Non-viral approaches to gene therapy

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Several advances in non-viral gene transfer technology have been reported over the past year. Cationic lipids have been successfully used to deliver genes *in vivo*, providing a clear alternative to recombinant viruses. In addition, investigators have demonstrated that direct application of DNA via injection or particle bombardment can be used for vaccination. Analysis of the mechanisms employed by viruses to invade cells has demonstrated a crucial role for membrane-active proteins or peptides in the entry process. Several non-viral systems that include membrane-active elements are now available.

Introduction

Many laboratories are developing non-viral methods of introducing genes into mammalian cells for applications in human gene therapy. To support these efforts, there is a practical argument favoring the simplicity and increased safety of non-viral systems. Recombinant viruses are fine in the laboratory and are probably safe in patients, but the safety certification of these vectors is extremely time-consuming and labor intensive. Non-viral systems provide a great flexibility in the size and sequence of DNA molecules that can be delivered. A second argument suggests that the generation of simple and efficient non-viral delivery systems requires an understanding of all the limiting steps in the DNA-delivery process. Learning to deliver DNA effectively to the target cell will teach us many things about endocytosis, membrane destabilization, viral entry and gene maintenance in transfected cells.

Successful delivery of a long, slender, hydrophilic and polyanionic DNA molecule (of micrometer dimensions) through three lipid bilayers (the cell membrane, as well as the double nuclear membrane) requires a number of obstacles to be overcome. First, the DNA must avoid mechanical shearing and enzymatic degradation. Second, it must survive a transient stay in the extracellular environment, either *ex vivo* in cell culture medium/serum (not harsh, and quite simple to alter) or *in vivo* in blood (more harsh) and other body fluids (e.g. bile, lymph and lung fluid) with exposure to the defense cells of the immune system as well as to components of the complement system. Third, DNA complexes must bind to the target cell (and perhaps not to other cells) and be of an internalizable size. Fourth, the chemistry of the complex must both allow or enhance release of the DNA from entrapment in vesicles and enable DNA to escape endosomes or lysosomes to the cytosol. Fifth, once the DNA is within the cell, it must find its way to the nucleus. Lastly, when the DNA has entered the nucleus, long-lived gene expression must be ensured.

In this review, we briefly discuss direct approaches for delivering DNA either by injection into muscle or by particle bombardment. In addition, we concentrate on two general strategies adopted in current non-viral approaches to gene therapy. First, cationic lipid-based systems, which generate positively charged DNA complexes that bind target cells and contain lipids with membrane-destabilizing properties to facilitate the entry of DNA into the target cell. Second, viral mimetic systems, which employ DNA condensed with conjugates containing both a ligand for cell attachment/endocytosis and an endosomolytic agent (e.g. adenovirus or synthetic peptides) to facilitate escape from the vesicle into the cytoplasm.

Truly non-viral gene delivery devices

A number of groups have taken a pragmatic approach to DNA delivery. Rather than attempt to mimic virus entry behavior (see below), they have analyzed the delivery problem and used cationic lipid chemistry, direct physical methods of injection, or particle bombardment to introduce the DNA into cells. Initially, these approaches yielded reagents that were only useful for cell culture experiments, but researchers have recently identified several applications where these approaches and reagents may have useful functions *in vivo*.

Direct DNA injection and particle bombardment
Although expression levels are probably too low for standard gene correction applications, direct DNA in-
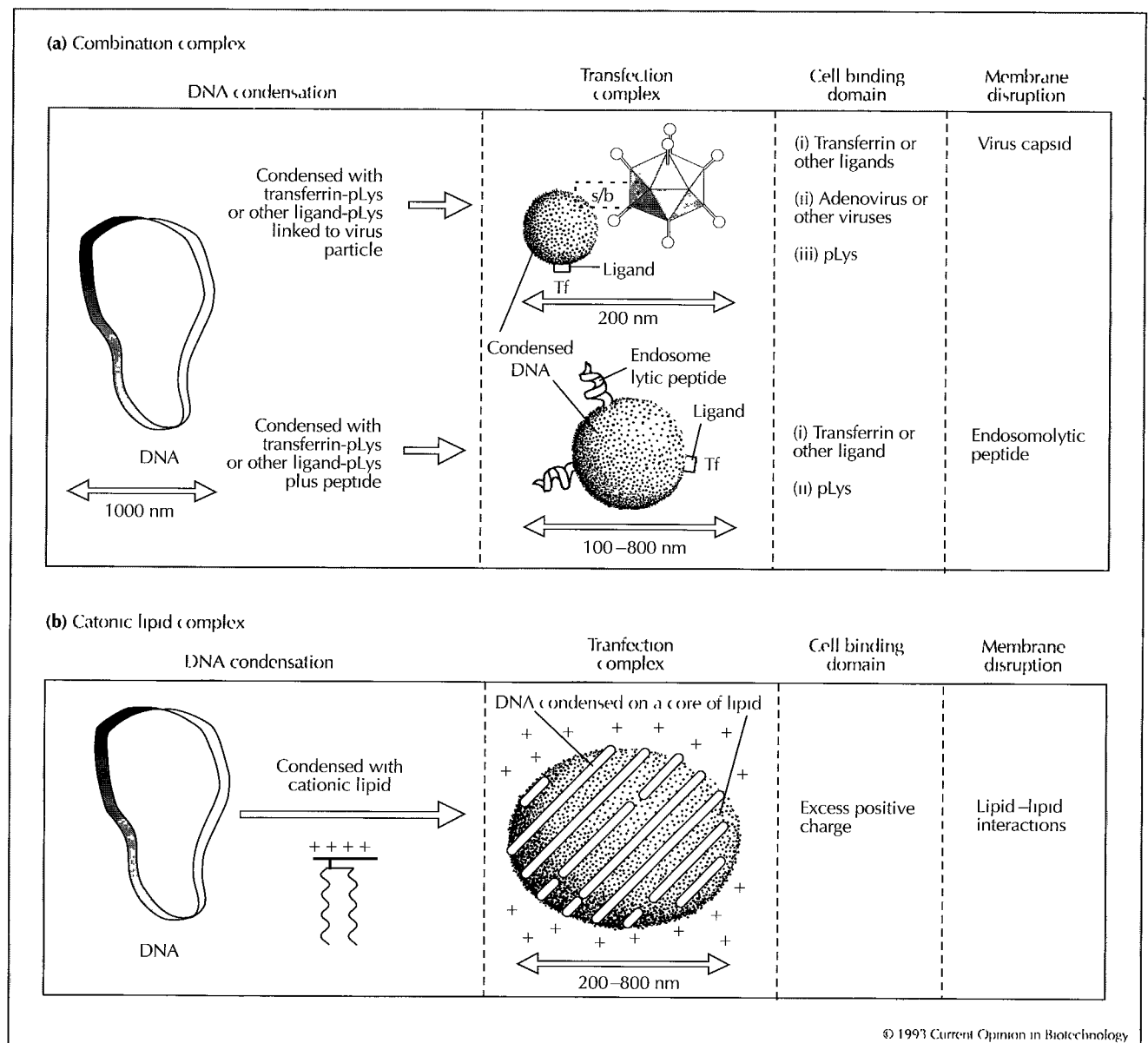
Fig. 1. The two major groups of DNA delivery reagents: **(a)** combination complexes and **(b)** cationic-lipid complexes. In each type of complex, the DNA is reacted with cationic reagents. This reagent serves to condense the DNA as well as to attach both cell-binding and membrane-disrupting activities. Abbreviations include: pLys, polylysine; s/b, streptavidin/biotin; Tf, transferrin.

jection will be very powerful as a vaccination method, because low-level gene expression is sufficient (and perhaps beneficial) for the triggering of immune responses [1,2•,3•].

Lipid-based systems

The two original cationic lipids, DOTMA [4] and DOGS [5,6], as well as several new variations (DC-chol [7,8], DMRIE [9], DORIE [9] and lipofectamine [10]) have been used for delivery of DNA. These lipids are designed to attach hydrophobic aliphatic groups to DNA using a head group containing one to four positive charges. Further analysis of these compounds suggests that their function in DNA delivery involves additional ac-

tivities including the condensation of the DNA structure and non-specific targeting of the DNA–lipid complexes (which carry a net positive charge; see Fig. 1b) to negatively charged (sialic acid bearing) components on the cell surface [11]. The inclusion of lipids such as DOPE, which have low hexagonal phase transition temperatures is thought to be required for the activity of these cationic lipids [8,12,13•]. Introduction of these destabilizing cationic lipids into the bilayer at the cell surface or in an endosome may be disruptive or fusogenic, serving a function similar to the adenovirus particle in adenovirus–DNA complexes (see below) that facilitates DNA entry into the cytoplasm. Inclusion of the cationic cyclic antibiotic peptide gramicidin S, which has an amphipathic structure, also aids in DNA delivery [13•].

Successful applications of these compounds include: DOTMA/DOPE delivery of DNA to endothelial cells in washed and occluded arteries [14]; DC-chol/DOPE delivery of DNA directly to tumor masses [15]; DOTMA/DOPE delivery of DNA to the lung [16,17•]; and a surprising claim for systemic delivery of DNA via an intravenous route using DOTMA/DOPE [18].

Non-viral gene delivery devices that resemble viruses

Many viruses successfully enter mammalian cells and have undergone many rounds of evolution to refine this process. If we can mimic viral entry functions without undesirable viral functions, we may have a useful vector.

Receptor-mediated gene delivery with polylysine–ligand conjugates

This strategy involves condensing delivered DNA with a polycation such as polylysine, chemically linked to a cell-binding ligand such as asialoorosomucoid for hepatocytes [19] or transferrin for dividing cells [20] (see Fig. 1a). The ligand triggers binding to the target cell and the DNA is internalized upon endocytosis of the ligand–DNA complex bound to the target receptor [21]. A wide variety of conjugates for receptor-mediated gene delivery are now available ([7,8,19,20,22–26]; see Table 1).

The naïve assumption is that a ligand specific for a certain cell type will be useful for the targeted delivery of DNA exclusively to that particular cell type and no other. One problem with this assumption is that the strong affinity of polylysine for many cell types [27,28] limits the targeting ability of these conjugates. A major barrier to receptor-mediated gene delivery is that most endocytosed material remains entrapped within the vesicles. Even so, the low (but significant) levels of gene delivery obtained with some of these conjugates in the absence of an endosomolytic activity demonstrates that the cell biologist's view of endocytosis as a tightly controlled, leak-free process is not universally true. Alternate methods of attaching ligands to DNA have been developed using intercalating conjugates [23,29]; however, these conjugates lack DNA condensation activity, which must be added separately [29].

Combination complexes

Receptor-mediated gene delivery has improved with the recognition that an endosomolytic agent is required to release material from the endocytosis pathway and gain entry into the cytoplasm. Two types of delivery complexes have demonstrated this (see Fig. 1). The

Table 1. Conjugates employed recently in receptor-mediated gene delivery

Ligand	Receptor	Target cell	Reference
Asialoorosomucoid	Asialoglyco-protein receptor	Hepatocytes	[19]
Gal4-peptide	Asialoglyco-protein receptor	Hepatocytes	[22]
Trigalactosylated bisacridine	Asialoglyco-protein receptor	Hepatocytes	[23]
Lactosylated polylysine	Asialoglyco-protein receptor	Hepatocytes	[24]
Insulin	Insulin receptor	Wide range	[25,26]
Transferrin	Transferrin receptor	Even wider range	[2•]
Anti-CD3	CD3 (TCR-associated)	T cells	*
Anti-CD4, HIV gp120	CD4	T cells	*
Anti-CD7	CD7	T cells	*
Anti-Tn	Tn antigen (O-glycosylation)	Cells of patients with a rare glyco-sylation disorder	**
Epidermal growth factor	Epidermal growth factor receptor	Epithelial cells	†
Anti-thrombo-modulin	Thrombomodulin	Lung endothelial cells	[7,8]
Anti-Ig	Surface Ig	B cells	†
Anti-secretory component	Polymeric Ig receptor	Epithelial cells	‡
Lung surfactant proteins A, B or C	Variety	Pulmonary cell types	§

*M Cotten, E Wagner, ML Birnstiel, unpublished data; **M Thurnher, E Wagner, K Mechler, M Cotten, unpublished data; †E Wagner, unpublished data; ‡T Ferkol, unpublished data; §GF Ross, JE Baetz, TR Korfhagen, J Whitsett, unpublished data. Abbreviations include: HIV, human immunodeficiency virus; Ig, immunoglobulin; TCR, T-cell receptor.

addition of adenovirus particles, which are naturally endosomolytic [30–32], enhances gene delivery [33–35] and is particularly effective when the DNA to be delivered is linked directly to the exterior of the adenovirus particle [36]. Other, more inert, viruses can also be used

for this purpose: the chicken adenovirus (CELO virus) is naturally defective in the mammalian cell and provides a potent endosome disruption activity [37]. In addition, the human rhinovirus has also been shown to augment DNA delivery (W Zauner, D Blaas, E Küchler, E Wagner, unpublished data).

Gene delivery can also be enhanced by the inclusion of membrane-disruptive acid-stabilized amphipathic helix peptides in polylysine–DNA complexes [22,24,38•]. Synthetic positively-charged polyamidoamine cascade polymer particles (50–70 nm in size) have been found to possess useful DNA delivery activity when used in charge-excess over the delivered DNA; coupling the membrane-active GALA peptide [39,40] to these particles allows DNA delivery with charge-neutral polymer–DNA complexes [41•]. The genetic addition of endosomolytic peptides to the surface of either virus-like particles from yeast or self-assembling structures based on the bacteriophage MS2 capsid can also generate endosomolytic elements useful for DNA delivery (S Chiocca, E Wagner, M Cotten, unpublished data).

Conclusions and future directions

Major successes have now been achieved in attempts to import DNA into the cell using non-viral approaches. Many of these efforts have focused on methods either to penetrate the cytoplasmic membrane or to release endocytosed material into the cytoplasm. At present, it is unclear how DNA, once released into the cytoplasm, enters the nucleus. A simple explanation could be that the cell has mechanisms for gathering chromosomal material into the two nascent daughter nuclei after the breakdown of the nuclear envelope at mitosis. Cation–DNA complexes are simply included in this passive round-up. Efforts to include proteins with nuclear localization signals such as HMG proteins [26,42–44] or histone H4 [45] have provided modest improvements in DNA delivery, but nothing to suggest that a new barrier has been surmounted. The high efficiency of combination complexes containing adenovirus particles may result from the virus functioning at both the endosome-disruption step and the nuclear-localization step. Yet, the capacity of combination complexes containing rhinovirus, which is a cytoplasmic virus, to potentiate DNA delivery argues against this role.

The next challenge to be met is that of stable gene maintainance. If gene therapists are to advance medicine and provide an alternative to pharmaceuticals, then they will often need to achieve long-term gene expression. The exodus from the recombinant retrovirus field does not mean that these viruses have no future in gene therapy. A method employing the retroviral functions that fix introduced genes into the chromosomes of target cells may soon be developed. Similarly, the adenovirus-associated virus internal transcribed regions (ITRs) may also be exploited

to integrate genes, possibly with the help of the viral replication (REP) functions.

Acknowledgements

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