A Short Course on Virology / Vectorology / Gene Therapy

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Abstract: For people starting off in the field of gene therapy, the encountered terminology is often quite confusing. Moreover, the background on basic virology may be modest. The following introduction provides a head start to any novice willing to gain more in-depth knowledge on the subject. The development of gene therapy is also addressed from a historical perspective.

INTRODUCTION

For people starting off in the field of gene therapy, the encountered terminology is often quite confusing. Moreover, the background on basic virology may be modest. The following introduction provides a headstart to any novice willing to gain more in-depth knowledge on the subject.

TERMINOLOGY (SEE ALSO TABLE 1)

Prokaryotic cells, such as bacteria, lack cell organelles and a nucleus but are surrounded by a cell wall. They have a metabolism that is distinct from *eukaryotic* cells, which have cell organelles and a nucleus and are surrounded by a cell membrane. Eukaryotic cells have a limited life span; primary cells can only be propagated for a limited amount of passages. That is why immortalized *cell lines*, which are originally derived from tumors and constructed in the laboratory, are used. These cell lines can have different origins (insect, mammalian, human). When targeting human cells in gene therapy, one has to make a clear distinction between somatic and germ cells. The *somatic cells* are the cells that make up the different organs of the human body. From a moral point of view, gene transfer in somatic cells is allowed, especially for curative purposes. The *germ cells* are the cells involved in reproduction. A genetic modification of those cells will result in genetically modified gametes and after fecundation in genetically altered offspring. Altering the human species by genetic modification of germ cells is not allowed and care should be taken to avoid inadvertent transduction of germ cells.

A *plasmid* is a circular form of extrachromosomal DNA that can be propagated in bacteria. A *eukaryotic expression vector* (often called an expression vector for short) is a plasmid that drives the expression of proteins from a eukaryotic promoter inside eukaryotic cells; through the presence of a bacterial origin of replication (*ori*) these plasmids can be propagated in bacteria as well. A *viral vector* is a protein particle derived from a replicative virus that contains genetic information in the form of RNA or DNA. A viral envelope may be present as well.

Gene transfer is the introduction of genetic material in cells that can be of different origin (bacteria, yeast, plants, insect cells, mammalian cells); gene therapy is the introduction of genetic material in cells with the purpose of curing a disease (e.g. X-SCID, hemophilia). *Gene therapy* typically involves gene transfer into animal models and patients. Expression of a disease protein in a cell is thus the result of a gene transfer and not of a gene therapy experiment. *DNA vaccination* is gene transfer into animal models and humans in order to prevent a disease by eliciting an immune response against the expressed gene product.

Gene transfer of DNA in bacteria, to express a recombinant protein in E. coli for example, is called *transformation*, whereas gene transfer of DNA in eukaryotic cells is called *transfection*. Using a viral vector (as defined above) to transfer a gene into a cell is referred to as *transduction*. When a virus that typically replicates and spreads into neighbouring cells, mediates a transfer of genes belonging to its own genome or extra genes it may carry, we talk about *infection*. It is important to make the following distinction: a viral vector *transduces* the target cells as opposed to the wild type (parental) virus that *infects* the target cells.

AN INTRODUCTION TO VIROLOGY

Viruses are small particles (10-300 nm) that depend on a host cell for their replication. This host cell can be a bacterium (for a bacteriophage), a plant cell or an animal cell. Viruses are composed of nucleic acids, which contain the genetic information of the virus, inside a protein shell named “capsid”. Some viruses are additionally surrounded by an envelope: a lipid membrane containing glyco-or lipoproteins, derived from the membrane of the host cell. This characteristic distinguishes enveloped from non-enveloped viruses. Viruses contain RNA or DNA that can be single- or double-stranded. The classification of viruses is traditionally based on nucleic acid type and presence or absence of an envelope (Table **2**).

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Table 1. (Retro) Viral Terminology

vector	Virus devoid of genetic information required for replication, encodes foreign (marker, therapeutic) genes instead
LTR	Long Terminal Repeat, contains the retroviral promoter (U3), repeat region (R), and polyadenylation region (U5). The LTR is present as a tandem repeats at the 5' and 3' ends of the DNA genome.
Gag	Group specific antigen, a polyprotein made up of the viral structural protein matrix (MA), capsid (CA) and nucleocapsid (NC).
Pol	Polymerase, a polyprotein containing the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN).
Env	Envelope, the retroviral surface protein that directs binding of the virion to a specific cellular receptor.
amphotropic	An amphotropic env protein can bind to a wide variety of cells (broad tropism).
ψ	Packaging signal, region in the viral RNA that is required for incorporating the RNA in the virion.
packaging cell	Cell that encodes viral proteins (gag, pol, env), but produces empty viral particles since ψ has been deleted.
transfection	Transfer of DNA into cell
transduction	Vector-mediated transfer of DNA into cell
infection	Virus-mediated transfer of DNA into a cell
lysis	Destruction of the cellular envelope of the host cell in order to free the viral particles after completion of their replication process.

Table 2. Classification of Animal Viruses

Without envelope	With envelope	Form
Papovaviridae Adenoviridae	Herpesviridae Poxviridae Hepadnaviridae (partially single-stranded DNA)	Double-stranded DNA
Parvoviridae		Single-stranded DNA
Reoviridae		Double-stranded RNA
Picornaviridae	Paramyxoviridae Orthomyxoviridae Rhabdoviridae Retroviridae Arenaviridae Coronaviridae Bunyaviridae Togaviridae	Single-stranded RNA

Viral replication always more or less depends on the host cell metabolism. The consecutive stages of the viral replication cycle can be schematically arranged. The early stages are similar for most viruses. The later stages of replication vary greatly between RNA and DNA viruses. The first step is the adsorption of the virus to the target cell. It involves interactions between the capsid or envelop of the virus and components of the cell membrane, called receptors that are often virus-specific. Those receptors have a role during the normal cell metabolism. Adsorption is followed by entry. There are two main mechanisms by which this can be realized: fusion of (enveloped) virus and cell membranes or receptor-mediated endocytosis. Endocytosis is followed by uncoating in the lysosome and the same goes for fusion, although only partial uncoating takes place there.

For DNA viruses (Fig. **1**), viral replication can be divided into an early and a late phase. The early phase takes place prior to DNA replication. Early mRNA's are synthesized using the cellular RNA polymerase. They encode early, non-structural proteins such as replication enzymes. After DNA replication, late mRNA's are synthesized. These encode the structural proteins of the virus. DNA replication in the nucleus is carried out by an enzyme complex of cellular and viral origin. The structural proteins are transported into the nucleus, where the new viral particles are assembled. Virus is then set free by lysis of the infected cell.

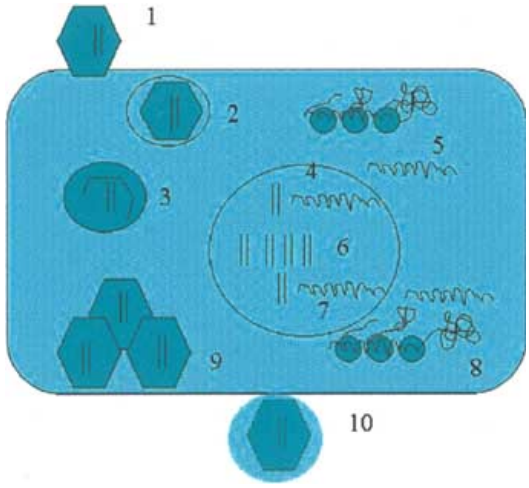

Fig. (1). Schematic representation of the replication cycle of a DNA virus.
The different steps of the viral life cycle are indicated: (1) binding, (2) entry, (3) uncoating, (4) transcription of (immediate) early genes, (5) translation of early proteins, (6) DNA replication in the nucleus, (7) transcription of late genes, (8) translation of late proteins, (9) assembly, (10) lysis of host cell.

The process of viral replication is very efficient. One infectious particle leads to formation of 10^5 to 10^7 new virus particles, although not all particles formed will be infectious. Replication of adenoviruses and herpesviruses is characterized by an additional immediate-early phase in the replication cycle. Replication of poxviruses (such as vaccinia) occurs in the cytoplasm using a viral RNA polymerase. During viral replication, host cell metabolism is suppressed to favor replication of the virus.

For RNA viruses, different replication mechanisms exist after adsorption, entry and uncoating. Positive strand viruses, such as picornaviruses, carry a single-stranded RNA genome that functions as mRNA. The mRNA is translated into a large polyprotein that is subsequently cleaved into the different structural and enzymatic proteins. Important is the viral RNA-dependent RNA polymerase, which drives the machinery needed for copying the genomic RNA. From the positive strand a negative strand is made, which serves as a template for the synthesis of new positive RNA strands that are assembled together with the structural proteins into new viral particles. The negative strand RNA viruses, such as the myxo and paramyxoviruses, contain an RNA polymerase that copies the negative RNA genome into a positive copy that serves as mRNA. In the myxoviruses the genome is composed of 8 fragments, each encoding one gene; each fragment is copied separately.

Retroviruses carry an RNA-dependent DNA polymerase (reverse transcriptase) that copies the two copies of the RNA genome into one double-stranded DNA molecule. This DNA molecule is then integrated in the host chromosome through the viral integrase. Consecutively, cellular RNA polymerase transcribes the viral genes into mRNA's that encode for structural and enzymatic viral proteins. The viral DNA is stably integrated in the host cell and copied together with the genome of the cell upon cell division.

Most RNA viruses leave the host cell by a budding process. At budding, the viral particles are surrounded by part of the cell membrane containing cellular and viral membrane proteins.

Adeno-associated virus is a parvovirus with a single-stranded DNA molecule that can only replicate in the presence of a helper virus, an adenovirus or a herpes virus. Hepatitis D virus is another defective virus. It contains a small circular RNA molecule and can only replicate in the presence of Hepatitis B virus.

WHAT VECTOROLOGY IS ABOUT?

Viruses have evolved by Darwinian selection over billions of years into efficient vehicula infecting cells and introducing their genetic material. Vectors derived from replicating viruses are the most frequently used vehicles to transfer genes into somatic cells. In a non-replicating viral vector, the viral genome has been deleted and replaced by the genes of interest. In result thereof, the vector will enter the target cell and introduce the therapeutic genes but will no longer be able to propagate itself due to the absence of the viral genome. Inadvertently, vectors may arise during production that has acquired the genes required for replication through recombination. These replication-competent vectors may have the capacity to spread to other cells. Production of viral vectors should be monitored for accidental contamination with replication competent vectors. For the different viral vector systems, strategies have been designed that limit the chance of recombination and assays for the detection of recombinants have been developed.

Retroviral vectors, derived from murine oncoretroviruses, were the first vectors used for experimental gene transfer *in vitro* and *in vivo* (and in patients). To date, vector systems have been derived from different viruses. All viral vectors have their advantages and disadvantages so that no single viral vector is applicable to all purposes in gene transfer (Table **3**).

Table 3. Characteristics of Gene Transfer Vectors

	Adenovirus	AAV	Retrovirus	Lentivirus	HSV	Naked DNA
Maximum insert size	30 kb	3.5-4 kb	7-7.5 kb	7-7.5 kb	35 kb	unlimited
Titers (viral particles/ml)	10^{12}	10^{12}	10^8	10^8	10^8	unlimited
Infectivity	broad	broad	dividing cells	broad	broad	broad
Applications for CNS	ex/in vivo	ex/in vivo	ex vivo	ex/in vivo	ex/in vivo	ex/in vivo
Integration	no	yes/no	yes	yes	no	poor
Stability of expression	short	long	long (silencing)	long	latency?	short
Immune response	extensive	unknown	low	low	medium	none
Preexisting host immunity	yes	yes	unlikely	unlikely (AIDS patients?)	yes	no
Safety	I, T	I, T	IM	IM ?	I, T	none

I = inflammation
T = toxicity
IM = insertional mutagenesis

Choice of viral vector is determined by the size of the gene of interest, the required duration of gene expression, the target cell and biosafety issues. In the following chapters, different viral vector systems will be explained starting from the virology of the parental virus. Those vector properties, which affect biosafety (for the scientist, the patient and the environment) will be accentuated.

EPIDEMIOLOGICAL ASPECTS OF THE VIRAL INFECTION

Viral infection of humans can have different consequences. Infection with viruses such as the virus of common cold (rhinovirus) leads to an acute self-limiting infection. An acute infection can be followed by a latent phase as seen in herpes viruses and many hepatitis viruses that maintain their viral DNA under episomal conditions or in integrated form in the nucleus of the target cell. The latency phase can be interrupted by acute exacerbations. A typical example is recurrent herpes labialis caused by herpes simplex or shingles, the exacerbation of latent varicella zoster virus. In the case of the human immunodeficiency virus (HIV) – a retrovirus – chronic infection will lead to a progressive destruction of the host lymphocytes, which will ultimately lead to immune deficiency. The pathogenicity of viruses is determined by their own (lytic) replication cycle but also by the immune response of the host that is destroying cells, which carry viral epitopes. Viruses with a latency phase have developed different tricks to evade the immune response of the host.

Viruses can be transmitted directly or indirectly. Determining factors are the nature of the virus, the site of infection and environmental conditions. Resistance of viruses against environmental conditions varies. Very resistant are viruses that infect their host through the gastrointestinal tractus (faeco-oral contamination) such as enteroviruses, hepatitis A and reoviruses. These viruses are liable to survive in surface waters for a long time and cause epidemics, which can be prevented by a proper hygiene. HIV, hepatitis B and C virus are transmitted through sexual intercourse and contamination with blood products (blood transfusion, intravenous drug use). Viruses that cause respiratory diseases can be spread by aerosol. Adenovirus is very stable in the environment at temperatures below 37°C and at pH 6-9. Arboviruses (arthropode born) are spread by blood-sucking insects. An important feature is that all enveloped viruses can be inactivated by ether or detergents. They are also sensitive to complement-based inactivation. Antiviral defense mechanisms can be non-specific or specific. Interferon, for example, is produced by the virus-infected cell and can protect neighbouring cells against viral infection. This particular immune reaction is based on production of virus-specific antibodies and stimulation of the cytocidal effect of cytotoxic lymphocytes. Cellular immunity plays an important role in non-cytolytic infections. Immune suppression on the other hand, can lead to exacerbations of latent virus infections.

GENE THERAPY THEN AND NOW

Retroviral Vectors

Virologists have been at the forefront of many new developments in molecular biology. Oncogenes and tumor-suppressor genes were first discovered in the context of RNA and DNA tumor viruses, respectively. Likewise, retrovirology has been the historical base for vectorology (For reviews see Miller, 1997 or Friedmann, 1999). In 1981, several groups independently reported the development of replication-defective, recombinant, murine retrovirus-based retroviral vectors in order to correct gene defects in mammalian cells (Shimotohno and Temin, 1981 ; Tabin *et al.*, 1982; Wei *et al.*, 1981). The first demonstration of the correction of a genetic defect (of Lesch Nyhan syndrome) in cell culture conditions was in 1983 (Willis *et al.*, 1984). Clinical trials on gene transfer into humans began in 1989-90. After a first gene marking study, to determine if a foreign gene could be transferred *ex vivo* into human tumor-infiltrating lymphocytes (Rosenberg *et al.*, 1990), a clinical trial was carried out with patients suffering from severe combined immunodeficiency disorder (SCID) due to a deficiency in the enzyme adenosine deaminase (Ada). Using a retroviral vector the normal Ada gene was transferred into patients' T cells. Evidence for gene transfer and gene expression was evident in these studies, and there were some indications of clinical efficacy.

High Days of Clinical Trials: 1990-1995

These first clinical trials got a tremendous amount of media attention. They were the start of many other human gene therapy trials over the following years. In the meantime, other viral vector systems had been developed (Table **3**) including vectors derived from human adenovirus, herpes simplex virus and adeno-associated virus (Mulligan, 1993; Smith, 1995; Berns and Giraud, 1995). Non-viral gene transfer systems, such as liposomes were designed as well. All possible viral and non-viral vector systems were tested for gene therapy applicability on many forms of cancer, metabolic diseases such as cystic fibrosis, familial hypercholesterolaemia, hemophilia, neuromuscular disorders and AIDS. By 1995, over 200 approved gene therapy studies were on their way.

Orkin-Motulsky Report 1995: "STOP, Go Back to the Bench"

By fall 1995, it became clear that the expectations of clinical benefit due to gene therapy had been too high and premature. The NIH committee chaired by Drs Stuart Orkin and Arno Motulsky stated that the clinical potential of human gene therapy with current vectors had been overstated to the public and that they recommended a renewed emphasis on the basic study of vector design. A second committee, chaired by Dr. I. Verma, proposed changes to the review process of clinical studies on gene therapy. As a result of the disappointing clinical results and the renewed efforts made to improve vector design, new generations of vectors were developed such as lentiviral vectors and gutless adenoviral vectors. It was clear, though, that no single vector was going to be perfect. Each vector had advantages and disadvantages, making it appropriate for one particular application. Finally, strict regulations for the approval process of clinical protocols came into existence.

1999-2001: Cautious Optimism

During the fall of 1999, the gene therapy field was struck by the Jesse Gelsinger case. Jesse was the first patient who

died during a gene therapy trial at the University of Pennsylvania, following an intravenous injection of a high dosage of adenoviral vector from an older generation. Afterwards, it became clear that the death should rather be ascribed to "malpractice" than to inherent risks associated with viral vector technology. Nevertheless, this turmoil pointed again to the need for close scrutiny of gene therapy trials and public awareness of side-effects during ongoing trials (Teichler Zallen, 2000; Somia and Verma, 2000).

In spring 2000, the gene therapy field applauded the first demonstration of unambiguous clinical benefit following retroviral vector-mediated ex vivo gene therapy on two babies suffering from X-linked SCID (Cavazzana-Calvo *et al.*, 2000). Promising clinical trials for cancer (using p53-based gene therapy) and hemophilia are being developed as we speak.

Leukemia in 2 out of 11 children in this trial has been attributed to the insertion of the retroviral vector in the *LMO2* gene, again pointing to the caution required for clinical gene therapy trials.

REFERENCES

Berns, K. I., and Giraud, C. (1995). Adenovirus and adeno-associated virus as vectors for gene therapy. *Ann. NY. Acad. Sci.,* **772**: 95-104.

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Deist, F. L., and Fischer, A. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science,* **288**: 669-672.

Miller, A.D. Development and applications of retroviral vectors. In Retroviruses (1997). Coffin, J., Hughes, S., and Varmus, H., Eds. (Cold Spring Harbor Laboratory Press USA). pp 437-473.

Friedmann, T. The origins, evolution and directions of human gene therapy. In The development of human gene therapy. Fiedmann, T. Ed. (1999) (Cold Spring Harbor Laboratory Press USA).pp 1-20.

Mulligan, R. C. (1993). The basic science of gene therapy. *Science*, **260**: 926-932.

Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R., Karson, E. M., Lotze, M. T., Yang, J. C., Topalian, S. L., Merino, M.J., Culver, K., Miller, A.D., Blaese, R.M. and Anderson, W.F. (1990). Gene transfer into humans-immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med.,* **323**: 570-578.

Shimotohno, K., and Temin, H. M. (1981). Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. *Cell*, **26**: 67-77.

Smith, A. E. (1995). Viral vectors in gene therapy. *Annu. Rev. Microbiol.,* **49**: 807-838.

Somia, N., and Verma, I. M. (2000). Gene therapy: trials and tribulations. *Nat. Rev. Genet.,* **1**: 91-99.

Tabin, C. J., Hoffmann, J. W., Goff, S. P., and Weinberg, R. A. (1982). Adaptation of a retrovirus as a eucaryotic vector transmitting the herpes simplex virus thymidine kinase gene. *Mol. Cell Biol.,* **2**: 426-436.

Teichler Zallen, D. (2000). US gene therapy in crisis. *Trends Genet.,* **16:** 272-275.

Wei, C. M., Gibson, M., Spear, P. G., and Scolnick, E. M. (1981). Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. *J. Virol.,* **39**: 935-944.

Willis, R. C., Jolly, D. J., Miller, A. D., Plent, M. M., Esty, A. C., Anderson, P. J., Chang, H. C., Jones, O. W., Seegmiller, J. E., and Friedmann, T. (1984). Partial phenotypic correction of human Lesch-Nyhan (hypoxanthine-guanine phosphoribosyltransferase-deficient) lymphoblasts with a transmissible retroviral vector. *J. Biol. Chem.,* **259**: 7842-7849.