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**Chapter 18**

The Advantages of Liposome-Based Gene Therapy: A Comparison of Viral Versus Liposome-Based Gene Delivery

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| Section                                      | Page |
|----------------------------------------------|------|
| Introduction                                 | 345  |
| General Requirements for Gene Therapy Vectors| 346  |
| Different Methods of Gene Delivery           | 346  |
| Viral Vectors                                | 347  |
| Liposomal Vectors                            | 351  |
| Conclusions                                  | 359  |
| Note                                         | 360  |

**INTRODUCTION**

Gene therapy as a treatment for human disease is in a stage of intensive development. Already in limited use as an experimental therapy for cancer (Oldfield et al., 1993; Nabel et al., 1993) and inborn errors of metabolism (Hoogerbrugge et al., 1992; Morgan and Anderson, 1993), it will become a tool to treat diseases that are currently difficult to manage. There are many competing strategies being developed to introduce exogenous genes into a subset of cells of a patient. Several of these are likely to proceed to clinical application. Deciding which therapeutic strategy is
best suited for a given disease will require an understanding of the underlying biology of the disease, as well as the therapy.

In using this chapter to learn about liposomal methods of gene therapy, one should remember that the material presented here is only current as of January, 1994. For more recent developments and greater detail, it will be necessary to consult the original literature.

GENERAL REQUIREMENTS FOR GENE THERAPY VECTORS

Regardless of the specific disease being addressed by gene therapy, several factors must be considered before one treats patients. The first is that the method and the treatment itself must be commensurate with the severity of the disease. Gene therapy will not be the preferred treatment for diseases such as phenylketonuria or galactosemia, both of which can be treated adequately by diet modification. A more widely recognized problem is that the treatment may, while curing the disease, create a new one in the process. The archetypal concern is the generation of insertional mutations by the integration of the curative gene into a cellular proto-oncogene, leading to cancer in the “cured” patient.

Once a method has been determined to be reasonably safe for the patient, it must be shown not to present a danger to others. This problem has hampered the widespread use of viral transfection vectors. Substantial progress has been made in the construction of replication-defective retroviral and adenoviral vectors. However, the possibility remains that superinfection with wild-type adenovirus, or insertion of a wild-type retrovirus into an integrated defective retroviral genome, can generate new, infectious viral particles that could contain an oncogenic fragment of the therapeutic gene. These viral particles may have the ability to transfer genes between a patient’s cells, and between individuals.

In order for a method of gene therapy to be useful, it must significantly ameliorate the symptoms of the disease, and halt the progression of the disease state for a significant period of time. Ideally, the disease would be cured permanently by one treatment. Although periodic retreatments might be acceptable, every treatment brings with it the possibility of insertional mutagenesis or the generation of an immune response to the therapeutic vector.

The final considerations involve ease of preparation and use. Many candidate vectors, although potentially capable of introducing foreign genes into an organism, are so difficult to generate, or administer, that it is difficult to envision their widespread adoption. This aspect of vector design is often not considered until after the vector is developed.

DIFFERENT METHODS OF GENE DELIVERY

Bearing in mind the above considerations, let us now consider the various approaches being developed to create vectors for gene therapy. In general, these can be divided into three groups: (i) active viruses, (ii) viral mimics that attempt to
reproduce a subset of viral activities in a synthetic construct, and (iii) artificial delivery systems. Given the amount of effort being expended on the development of these systems, and the highly varied nature of the intended target diseases and organs, it is likely that there will never be a single best choice of method. Every disease will call for a tailored strategy that can be optimized to best fit the needs of the situation. Table 1 contains a summary and comparison of various gene therapy delivery vectors.

**Viral Vectors**

Ten years ago, it seemed a foregone conclusion that retroviruses were the only serious candidate for an eventual therapeutic vector. Today, many different viral vectors are being considered for use in gene therapy. Because different viruses target different cell types and have different modes of replication, each viral delivery system may be best suited for treatment of different diseases. The viral vectors under most active development are retroviruses, adenoviruses, adeno-associated virus, and herpesviruses. Some general characteristics of the viruses currently being developed as gene therapy vectors are presented in Table 2.

**Retroviruses**

Wild-type retroviruses are contained within a lipid membrane and have two identical copies of an RNA genome. After the viral particle's entry into the cell, the RNA molecule is reverse-transcribed into a DNA copy, which is then integrated into the host's genome. mRNAs for the various viral proteins synthesized during the course of the infection are transcribed from this integrated DNA. The integration of the retroviral DNA genome into the host genome forms the basis for the use of retroviruses as vectors for the introduction of exogenous DNA into eukaryotic cells (Hoeben et al., 1992).

To convert a wild-type retrovirus into a retroviral gene therapy vector, two steps are necessary: first, the therapeutic gene must be added to the retroviral and, genome second, the potential of the retrovirus to remain infectious after integration into the target cell must be eliminated. Presently, this is accomplished by inserting a therapeutic gene into a retroviral genome that has had most of the genes necessary for packaging the virus removed or mutated. Deletion of the viral genes eliminates infectivity in the treated cells by preventing the newly integrated genome from making infectious copies of itself that could complete further rounds of infection. It also has the added benefit of increasing the maximum length of a therapeutic gene that can be delivered by a retrovirus.

Some advantages of retroviruses as gene delivery vectors include: (i) the efficient and stable integration of the introduced gene into the host genome, (ii) a wide host range, and (iii) the ability to infect large numbers of cells. Potential
Table 1. Comparison of Different Gene Therapy Delivery Vectors

| Vector                    | Advantages                                                                 | Disadvantages                                                                 |
|---------------------------|----------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Retrovirus                | Efficient integration of gene into chromosomal DNA                         | Low expression of genes                                                      |
|                           |                                                                            | Possibility of superinfection and reactivation of virus                      |
|                           |                                                                            | Size of delivered gene limited to 7-13 kg                                    |
| Adenovirus                | High expression in some animal systems                                      | Viral genome remains episomal and is lost after time, leading to necessity for constant reinfection |
| Adeno-associated virus    | Site-specific integration of genome                                          | Size of insert limited to 5 kb                                               |
| Herpes simplex virus      | Stable expression in neuronal and other postmitotic cells                   | Trouble maintaining high levels of expression                               |
|                           |                                                                            | Possibility of reactivation of infectious virus                              |
| DNA-lipopolyamine complex | Technically simple                                                          | Cannot be targeted to a subset of cells                                      |
|                           | Can be used for large DNA fragments                                         |                                                                              |
| Cationic liposome-DNA     | Technically simple                                                          | Cannot be targeted to specific cells                                         |
| complexes                 | Works both in vitro and in vivo                                             | Does not usually work on postmitotic cells                                  |
|                           | Generally nontoxic to cells                                                 | Low efficiency with some cell types                                          |
|                           | Can be used for large DNA fragments                                         |                                                                              |
| Liposomes                 | Nontoxic                                                                    | Relatively low efficiency                                                    |
|                           | Technically simple                                                          | Not all tissues can be transfected in vivo                                   |
|                           | Can be used for large DNA fragments                                         |                                                                              |
|                           | Can deliver DNA for proteins                                                |                                                                              |
|                           | Can be targeted to specific cells                                           |                                                                              |
| Virosomes                 | All advantages listed for liposomes                                         | More complex preparation                                                    |
|                           | Much higher efficiency                                                      | Lower DNA entrapment efficiency than REV liposomes                           |
|                           |                                                                            | Viral protein may produce immune response                                    |
Table 2. Comparison of Viruses Being Developed as Gene Therapy Vectors

| Characteristic          | HSV   | Adenovirus | AAV  | Retrovirus |
|-------------------------|-------|------------|------|------------|
| Nucleic Acid            | dsDNA | dsDNA      | ssDNA| ssRNA      |
| Chromosomal integration | No    | No         | Yes  | Yes        |
|                         |       |            | (70% at one site) (random) |
| Size                    | 150-200| 70-100     | 18-26| 100-210    |
| Genome length (kb)      | 120-230| 36         | 5.5  | \(\sim\) 10 |
| Surrounded by envelope  | Yes   | No         | No   | Yes        |
| Site of fusion          | Cell membrane or endosome | Cell membrane or endosome | Cell membrane or endosome | Endosome |

Notes: HSV, Herpes simplex virus; AAV, adeno-associated virus.

problems associated with the use of viral vectors include: (i) the possibility of recombination events that could convert a replication-defective vector into an infectious agent, (ii) the possibility that superinfection with another retrovirus may allow unwanted transfer of the introduced gene between individuals, (iii) a 7-13 kb limit on the amount of DNA that can be packaged, (iv) potential problems in targeting the virus to specific cells, and (v) difficulty in maintaining high-level expression of the exogenous gene.

Adenoviruses

Wild-type adenoviruses cause respiratory disease in humans. The adenovirus genome is a linear double-stranded DNA molecule, about 36 kb in length. Although the virus does not have a lipid coat, it is taken up by cells via endocytosis and enters the cytoplasm in a manner analogous to the retrovirus. Once inside the cell, the viral DNA moves to the nucleus and begins its replication cycle without becoming part of the host cell's genome.

Adenoviruses that are being considered for use as gene therapy vectors have had substantial parts of their genome removed, rendering them unable to replicate except in specially developed cell lines (i.e., packaging cell lines) that express the removed proteins (Kozarsky and Wilson, 1993). The therapeutic gene to be introduced is added to the adenoviral genome by homologous recombination with a plasmid containing the therapeutic gene flanked by adenoviral DNA.

Although possible target organs for adenoviral gene therapy include liver, central nervous system, vascular endothelium, and muscle, the primary target organ is the lung. Clinical trials of adenoviral gene therapy for treatment of \(\alpha_1\)-antitrypsin deficiency and cystic fibrosis are underway (Crystal, 1992).
Advantages of adenovirus-based vectors include: (i) the ability to infect large numbers of cells, (ii) relative ease of constructing the vector and obtaining large amounts of virus, and (iii) absence of genomic integration, obviating the problem of insertional mutagenesis. Some disadvantages are: (i) the necessity to periodically reinfect the patient, as the genome is lost from dividing cells; (ii) the concomitant development of an allergic response; and (iii) possible toxicity of high doses of virus.

**Adeno-Associated Virus**

Adeno-associated virus is a naturally occurring defective virus that infects human lungs. Although it is unable to go through lytic infection of cells independently, it can do so in the presence of a separate infection by adenovirus or herpes simplex virus. In the absence of a helper infection by one of these viruses, the adeno-associated virus establishes a latent infection of the host cell by integrating itself into the host genome. About 70% of the integrations of the wild-type virus occur at a single site on chromosome 19 (Samulski et al., 1991). Because these integrations do not seem to be associated with any disease, this virus may eventually be used to add therapeutic genes to the human genome with a lower risk of insertional mutagenesis. To date, however, the targeted nature of the insertional events is not retained when the native viral genome is replaced by a therapeutic or marker gene. In addition, packaging constraints limit the size of any insert to 4.5-5 kb of DNA.

**Herpesviruses**

The genomes of herpesviruses are very large and complex. This fact alone might argue against the use of herpes simplex virus I (HSV-I) as a gene therapy vector, since a complex genome will be more difficult to effectively “tame.” However, HSV-I has an advantage that compensates for this problem, in that the virus can exist and mediate persistent expression of its genes in neurons and other postmitotic cells (Geller, 1993). In the normal course of an infection, HSV-I will enter neuronal cells and remain latent, sometimes for years, before reactivating and starting a lytic cycle that results in a small patch of endothelial cell death (e.g., a cold sore). During latency, the host cell transcribes some viral genes, producing what are termed latency associated transcripts (LATs). HSV-I deletion mutants have been isolated that are incapable of reactivation, and that once inserted into a neuron will remain latent indefinitely. If therapeutic genes added to these vectors have LAT promoter elements, they will be expressed even though the virus itself remains latent. These vectors are being developed for the introduction of genes into cells of the central nervous system (Geller, 1993).

In summary, all viral methods of gene therapy share several strengths, as well as numerous weaknesses. It is relatively easy to produce the large amounts of virus
necessary to treat patients. Viruses efficiently infect a large fraction of the cells with which they come in contact. Although this may be a drawback in some cases where expression of the therapeutic gene must be restricted to specific cells, it may be possible to limit unwanted expression of the inserted gene by the use of tissue-specific promoters. All viral vectors for human gene therapy are derived from organisms that cause human disease. The possibility of reactivation of the vectors’ infectivity or pathogenicity is hard to dismiss. In the end, it may prove easier and safer to construct a gene therapy vector having the elements of a successful virus, but derived from completely synthetic components. One such strategy involves the use of liposomes.

**Liposomal Vectors**

Liposomes are small vesicles usually made from pure preparations of phospholipids. Because they can be made from completely pure components, they have been used extensively to characterize and define the physical properties of membranes and membrane proteins. Liposomes have also been used as a means of delivering substances to cells, both *in vitro* and *in vivo*, including DNA (Hug and Sleight, 1991).

Before discussing factors to be considered in preparing liposomes for gene therapy of a given disease, it is worthwhile to review some general aspects of liposome preparation and design. Liposomes are generally classified by the number of bilayers (lamellae) they contain, by size, and by the composition of their membrane. The earliest liposomes were formed by hydration of a dried lipid film, followed by vortexing. This results in a cloudy solution of liposomes having many lamellar layers, resembling an onion. These are called multilamellar vesicles, or MLVs. Although MLVs are effective for the packaging of lipophilic drugs, they have very little aqueous volume in their interiors, and are not efficient at entrapping water-soluble compounds or large DNA fragments. Because of these deficiencies, MLVs were quickly superseded by unilamellar vesicles, which have only a single lipid bilayer between the external fluid and the lumen. Several methods are used to prepare unilamellar vesicles, each having its own advantages and disadvantages. Because of space limitations, we will only briefly describe those methods of liposome preparation that are directly relevant to the construction of gene therapy vectors. Table 3 gives a direct comparison of these methods of preparation as related to transfection.

**Methods of DNA Encapsulation**

*Cationic Liposomes.* Liposomes made entirely or partially of a cationic lipid have a charge-based affinity for DNA. When such liposomes are incubated in solution with DNA, they form complexes that can transfect cells. Several types of cationic lipid are available which in general have similar characteristics (Leventis
Table 3. Methods of Liposome Preparation for Transfection

| Method                  | Tested DNA Encapsulation Efficiency (%) | Viral Particles Used as Fosogens                                                                 |
|-------------------------|-----------------------------------------|--------------------------------------------------------------------------------------------------|
| Ca/EDTA chelation       | No                                      | Made with Sendai or influenza viral envelopes                                                   |
| Chimerasome             | Yes                                      | 36                                                                                               |
| Detergent dialysis      | No                                      | 2-15                                               Reconstituted Sendai virus envelopes             |
| Extrusion               | Yes                                      | 30                                                                                               |
| Minimal volume entrapment | No                                      | No determined                                                                                    |
| Reverse-phase evaporation | Yes                                      | 25-40                                               Reconstituted Sendai virus envelopes             |
|                         |                                         |                                                   Inactivated Sendai virus                                                                      |
|                         |                                         |                                                   Reconstituted influenza virus envelopes                                                       |
|                         |                                         |                                                   Inactivated influenza virus                                                                  |
| Sonication              | Yes                                      | Not determined                                     Inactivated Sendai virus                          |
| Lipofection             | Yes                                      | DNA external to vesicles                                                                         |

Note: All methods described here have been used in vitro. In some cases the vesicles are prepared with solubilized viral membranes to form virosomes. In others, the liposomes are co-incubated with virosomes or intact viruses to promote fusion with cells. Commonly used viral material is indicated in the column labeled “Viral particles used as fosogens.” DNA encapsulation efficiency refers to the fraction of DNA in solution that becomes encapsulated during liposome formation.

and Silvius, 1990; Ito et al., 1990; Gao and Huang, 1991; Hazinski et al., 1991; Farhood et al., 1992). This technique was initially intended exclusively for in vitro use. However, in vivo transfections have been performed with several commercial preparations of cationic lipids.

There are several reasons why cationic lipid-DNA complexes have proved so popular for transfections: (i) They are very easy to prepare. The liposomes and the DNA are incubated together, and then used without further preparation. (ii) The complexes are relatively non-toxic. (iii) Transfections using this method are efficient with most cell types. And (iv) because the DNA remains outside the liposome, questions of entrapment efficiency do not apply.

In vivo transfections using cationic lipid-DNA complexes are completely uncontrolled. That is, there are no targeting or specific binding agents present, and the complexes probably deliver DNA to the first cell they contact. This occurs most often in the first capillary bed through which the injected complexes pass. When DNA-lipid complexes are intravenously injected, the highest levels of gene expression are usually seen in the pulmonary vascular endothelium (Brigham et al., 1993).
DNA-lipid complexes introduced into the airway cause a generalized transfection of the lung. Because the site of transfection is completely uncontrolled, expression of the inserted gene may need to be regulated by means of tissue-specific promoters (Hazinski et al., 1991). Cell specific promoters are not available for all tissues, and those currently available are sometimes less specific than desired. While both cationic liposomes and retroviral gene delivery vectors lack the ability to target specific cells, the lack of length constraints makes tissue-specific expression easier to achieve using DNA-lipid complexes as compared to retroviruses.

Ca$^{2+}$/EDTA Chelation. In the presence of calcium, small unilamellar vesicles (SUVs) composed of acidic phospholipids (typically phosphatidylserine) aggregate and fuse into large cochleate structures. When EDTA is added to chelate the calcium, these structures form large unilamellar vesicles (LUVs) (Papahadjopoulos et al., 1975). In the process, the vesicles encapsulate a substantial fraction of the surrounding solvent and solutes. If DNA is present, some of it becomes encapsulated in the vesicles. Two advantages of this technique are: (i) the DNA is not exposed to harsh conditions that would degrade it, and (ii) the entrapment is relatively efficient. However, the requirement for phosphatidylserine makes this technique impractical for most in vivo applications, with the exception of transfecting macrophages which have cell surface receptors for phosphatidylserine (PS).

Detergent Dialysis (and Virosomes). When membranes are mixed with detergents at concentrations above their critical mixed micellar concentration (CMMC), they combine with the membrane’s phospholipids and proteins to form mixed micelles. Because detergents are relatively water soluble, they can be removed from the mixed micelles by dialysis. When their concentration in the micelles falls below the CMMC, membrane bilayers regenerate and form liposomes.

Enveloped viruses have membrane proteins that promote the fusion of the viral membrane with cellular membranes. When an enveloped virus fuses to a cell, the nucleocapsid is released into the cytoplasm, and the infection proceeds. Detergent solubilization of enveloped viruses produces a micellar mixture containing detergent, viral phospholipids, and viral membrane proteins. Dialysis of these detergent mixtures results in the production of liposomes that are often called virosomes to indicate the presence of viral proteins. If the dialysis is performed properly, the fusion proteins of many viruses can be reconstituted in an active conformation (White et al., 1983). Viral fusion proteins having a variety of different characteristics have been identified. For example, some are only active at low pH and fuse with endosomal membranes, while others are active at physiological pH and fuse to the plasma membrane. Some viral fusion proteins, such as the HA protein of influenza, bind to specific receptors before fusion. A few viral fusion proteins will only promote fusion to target membranes containing a specific mixture of phospholipids (Cervin and Anderson, 1991; Nussbaum et al., 1992).
The major advantage of detergent dialysis is that it is very gentle since no sonication, vortexing, or organic solvent is used. This results in only a small amount of DNA fragmentation during encapsulation. Unfortunately, the efficiency of entrapment is very low, causing much of the DNA to be wasted and resulting in a high fraction of empty liposomes being formed. Loyter and co-workers have fused DNA-containing liposomes with cells by making empty virosomes and DNA-containing liposomes separately, and then fusing the two populations of vesicles in the presence of target cells (Lapidot and Loyter, 1990). To date, this technique has not been used *in vivo*.

*Reverse-Phase Evaporation.* Currently, the highest DNA entrapment efficiencies can be achieved when vesicles (REVs) are prepared by reverse-phase evaporation (Fraley et al., 1980). REVs are prepared by bath sonication of a mixture of lipid, DNA, ether, and buffered saline, followed by evaporation of the ether to form a paste of phospholipid inverted monolayers surrounding aqueous droplets. These are resolved into liposomes by vortexing the paste. Encapsulation efficiency is high (up to 40 or 50%) because there is only a small amount of aqueous buffer present in relation to the amount of lipid. Presumably, DNA molecules present in the buffer are forced to curl up in small aqueous droplets, a conformation that is easily entrapped (Szelei and Duda, 1989). Because the size distribution of the REVs is heterogeneous and hard to control, they are often sized by extrusion through a filter before use.

*Extrusion.* Multilamellar vesicles formed by vortexing a hydrated lipid film are repeatedly passed (extruded) through a filter under pressure. As the liposomes are forced through the pores of the filter, they are converted from multilamellar to unilamellar vesicles, and the buffer and anything present in it is trapped inside the liposomes (Mayer et al., 1986). To a certain extent, the size of the liposomes corresponds to the pore size of the filter used. Advantages of this technique are: (i) nearly any lipid or lipid mixture can be used, (ii) lipid size from 25 to 100 nm in diameter can be controlled, and (iii) the encapsulation efficiency is excellent (up to 30%). The main disadvantage is that the high pressures used in the extrusion process can fragment the DNA, and denature or dissociate DNA-protein complexes.

*Entrapment Efficiencies*

A central difficulty in using liposomes for gene therapy is the entrapment of the DNA. The ideal encapsulation method would efficiently entrap but not degrade the DNA. At the same time, the method would be sufficiently flexible with regard to size and vesicle composition to allow targeting of liposomes based on these properties. DNA in solution has a linear conformation. In general, DNA restriction fragments containing an entire gene are much longer than the diameter of com-
monly used liposomes. It should not be surprising then that most methods of liposome formation entrap DNA only at low efficiency, and that the efficiency is inversely related to the length of the DNA (Fraley et al., 1980).

Two methods of increasing the entrapment efficiency of DNA have been developed. In the first, the liposomes are formed in such a way that the aqueous phase, containing the DNA, is broken into droplets small enough such that the DNA is forced to curl in upon itself to remain within the droplet. This probably occurs during REV formation. The other method achieves the same result by complexing the DNA with some substance in a tight and compact aggregate. To date, this has been done with spermine (Tikchonenko et al., 1988) and basic proteins (Jay and Gilbert, 1987). The efficiency of encapsulation can also be increased by compacting the DNA in phage head particles (Szelei and Duda, 1989) before liposomal entrapment.

In Vivo Survival

The basis for successful transfections by liposomal vectors delivered intravenously lies in the maximization of the in vivo circulation time of the liposomes. Most liposome formulations are cleared rapidly from the circulation by the reticuloendothelial system (RES), with large amounts accumulating in macrophages (Allen et al., 1989). If the intended target of the gene therapy is the RES, as in Gaucher's disease, this is a benefit. In most cases, however, this fate will only serve to reduce the number of liposomes being exposed to their intended target cell population.

While several different strategies have been explored to lengthen the circulation half-life of liposomes, they all have certain aspects in common (Allen et al., 1989; Gabizon and Papahadjopoulos, 1992). First, they seek to make the liposomes as small as possible. This seems to reduce their interaction with the RES. Second, they are designed to make the liposomal surface as much like the surface of a normal cell as possible. This can be accomplished by using lipid formulations containing a high fraction of cholesterol, high transition temperature phospholipids, and a substantial fraction of sphingomyelin or the ganglioside GM1. A different approach has been to modify the liposomal phospholipids by addition of polyethylene glycol (PEG) to the polar headgroup (Lasic et al., 1991; Blume and Cevc, 1993). PEG-modified lipids have a higher hydrophilicity than native lipids, and appear to escape recognition by macrophages. A potential disadvantage of this approach is that targeting of liposomes with covalently attached antibodies (see below) is inhibited by the presence of PEG-modified lipids (Klibanov et al., 1991).

Targeting to the Proper Cell Type

Once a liposome formulation has been settled upon, attention must be paid to limiting the number of cells exposed to the DNA delivered by the liposomes. This
is necessary for several reasons. First, if the DNA will be integrated into the host cell's genome, there is a chance that insertional mutagenesis will lead to activation of a proto-oncogene and the development of cancer by the host. By limiting the sites of DNA delivery, the chance of insertional mutagenesis is lowered. Second, limiting the number of cells that are transfected reduces the amount of DNA, lipid, and other proteins needed to perform the gene therapy. This reduction can be substantial, and can reduce potential systemic toxicities, as well as cost. Table 4 compares some different methods of targeting liposomes to specific cell types.

There are three general methods by which liposomes may be directed to a subset of cells within an organism. First, the liposomes may be introduced into a compartment of the body that is sequestered from the general circulation. One example of this is the addition of liposomes to the airway in a fluid or an aerosol (Hazinski et al., 1991; Stribling et al., 1992). Because liposomes cannot cross the lung epithelial barrier, entrance into the general circulation is prevented. Therefore, it is anticipated that liposome vectors will transflect only those cells present on the epithelial surface. Another site where this approach may be applicable is in the central nervous system (CNS). In the cerebral vasculature, blood is separated from the CNS by the blood-brain barrier, which would normally be completely impermeable to any liposomes. However, if the cerebral vasculature is isolated, and perfused with a hyperosmotic solution, the blood-brain barrier is damaged, leaving holes large enough to admit liposomes into the CNS (Johansson, 1992). The blood-brain barrier reconstitutes itself shortly thereafter, leaving the liposomes trapped within the CNS and unable to interact with any other cells.

| Targeting Method      | Advantages                               | Disadvantages                                                                 |
|-----------------------|------------------------------------------|-------------------------------------------------------------------------------|
| Antibody              | High degree of flexibility                | Unique epitope must be found to take advantage of specificity                  |
|                       | Extreme specificity                       | Antibody to desired target must be generated if it does not already exist    |
|                       |                                         | Incompatible with PEG-coated liposomes                                        |
| Derivatized proteins  | High degree of flexibility                | Protein must be modified and characterized                                    |
|                       | Low degree of immunogenicity             | Not all derivatized proteins retain function                                   |
|                       |                                         | Not all derivatized proteins partition into membranes                         |
| Injection site        | Good specificity                         | Target must be isolated from general circulation                              |
| Lipid composition     | High degree of flexibility                | May not be specific enough                                                    |
|                       | Simplicity                               |                                                                               |
Second, the lipid composition of the vesicles can be adjusted to cause them to be taken up preferentially by some types of cells. For example, macrophages have a very high affinity for liposomes containing (PS) on their surface (Lee et al., 1992). Consequently, liposomes containing a substantial fraction of PS are quickly cleared from the circulation by macrophages (Allen et al., 1989). A similar targeting strategy uses lactosylceramide as a membrane component. This lipid binds to the asialoglycoprotein receptors found on hepatocytes, causing these cells to be preferentially targeted by the vesicles (Grosse et al., 1984).

The final method involves the addition of antibodies or other proteins to the surface of the liposome after it has been formed (Loughrey et al., 1990). These “proteoliposomes” are taken up by cells to which they can bind. Table 5 gives a representative list of proteins that have been used to target liposomes to cells. Many proteins can be modified by the addition of phospholipid molecules or acyl chains and retain their native function. These modified proteins can be coupled to a preexisting bilayer, endowing the liposome with their binding properties.

All three methods described above have been used successfully to direct the uptake of liposomes to specific cell types. The methods are not mutually exclusive and, in principle, it should be possible to combine them for more specific targeting.

**Successful Introduction of DNA into Cytoplasm**

The central problem of any liposome-based gene therapy system is that DNA must be introduced into the cytoplasm of the target cell. When DNA-loaded liposomes are added to cells they are endocytosed, and most are carried to lysosomes where they are degraded by digestive enzymes. Somehow, a small fraction of encapsulated DNA escapes this pathway, enters the cytoplasm, and is expressed by the cells (Straubinger et al., 1990). Several methods have been developed to increase the proportion of DNA delivered to the cytosol. Two general approaches that have been used successfully are: (i) bypassing the endocytic

| Protein          | Hydrophobic Moiety          | Molecular Target                        |
|------------------|----------------------------|----------------------------------------|
| Antibody         | PE                         | many                                   |
| Antibody         | PE, palmitoyl lysine       | many                                   |
| IgG              | stearylamine               | hepatitis B surface antigen            |
| Influenza HA     | Fatty acid                 | sialic acid residues                   |
| Transferrin      | Stearylamine               | transferrin receptor                   |
| Serum transferrin| PE                        | transferrin receptor                   |
| Streptavidin     | PE                         | biotinylated antibody                  |
| Glu-plasminogen | PE                         | fibrin clots                           |

*Table 5. Proteins Used to Target Liposomes*

*Notes: To associate the proteins with liposomes, hydrophobic moieties are covalently linked to the proteins. These hydrophobic moieties become associated with the vesicles’ bilayer, anchoring the proteins. PE, phosphatidylethanolamine.*
pathway completely, and (ii) using the initial stages of the endocytic pathway but escaping the pathway before delivery of the DNA to lysosomes.

The endocytic pathway of liposomal DNA entry into the cell is bypassed when liposomes are fused directly to the plasma membrane of the cell. This has been accomplished using virosomes containing a constitutively active viral fusion protein (Nakanishi et al., 1987). As soon as the virosole binds to the cell surface, the fusion protein acts to fuse the virosomal membrane with that of the cell, causing the vesicle contents to be introduced directly into the cytoplasm.

Some liposome delivery systems use the initial stages of the endocytic pathway in the delivery process. This strategy takes advantage of the low pH of endosomes, which can be used to trigger fusion of liposomes with the endosomal membrane. pH-dependent fusion of liposomes with endosomes has been achieved using vesicles composed of phosphatidylethanolamine (PE) and substances such as dipalmitoylsuccinylglycerol that leave the membrane at low pH (Liu and Huang, 1990). As the pH is lowered in the endosome, the effective concentration of PE rises. When the concentration rises above 60 mole percent, the vesicle membrane can no longer remain as a bilayer. As it converts to the inverted hexagonal phase, it destabilizes the adjacent endosomal membrane, and releases the liposomal contents into the cytoplasm. Similar results can be seen using virosomes containing pH-sensitive fusion proteins (Gould-Fogerite et al., 1989; Lapidot and Loyter, 1990).

**Nuclear Targeting**

Once the DNA has successfully been introduced into the cytoplasm of the target cell, the factors governing expression are much less well understood. However, proteins exist that are karyophilic, that is, they are actively transported into the nucleus. Two such proteins are the Semliki Forest Virus nucleocapsid protein (Michel et al., 1990) and the non-histone high mobility group I chromosomal protein (HMG I) (Kato et al., 1991; Tomita et al., 1992). If a gene is coencapsulated in a liposome with HMG I, expression increases as much as 10-fold over the levels seen without the protein. Delivery of DNA/protein complexes has the potential for greatly increasing transfection efficiency, and should prove to be compatible with most methods of liposomal preparation.

**Maintenance of High-Level Expression**

One shortcoming that all present approaches to gene therapy share is their inability to maintain a high level expression of the transfected gene for long periods of time. For some applications, such as the activation of the immune system to fight a cancer, this will probably not be a problem. However, to effectively cure many genetic diseases, the gene will need to be expressed at near normal levels throughout the patient’s life.
Successful transfection of cells with some currently available viral vectors may lead to expression of at least some viral proteins, resulting in the clearance of the cells by the host’s immune system. This process may be partially responsible for the loss of expression seen in transfections with retroviral and adenoviral vectors. Another possible cause of low levels of expression is that length constraints of viral vectors usually necessitate the use of cDNA copies of genes. These constructs do not have introns, greatly reducing their length. There is some evidence from transgenic mouse experiments that expression of exogenous genes is higher when at least some of the introns are present (Brinster et al., 1988; Palmiter et al., 1991). If this is true for genes inserted by viral or liposomal methods, then it may be necessary to include introns in the delivered DNA to produce an adequate therapeutic effect. Unfortunately, increasing the size of the therapeutic gene would prevent the use of most viral vectors.

**CONCLUSIONS**

While all the methods of gene therapy have specific advantages, none is suitable for all applications. In an environment as complex as a living organism, it is futile to hope that a single method might be ideal in all situations. However, the availability of many methods greatly increases the number and types of diseases that can be treated.

Viruses have evolved such that they are able to efficiently introduce and express exogenous genes (i.e., viral genes) in eukaryotic cells. However, it is important to remember that viruses have been optimized by selective pressure to meet criteria that are not precisely those of gene therapy. In particular, most viruses need to maintain high-level expression of their proteins for only a short time, and need not be concerned with the viability of the host cell after infection. Attempts to modify a virus into a gene therapy vector will be hampered by this conflict. Virus-based methods of gene therapy are likely to be most useful in applications that require a burst of high-level expression in many of the patient’s cells, such as in cancer therapy.

Cationic lipid-DNA complexes will never be as selective as either virus- or conventional liposome-based gene therapy. However, they will remain much easier to prepare and use. The complexes will probably find their greatest use in *in vitro* and animal gene transfer. Although they are currently more widely used in all areas of gene transfer than any other technique, their lack of control and cell-specificity will probably lead to their replacement by viral and liposomal methods of gene introduction in clinical areas.

Liposomal methods of gene therapy are flexible, in that all the components of the system are controlled by the designers. As these systems have become more sophisticated, they have begun to take on several characteristics of the viruses that they are intended to replace. The use of basic substances to condense the DNA has
increased the efficiency of encapsulation. The addition of nucleophilic proteins raises the efficiency of transfection. By adding antibodies or other targeting molecules to the surface of liposomes, preferential binding of vesicles to a desired cell type has been increased. The incorporation of viral fusion proteins into the membrane allows a greater fraction of liposomes binding to cells to deliver their contents into the cytoplasm. In short, many aspects of a virus from budding to delivery of the genetic material to the nucleus have been mimicked in liposomal vectors. Current methods of liposome-based gene therapy may be improved by modifying the structure of the introduced gene. Here, preliminary indications are that constructs resembling native genes, rather than cDNAs, will provide the best long-term expression.

Not all these enhancements to the basic liposome have been used together, and not all of them are mutually compatible. However, it seems inevitable that the best liposome-based gene therapy vectors will eventually combine many of these enhancements. The resulting liposome, with a condensed DNA-protein complex containing both histonelike proteins and nuclear targeting proteins, a membrane designed for long circulation within the blood, targeting molecules on the surface, and viral fusion proteins in the membrane, will in effect be a completely man-made nonreplicative virus.

NOTE

1. For a detailed description of liposome preparation and use, the reader should consult the following two books: Liposome Technology, edited by G. Gregoriadis, 1993, CRC Press, Boca Raton, FL, and Liposomes: A Practical Approach, edited by R.R.C. New, 1990, Oxford University Press, New York, NY. Both describe in detail methods for making different types of liposomes.

REFERENCES

Allen, T.M., Hansen, C., & Rutledge, J. (1989). Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. Biochim. Biophys. Acta 981, 27-35.
Blume, G., & Cevc, G. (1993). Molecular mechanism of the lipid vesicle longevity in vivo. Biochim. Biophys. Acta 1146, 157-168.
Brigham, K.L., Meyrick, B., Christman, B., Conary, J.T., King, G., Berry, L.C., Jr., & Magnuson, M.A. (1993). Expression of human growth hormone fusion genes in cultured lung endothelial cells and in the lungs of mice. Am. J. Respir. Cell. Mol. Biol. 8, 209-213.
Brinster, R.L., Allen, J.M., Behringer, R.R., Gelinas, R.E., & Palmiter, R.D. (1988). Introns increase transcriptional efficiency in transgenic mice. Proc. Natl. Acad. Sci. USA. 85, 836-840.
Cervin, M., & Anderson, R. (1991). Modulation of coronavirus-mediated cell fusion by homeostatic control of cholesterol and fatty acid metabolism. J. Med. Virol. 35, 142-149.
Crystal, R.G. (1992). Gene therapy strategies for pulmonary disease. Am. J. Med. 92, 44S-52S.
Farhood, H., Bottega, R., Epand, R. M., & Huang, L. (1992). Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity. Biochim. Biophys. Acta 1111, 239-246.
Liposomal Gene Therapy

Fraley, R., Subramani, S., Berg, P., & Papahadjopoulos, D. (1980). Introduction of liposome-encapsulated SV40 DNA into cells. J. Biol. Chem. 255, 10431-10435.

Gabizon, A., & Papahadjopoulos, D. (1992). The role of surface charge and hydrophilic groups on liposome clearance in vivo. Biochim. Biophys. Acta 1103, 94-100.

Gao, X.A., & Huang, L. (1991). A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochem. Biophys. Res. Commun. 179, 280-285.

Geller, A.I. (1993). Herpesviruses: expression of genes in postmitotic brain cells. Curr. Opin. Gen. Devel. 3, 81-85.

Gould-Fogerite, S., Mazurkiewicz, J.E., Raska, K., Jr., Voelkerding, K., Lehman, J.M., & Mannino. (1989). Chimerasome-mediated gene transfer in vitro and in vivo. Gene 84, 429-438.

Grosse, E., Kieda, C., & Nicolau, C. (1984). Flow cytofluorometric investigation of the uptake by hepatocytes and spleen cells of targeted and untargeted liposomes injected intravenously into mice. Biochim. Biophys. Acta 805, 354-361.

Hazinski, T.A., Ladd, P.A., & DeMatteo, C.A. (1991). Localization and induced expression of fusion genes in the rat lung. Am. J. Respir. Cell. Mol. Biol. 4, 206-209.

Hoeben, R.C., Valero, D., van der Eb, A.J., & van Ormondt, H. (1992). Gene therapy for human inherited disorders: techniques and status. Crit. Rev. Oncol. Hematol. 13, 33-54.

Hoogerbrugge, P.M., Vossen, J.M., Beusechem, V.W., & Valerio, D. (1992). Treatment of patients with severe combined immunodeficiency due to adenosine deaminase (ADA) deficiency by autologous transplantation of genetically modified bone marrow cells. Hum. Gene Ther. 3, 553-558.

Hug, P., & Sleight, R.G. (1991). Liposome-mediated transformation of eukaryotic cells. Biochim. Biophys. Acta 1097, 1-17.

Ito, A., Miyazoe, R., Mitoma, J., Akao, T., Osaki, T., & Kunitake, T. (1990). Synthetic cationic amphiphiles for liposome-mediated DNA transfection. Biochem. Int. 22, 235-241.

Jay, D.G., & Gilbert, W. (1987). Basic protein enhances the incorporation of DNA into lipid vesicles: model for the formation of primordial cells. Proc. Natl. Acad. Sci. USA. 84, 1978-1980.

Johansson, B.B. (1992). Experimental models of altering the blood-brain barrier. Prog. Brain. Res. 91, 171-175.

Kato, K., Kaneda, Y., Sakurai, M., Nakanishi, M., & Okada, Y. (1991). Direct injection of hepatitis B virus DNA into liver induced hepatitis in adult rats. J. Biol. Chem. 266, 22071-22074.

Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P., & Huang, L. (1991). Activity of amphiphatic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. Biochim. Biophys. Acta 1062, 142-148.

Kozarsky, K.F., & Wilson, J.M. (1993). Gene therapy: Adenovirus vectors. Curr. Opin. Gen. Devel. 3, 499-503.

Lapidot, M., & Loyter, A. (1990). Fusion-mediated microinjection of liposome-enclosed DNA into cultured cells with the aid of influenza virus glycoproteins. Exp. Cell. Res. 189, 241-246.

Lasic, D.D., Martin, F.J., Gabizon, A., Huang, S.K., & Papahadjopoulos, D. (1991). Sterically stabilized liposomes: A hypothesis on the molecular origin of the extended circulation times. Biochim. Biophys. Acta 1070, 187-192.

Lee, K.D., Hong, K., & Papahadjopoulos, D. (1992). Recognition of liposomes by cells: In vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density. Biochim. Biophys. Acta 1103, 185-197.

Lenthen, R., & Silvius, J.R. (1990). Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. Biochim. Biophys. Acta 1023, 124-132.

Liu, D., & Huang, L. (1990). pH-sensitive, plasma-stable liposomes with relatively prolonged residence in circulation. Biochim. Biophys. Acta 1022, 348-354.

Loughrey, H.C., Choi, L.S., Cullis, P.R., & Bally, M.B. (1990). Optimized procedures for the coupling of proteins to liposomes. J. Immunol. Methods 132, 25-35.
Mayer, L.D., Hope, M.J., & Cullis, P.R. (1986). Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim. Biophys. Acta 858, 161-168.

Michel, M.R., Elgizoli, M., Dai, Y., Jakob, R., Koblet, H., & Arrigo, A.P. (1990). Karyophilic properties of Semliki Forest virus nucleocapsid protein. J. Virol. 64, 5123-5131.

Morgan, R.A., & Anderson, W.F. (1993). Human gene therapy. Ann. Rev. Biochem. 62, 191-217.

Nabel, G.J., Nabel, E.G., Yang, Z.-Y., Fox, B.A., Plauty, G.E., Gao, X., Huang, L., Shu, S., Gordon, D., & Chang, A.E. (1993). Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. Proc. Natl. Acad. Sci. USA. 90, 11307-11311.

Nakanishi, M., Uchida, T., Sugawa, H., Ishiura, M., & Okada, Y. (1987). The improved efficient method for introducing macromolecules into cells using HVJ (Sendai virus) liposomes with gangliosides. Exp. Cell Res. 173, 56-59.

Nussbaum, O., Rott, R., & Loyter, A. (1992). Fusion of influenza virus particles with liposomes: Requirement for cholesterol and virus receptors to allow fusion with and lysis of neutral but not negatively charged liposomes. J. Gen. Virol. 73, 2831-2837.

Oldfield, E.H., Ram, Z., Culver, K.W., Blaese, R.M., DeVroom, H.L., & Anderson, W.F. (1993). Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. Hum. Gene Ther. 4, 39-69.

Palmiter, R.D., Sandgren, E.P., Avarbock, M.R., Allen, D.D., & Brinster, R.L. (1991). Heterologous introns can enhance expression of transgenes in mice. Proc. Natl. Acad. Sci. USA. 88, 478-482.

Papahadjopoulos, D., Vail, W.J., Jacobson, K., & Poste, G. (1975). Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. Biochim. Biophys. Acta 394, 483-491.

Samulski, R.J., Zhu, X., Xiao, X., Brook, J.D., Housman, D.E., Epstein, N., & Hunter, L.A. (1991). Targeted integration of adenov-associated virus (AAV) into human chromosome 19. EMBO J. 10, 3941-3950.

Straubinger, R.M., Papahadjopoulos, D., & Hong. K. (1990). Endocytosis and intracellular fate of liposomes using pyranine as probe. Biochemistry 29, 4929-4939.

Stirling, R., Brunette, E., Liggitt, D., Gaensler, K., & Debs, R. (1992). Aerosol gene delivery in vivo. Proc. Natl. Acad. Sci. U. S. A. 89, 11277-11281.

Szelei, J., & Duda, E. (1989). Entrapment of high-molecular-mass DNA molecules in liposomes for the genetic transformation of animal cells. Biochem. J. 259, 549-553.

Tikchonenko, T.I., Glushakova, S.E., Kisliina, O.S., Grodnitskaya, N.A., Manykin, A.A., & Naroditsky, B.S. (1988). Transfer of condensed viral DNA into eukaryotic cells using proteoliposomes. Gene 63, 321-330.

Tomita, N., Higaki, J., Morishita, R., Kato, K., Mikami, H., Kaneda, Y., & Ogihara, T. (1992). Direct in vivo gene introduction into rat kidney. Biochem. Biophys. Res. Commun. 186, 129-134.

White, J., Kielian, M., & Helenius, A. (1983). Membrane fusion proteins of enveloped animal viruses. Quarterly Rev. Biophys. 16, 151-195.