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Minireview

On the strategy of using nonviral carriers in cancer gene therapy

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Effectiveness and mode of therapeutic gene delivery *in vivo* as well as biological safety of such transfer must be improved before widespread application of gene therapy in the clinic becomes possible. Most research has so far focused on recombinant viral delivery systems. Clinical future seems to belong, however, to nonviral delivery systems. Such systems feature DNA complexed to lipid, protein, peptide or polymeric carriers with ligands allowing *in vivo* tissue targeting by the complex and nuclear translocation of the exogene. Nonviral gene carrier systems are discussed together with strategies of destroying cancer cells.

Transfer of therapeutic material in cancer gene therapy is accomplished with both viral and nonviral carriers (see [1, 2] for review).

Recombinant viral vectors mainly adeno- and retroviruses are highly efficient in terms of transfection; however, they are not "safe" biologically. Due to recombination of replication-defective forms inside transfected cells, various forms may arise that are replication-competent. Also, insertional mutations may in theory cause, for instance, oncogene activation. Viral vectors are mainly used in *ex vivo* experiments where cells taken from tumors are used for *in vitro* transfection and subsequently returned to the individual in order to trigger immunological response. Viral carriers, mainly adenoviruses, used *in vivo* are applied directly at the tumor site [3]. Poor targeting by viral vectors makes them unsuitable for systemic administration.

Nonviral carriers (liposomes [4] and molecular conjugates [5–8]) are biologically "safer" but their efficiency of transfection is, so far, inferior to that of viral vectors. Currently used nonviral carriers need to be improved: they are not sufficiently stable for systemic administration and they cannot be efficiently targeted to specific tissues. Present nonviral carriers do not function yet as efficient endosome membrane-destabilizing complexes which makes them ineffective in preventing destruction of endosome-encapsulated therapeutic material which they carry [9]. They do not assure long-term expression yet. These deficiencies remain unsolved. However, potential advantage of nonviral delivery systems lies in the fact that some of these carriers may be administered systemically and, owing to inherent modularity of their design they can incorporate useful molecular features of both viruses and nonviral systems.

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Abbreviations: ASOR, asialoorosomucoproteid; DC-Chol, 3β[*N*-(*N'*,*N'*-dimethylaminoethano)-carbamoyl]-cholesterol; DDAB, dimethyldioctadecylammonium bromide; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DOTMA, *N*-[1-(2,3-dioleoyloxy)propyl]*N*,*N*,*N*-trimethylammonium chloride; PC, phosphatidylcholine; PEG, polyethylene glycol; RES, reticuloendothelial system.

Such advantage could prove crucial for destroying metastatic cells.

This review aims at presenting basic information on nonviral gene carriers used in cancer gene therapy. Simplicity of their use suggests that gene therapy based on nonviral carriers may in the future approach chemotherapeutic modalities [10].

Genetic material is administered to the individual as a DNA-carrier complex, either systemically or locally. Provided that the complex avoids capture and destruction by the reticuloendothelial system (RES) its further fate consists of cellular internalization and release from endosomes into the cytoplasm or degradation in the endosomal pathway [4, 9, 10]. In the former case the complex subsequently translocates into the nucleus where expression of therapeutic material takes place.

Responsible for cellular internalization of the complex is, most likely, the process of endocytosis. Initial binding by the cells may result either from electrostatic interaction between the complex and unspecific cell or, alternatively, from selective interaction between ligand (linked with DNA-carrier complex) and specific cellular receptor. In other situations carrier-linked antibody may be used to induce interaction with appropriate antigen expressed by targeted cells.

Stability of endosomal membrane is among factors limiting transfection efficiency since it does not allow escape of therapeutic material from endosomes into the cytoplasm [9]. Only a minor fraction of endosomes undergo destabilization resulting in escape of their released contents from degradation by lytic enzymes. In cases where low gene expression is sufficient, e.g. to trigger immune response, the level of gene delivery may be satisfactory. Otherwise compounds which are endosomolytic agents should be included in the design of novel nonviral gene therapy carriers [9, 11]. The fate of therapeutic sequence-carrier complexes which escaped the endosomolytic pathway is not exactly known. However, complexes that include carrier associated with proteins possessing a nuclear localization signal (histones, replication-defective viruses) can translocate into the nucleus owing to a specific receptor directing such complexes to nuclear pores [9, 11].

From the foregoing description of the fate of gene-carrier complexes *in vivo*, both outside and within cells, it should be clear that the design of novel carriers for cancer gene therapy ought to combine, in a modular manner, useful properties of both types of carriers, that is primarily transfection efficiency of viruses and biological "safety" of nonviral systems [6, 11]. Such design should also depend on strategies used for destroying primary tumors, metastatic cells and residual disease, the strategy of administering therapeutic complexes either intratumorally or systemically and, finally, the strategy of directing the drug to neoplastic cells in order to increase the "therapeutic window".

Figure 1 shows strategies of destroying various kinds of cancer cells with the help of nonviral gene carriers. They had been divided into three arbitrary classes or "generations". Such classification stresses functional features of carriers rather than their structure.

First-generation nonviral carriers are not directly targetable or injectable. They are rapidly cleared from circulation if injected systemically. For this reason, as complexes with therapeutic material, they are used to destroy primary tumors only. They are applied directly at the tumor site. The therapeutic material carried may be a "suicide" gene which destroys the tumor by radio- or chemosensitizing it (Szala, S., Missol, E. & Sochanik, A., in preparation). Use of tissue-specific transcription promoters prevents exogene expression in incidentally transfected normal cells and confers to those carriers a certain degree of specificity in destroying tumor cells (transcriptional targeting).

In order to destroy metastatic cells circulating in blood or lymph and to treat established metastases, systemically injected long-lived carriers of second or third generation are needed.

Second-generation carriers represent an advance since they are not easily destroyed upon injection into the bloodstream and subsequent contact with the reticuloendothelial system (RES). Such carriers are sterically stabilized [12–14]. However, only transcriptional targeting is achievable with carriers of the second generation.

Third-generation cancer gene therapy carriers are not only sterically stabilized and thus injectable; they are additionally targetable i.e., capable of recognizing and delivering therapeutic material to specific cells (transductional targeting) [15]. Long-lived third-generation

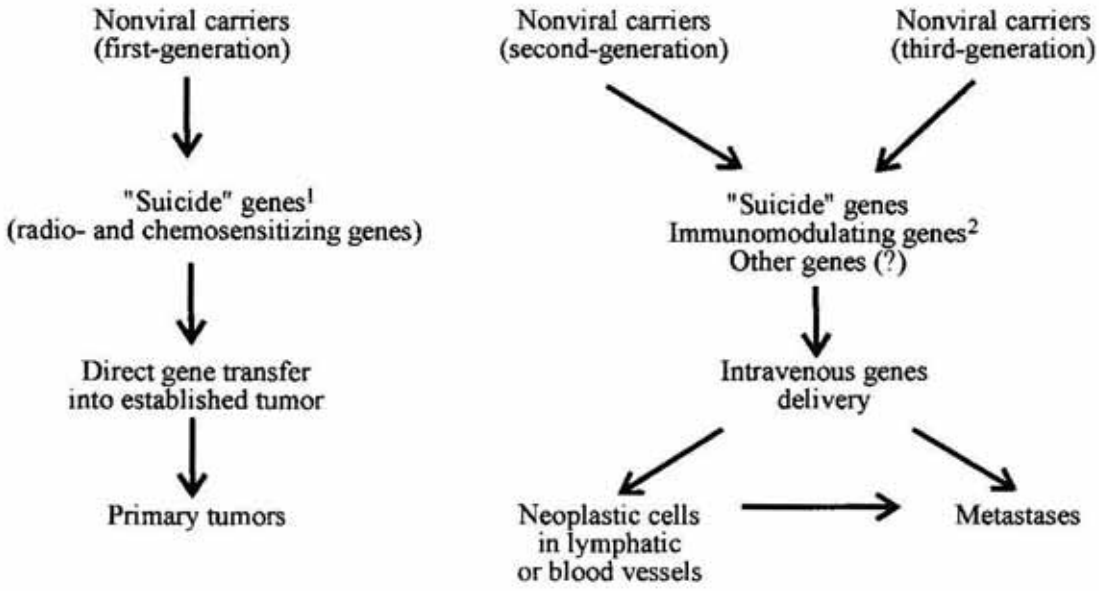

Fig. 1. Strategies of destroying cancer cells with the help of gene therapy using nonviral carriers of therapeutic material.

[1]Suicide genes are bacterial or viral genes coding for enzymes converting a nontoxic substrate (prodrug) into a toxic product (drug). Mammalian cells transfected with suicide enzymes are selectively killed upon co-administration of the prodrug. Examples of such genes include *E. coli* cytosine deaminase [38], *E. coli* nitroreductase [39], thymidine kinase from herpes simplex virus [40], *E. coli* DeoD gene [41]. [2]Examples of immunomodulatory genes include MHCI, MHCII, B7 or interleukin genes (see for review: [42–45]).

carriers thus promote selective expression of introduced gene. With these carriers both transcriptional and transductional targeting may be achieved simultaneously.

Examples of nonviral carriers used in cancer gene therapy trials and which represent all three generations discussed are listed in Table 1.

First-generation nonviral carriers. This group is made up of various so-called "cationic" liposomes [4, 16, 17]. Their distinguishing feature is a positively charged lipid allowing them to interact electrostatically with polyanions, e.g. upon mixing with DNA, RNA, oligonucleotides and also with negatively charged residues in cell membrane. The interaction results in complex formation between negatively charged phosphate groups from polynucleotide and positively charged headgroups of cationic lipids. Besides a charged lipid cationic liposomes contain an electrically neutral co-lipid, usually dioleoyl phosphatidylethanolamine (DOPE) or dioleoyl phosphatidylcholine (DOPC) [18]. The presence of a co-lipid is required because of its stabilizing effect on cationic liposome structure and, in the case of DOPE, also because of its fusogenic properties. Also, transfection activity of cationic liposomes depends on the co-lipid. Cationic lipids alone are much less active transfectionally than liposomes containing DOPE [18].

Cationic lipids cause DNA to be bound to the liposome surface. Association of DNA with liposome surface rather than its encapsulation distinguishes these cationic liposomes from "classical" ones. The former are also more efficient transfectionally.

Cationic liposome carriers are not cell-specific; they may penetrate both normal and cancer cells. Their usefulness as gene therapy carriers *in vivo* is limited for *in situ* administration, for example intratumoral (Szala, S., Missol, E. & Sochanik, A., in preparation) [19, 20] intratracheal [21] or intraarterial [17, 22]. This is because the formation of cationic liposome-DNA complexes is impeded by the presence of blood serum. Cationic liposomes with bound plasma proteins are rapidly removed from circulation *in vivo* by a very efficient reticuloendothelial system (RES) whereas liposome degradation *in vivo* is retarded by *in situ* administration. Cationic liposomes are well suited to *in vitro* transfection where serum concentration in culture media can be manipulated.

Due to nondestructive nature of complex formation between DNA and cationic lipids the latter might also find use in designs of modular

Table 1

Synthetic nonviral carriers used in gene therapy trials

Carriers	Properties	Examples
First-generation	Nonspecific; short life-time in blood; cationic amphiphiles binding to DNA and coating it with a cationic layer which in turn interacts with anionic residues on cell surface; do not require specific cell surface receptors;	Lipofectin [23] (DOTMA/DOPE - BRL) TransfectACE [25] (DDAB/DOPE-BRL) DC-Chol/DOPE [24] DMRIE/DOPE [18] DOTAP [11]
Second-generation	Nonspecific; longer life-time in blood; sterically stabilized; either true liposomes encapsulating transported material or DNA-compacting polycationic amphiphiles; do not require specific cell surface receptors;	AminoPEG-PE/PC/cholesterol [14] DPPE-lysine/PC/cholesterol [27]; lipopolyamine w/o ligands (DOSPA) [30]
Third-generation	Specific; long life-time in blood; receptor-targeted sterically stabilized liposomes; synthetic virus-like conjugates; require tumor cell surface specific receptors or antigen;	Sterically stabilized liposomes conjugated to ligand *via* PEG [12–14]; Lipopolyamine-condensed DNA particles presenting ligands (transferrin [34], insulin [35], folate [36], galactose [8]; Poly-L-lysine/galactose [28]; Immunoliposomes (encapsulated liposomes with Fab') [46, 47]

transfection systems overcoming present deficiencies. Three types of such lipids can presently be distinguished: a) DOTMA-type cationic lipids (DOTMA [23] is *N*-[1-(2,3-dioleoyloxy) propyl]*N*,*N*,*N*-trimethylammonium chloride) with a structural motif including dialkylglycerol "backbone" substituted with two identical alkyl chains, a "spacer" part with ester or ether internal bonds and a quartenary ammonium moiety with substituents of varying hydrophobicity; DOTMA-type cationic lipids which increase transfection efficiency of liposomes possess dimiristyl hydrophobic residues, "spacers" with ester bond and hydroxyethyl substituents in the quartenary ammonium group [18]; b) cationic lipids containing cholesterol which is minimally toxic to the cells and whose presence stabilizes lipid bilayers; an example of such a compound is DC-Chol [24], 3β[*N*-(*N'*,*N'*-dimethylaminoethano)-carbamoyl]cholesterol. The presence of metabolizable carbamoyl bond facilitates final degradation of lipid by cellular esterases and thus diminishes toxicity; transfection efficiencies of liposome preparations containing DC-Chol (with DOPE) appear better than those obtained with Lipofectin [24]; c) quartenary ammonium detergents; an example is DDAB [25] (dimethyldioctadecylammonium bromide); reasonable efficiency of transfection and cytotoxicity *in vitro* of liposomes containing DDAB in combination with true lipid DOPE has encouraged trials of *in vivo* gene therapy of solid tumors in laboratory animals [26].

Second-generation nonviral carriers. These are sterically stabilized true liposomes characterized by prolonged circulation time in blood [12–14]. Most of them are synthesized with polyethylene glycol (PEG) derivatives e.g. methoxyPEG, aminoPEG or hydrazidoPEG.

Removal of liposomes from circulation by RES depends, among other things, on chemical composition of their lipid bilayers. Interaction between negative charge-bearing blood components and sterically stabilized liposomes with PEG phospholipid derivatives in the membrane is diminished by shielding of positive charges from PE (phosphatidylethanolamine) part of liposome by terminal amino groups introduced with PEG. These, being protonated at the physiological pH, cause effective polarization of liposome surface charge. The resulting shielding significantly decreases fagocytic removal of sterically stabilized liposomes [13].

All liposomes based on such modified lipids, irrespective of polymer terminal group, show a much greater resistance to the action of serum proteins than liposomes containing a neutral polymer.

Also, the addition of dipalmitoyl phosphatidylethanolamine complexed with lysine to a standard liposome formulation containing cholesterol and PC allows forming liposomes that are much more stable in serum and culture medium than "classical" ones [27].

Another strategy of designing gene therapy carriers with increased life-time in blood relies upon the use of polycationic amphiphiles possessing a DNA-compacting headgroup [28]. The simplest polyamines spermine and spermidine form the so-called "double-sided sticky tape" aggregates able to compact DNA. The formation of such aggregates does depend on ionic strength of reaction mixtures [11]. Under physiological conditions it does not occur. However, lipid derivatives of polyamines, called lipopolyamines exhibit not only self-aggregation feature but are able to permanently condense DNA forming a lipid-coated DNA particle; examples of such compounds include DOGS [29] (dioctadecylamidoglycylspermine) or DOSPA [30] (2,3-dioleyloxy-*N*-2[(spermine-carboxamido)ethyl]-*N*,*N*-dimethylpropaminium trifluoroacetate). The net charge of the cationic headgroup of DOSPA is +5 at the physiological pH.

Polycationic carrier complexes appear *in vitro* more effective transfectionally than monocationic liposomes, e.g. DOTMA. Transfection efficiency of lipopolyamines is most likely a combined result of their three features: first, the ability to compact DNA; second, increased charge density in their NH^+ group compared to $N(CH_3)^{3+}$ group of monocationic carriers (in the latter charge is delocalized what causes weaker interaction with residues of compounds forming membranes); third, the least basic secondary amines (for example in DOGS) have $pK < 6$ and may buffer lysosomal acidity and thus indirectly protect DNA from degradation [11].

Third-generation nonviral gene carriers. They represent state-of-the-art of proposed gene therapy carriers. They are designed as sterically stabilized (and thus injectable) to extend their stay in the extracellular environment; functional groups included allow delivery of therapeutic material to a desired destination *in vivo*, making such carriers targetable. They should also be capable of condensing DNA to internalizable size, enhancing DNA release from endosomal compartment and promoting DNA transfer into the nucleus [6, 8, 11, 28]. Future carriers will also tackle the problem of long-term therapeutic gene maintenance either in extrachromosomal or in chromosome-integrated form.

Targeted gene transfer can be accomplished *via* interaction of cellular receptors with their ligands coupled to the carrier of therapeutic DNA, or, alternatively, *via* interaction of expressed antigen with antibody coupled to the carrier. Since it is chemically feasible to covalently bind ligands or antibodies to various gene carriers this strategy appears additionally justified owing to the increased density of various receptors on the surface of cancer cells.

Currently tried carriers of third generation include:

- a) positively charged polypeptides [28] covalently joined to ligands or antibodies. Their use is illustrated by interaction of polylysine/DNA-coupled ligand with asialoorosomucoproteid (ASOR) hepatocyte receptor [31, 32] or interaction of histone H1-linked galactose with asialoglycoprotein receptor [33]. The presence of histone molecule in such a system allows both DNA condensation and its transfer to cell nucleus. However, the immunogenicity of carriers containing protein components does not speak in favor of pursuing such strategy. Transferrin receptor presents another opportunity for targeted gene delivery to dividing cells [6]. The usefulness of polylysine-based constructs for intravenous delivery *in vivo* [28] seems to be limited by its strong affinity to cells despite the inclusion of cell-specific ligands;
- b) novel liposomes incorporating modular constructs such as distearoylphosphatidylethanolamine-polyethylene glycol-folate (DSPE-PEG-folate) in the lipid bilayer [12–14]; preparation of such construct is independent from formation of liposomes themselves; similar strategies widen the possibilities of modular nonviral gene carrier design;
- c) polycationic lipopolyamines equipped with additional capacity to direct therapeutic genes to defined tissues *in vivo*; they appear at

present as the most promising carriers of the third generation. These carriers might have as ligands transferrin [34], insulin [35], folic acid [36], galactose and other sugars [7], surfactant protein A [37], ASOR or other moieties [5]. Cell receptors for insulin [35], folate [36] or surfactant protein A [37] might be used to specifically direct therapeutic sequences to desired cell targets.

To summarize the progress in cancer gene therapy with nonviral carriers of therapeutic polynucleotides: new developments will most likely continue the trend towards synthetic, modular carriers that will be blood-injectable and targetable. The future of gene therapy in general probably depends on construction of such carriers.

In any case the following features of gene therapy carriers of the future should certainly be desired in every design:

- high efficiency of transfection; of degree comparable to viruses;
- safety of use, i.e. lack of genetic complications from the host; of degree comparable to liposomes;
- ability to target neoplastic cells, i.e. to recognize them and selectively deliver therapeutic materials;
- simplicity of manufacture on larger scale;
- economy of application in the clinical setting.

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