gag** region encodes genes for the capsid protein, the **pol** region encodes genes for reverse transcriptase enzyme and the **env** region encodes proteins needed for receptor recognition and envelope protein. For RMGT, these essential genes are generated to supply proteins in *trans* only to create a retrovirus which can not replicate autonomously. In order to make the system safer, the three genes are removed from the vector itself, being separated onto two different plasmids and transfected mouse fibroblast cell line, the so-called packaging cell line, without the Ψ packaging signal (the signal for packaging of viral RNA). The Ψ packaging signal remains on the retroviral vector which offers a large space for insertion of a transgene after the removal of almost the entire genome.

The above modifications lead to replication-incompetent RMGT which is based on the combination of packaging cell lines which carry the essential viral genes, and a retroviral vector which carries the Ψ packaging signal, space for transgene

insertion, promoter for the target DNA and a selective marker gene (some vectors have more than one marker or reporter gene).

Antibiotic resistance genes, such as the neomycin resistance gene (Neo^R), have been widely used as selectable markers of successfully transduced cells. Other genes have also been used as selection systems, like (i) enhanced fluorescent green protein gene (EFGP) when transduced cells will express green fluorescence, (ii) LacZ gene when tranduced cells will present as blue colonies in x-gal stained cultures due to the expression of β-galactosidase or (iii) some surface marker gene, such as the CD4 gene.[22-24] Compared with Neo^R, these marker genes allow a reduction in the length of the selection period since transduced cells can express them in two or three days following transduction and provide a non-toxic detection method for transduced cells. Selection of stably transduced cells using Neo^R as a reporter gene takes two weeks growth in tissue culture. Advantages and disadvantages of retroviral vectors are shown in Table 2.

Table 2. Advantages and disadvantages of retroviral vectors

Advantages	Disadvantages
Extremely well studied system	Requires dividing target cells
High transduction efficiency	Random integration
Integrates into host genome resulting in sustained expression of DNA	
Insert size up to 8 kb	
Convenient system due to the availability of packaging cell lines	
Vector proteins not expressed in host	

Haemopoietic stem cells, one of the most wanted targets

One of the major aims of gene transfer is its therapeutic application (gene therapy). Although this strategy has been attempted for some time, no satisfactory protocol has yet been proposed which succeeds in correcting the genetic defect in one operation. Permanent expression of the transferred gene is not achieved since the host cells or target cells will senesce and finally die. Therefore, haemopoietic stem cells have become an attractive target for gene transfer research since they are perceived as a permanently self-renewing population. The ideal haemopoietic targets for gene transfer are the most primitive stem cells because they have the greatest potential to maintain a long-lived clone of primitive, self-maintaining cells which will express the transferred genes.[25-27]

Almost all primitive haemopoietic stem cells are quiescent and are therefore theoretically unable to be transduced by a retroviral vector.[15] Whilst cytokine manipulation can drive them into cell cycle,[28-29] there is a risk that they will lose their primitive nature by differentiation.[18] Accordingly, the development of a new generation of viral vectors was undertaken. While studies of retroviral vectors continue to search for a solution to overcome its major obstruction, the new generation of vectors also shows some limitations.[9]

Studies of "*ex vivo* expansion" which focus on defining the *in vitro* culture conditions (i.e. a cytokine or a combination of cytokines) that drive haemopoietic stem cell expansion by increasing self-renewal rather than differentiation have not been universally successful. Piacibello et al in 1997[30] showed that with a simple combination of two growth factors, thrombopoietin (TPO) and fms-like tyrosine kinase (FLT-3) ligand, CD34+ cord blood cells could be sustained and greatly expanded in liquid culture for longer than 6 months without losing their

primitive marker (CD34) expression. If confirmed, this protocol could lead to an effective application of genetic manipulation in haemopoietic stem cells using retroviral vectors and open up the future of RMGT.[31]

From gene transfer to human gene therapy, only a promise?

It has been a decade since the first genetic manipulation was launched in medical treatment, on 14 September 1990.[32] Since then, there have been more than 500 clinical trials worldwide studying gene therapy against a wide variety of diseases.[32-33] Unfortunately, a few have worked. Moreover, there were some reports of serious adverse events with at least one death from genetic treatment reaction using adenoviral vector. *What is wrong?* Not only are clinical trails rarely working, but the protocols also seem hazardous. *Could the difficulty be an inadequate understanding of human body reaction to genetic treatment? And could another possibility be an incomplete comprehension of the basic biology of gene transfer itself?* As all gene transfer vectors are relatively new. Furthermore, it is hard to insert a new gene into billions of target cells in human body. Once inserted, the new gene needs to function. Frequently, the body suppresses the transgene expression and switches it off or even destroys the gene. Among the trials, RMGT participates in around 40% of gene delivery protocols and about 50% of all patients in trials received genetic treatment by RMGT.[33] Although no one can really confirm whether or not RMGT is definitely safe at the moment, at least retroviral vector is more extensively studied than any other vectors. Therefore we could assume that researchers are more familiar with its limitations and side effects. In addition, RMGT remains the most successful method for transferring foreign gene into haemopoetic target cells for *in vitro* investigations especially when being used in combination with an effective *ex vivo* expansion protocol.[30] In conclusion, all the major difficulties of gene transfer in clinical level need to be worked out. Until these significant problems are solved, human gene therapy will be only a dream.

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