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REVIEW

Gene Therapy Progress and Prospects: Nonviral vectors

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The success of gene therapy is largely dependent on the development of the gene delivery vector. Recently, gene transfection into target cells using naked DNA, which is a simple and safe approach, has been improved by combining several physical techniques, for example, electroporation, gene gun, ultrasound and hydrodynamic pressure. Chemical approaches have been utilized to improve the efficiency and cell specificity of gene transfer. Novel gene carrier molecules, which facilitate DNA escape from the endosome into the cytosol, have been developed. Several functional polymers, which enable controlled release of DNA in response to an environmental change, have also been reported. Plasmids with reduced number of CpG motifs, the use of PCR fragments and the sequential injection method have been established for the reduction of immune response triggered by plasmid DNA. Construction of a long-lasting gene expression system is also an important theme for nonviral gene therapy. To date, tissue-specific expression, self-replicating and integrating plasmid systems have been reported. Improvement of delivery methods together with intelligent design of the DNA itself has brought about large degrees of enhancement in the efficiency, specificity and temporal control of nonviral vectors.
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In brief

Progress

- Naked DNA delivery by physical method: to overcome safety issue and to realize efficient gene expression *in vivo*
- Gene delivery using a chemical carrier: to establish functional gene delivery *in vivo*
- Nonviral vector modifications with peptides to increase intracellular gene delivery
- Reduction of immune responses by modifying the administration protocol or the composition of the DNA
- Design of tissue-specific, self-replicating and integrating plasmid expression systems to facilitate long-lasting gene expression

Prospects

- Physical techniques for gene delivery into cells such as electroporation, with and without adjuvants, will be significantly optimized
- Knowledge of the interaction of naked DNA with serum components and cell surface receptors will continue to accumulate. Immune responses originating from CpG motifs and nonviral gene carriers will diminish
- The structure of gene carriers will be further optimized and tailored for specific uses such as systemic administration, local injection or organ-specific delivery
- Novel ligands for targeted delivery of DNA will be found
- Translocation mechanisms for plasmid DNA within the cell will be identified – these may provide novel strategies for efficient delivery
- More tissue-specific, site-specific integrating or self-replicating plasmid vectors are likely to appear

Introduction

The development of gene carriers for effectively delivering genes into cells has attracted a great deal of attention in recent years. Nonviral vectors should circumvent some of the problems occurring with viral vectors such as endogeneous virus recombination, oncogenic effects and unexpected immune response. Further, nonviral vectors have advantages in terms of simplicity of use, ease of large-scale production and lack of specific immune response. These techniques are categorized into two general groups: (1) naked DNA delivery by a physical method, such as electroporation and gene gun and (2) delivery mediated by a chemical carrier such as cationic polymer and lipid. In this review, we focus on

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the progress made over the last two years and discuss techniques in these two categories.

Naked DNA delivery by physical method: to overcome safety issue and to realize efficient gene expression in vivo

Many mechanical techniques are included in this section. The simplest way for administration of DNA is direct injection of naked plasmid DNA into the tissue or systemic injection from a vessel. Use of naked DNA without any carrier molecule is also the safest method. Little attention needs to be paid on issues of complex formation and its safety assessment. So far, site of the direct injection includes skeletal muscle, liver, thyroid, heart muscle, urological organs, skin and tumor.[1] Systemic injection is also a convenient route for gene administration. However, owing to rapid degradation by nucleases in the serum and clearance by the mononuclear phagocyte system, the expression level and the area after injection of naked DNA are generally limited. Various physical manipulations have been used to improve the efficiency. Electroporation, bioballistic (gene gun), ultrasound, hydrodynamics (high pressure) injection and others have been established (Figure 1).[2]

Electroporation, the application of controlled electric fields to facilitate cell permeabilization, is used for enhancement of gene uptake into cells after injection of naked DNA.[3] In addition, electroporation can achieve long-lasting expression and can be used in various tissues. Skin is one of the ideal targets because of the ease of administration. Drabick *et al*[4] established cutaneous transfection method for the purpose of DNA vaccination. To optimize the condition of electroporation, factors such as dose of DNA, electrode shape and number, electrical field strength and duration have been optimized for expression of hepatitis B surface antigen,[4] erythropoietin[5] and IL-12.[6] High ionic strength in the injection medium is also favorable for gene expression in the skin.[7] Muscle is also a good candidate for electroporation. Most of reports published recently relate to immunological applications. For DNA vaccination, potent immune responses against hepatitis B surface antigen and HIV gag protein were obtained by electroporation of muscle after intramuscular injection of naked plasmid DNA.[8] Therapeutic effect of cytokines, such as IL-12[9] and IFN-α,[10] for inhibition of tumor growth located at a distant site has been demonstrated. IL-12 was also employed for electroporation after intratumor injection.[11] Our laboratory recently reported the use of a syringe electrode, with which same transfection efficiency could be achieved by using much lower electric field strength than that of conventional electrode. Tissue damage by the electric field is thus minimized.[12] Electrically mediated DNA delivery to hepatocellular carcinoma in the liver was reported by Heller *et al*.[13] All of the electroporation protocols employ local injection of the plasmid DNA. However, our group recently demonstrated efficient gene transfer to the liver by electroporation following tail-vein injection of naked DNA.[14] Comparing with local injection of DNA to the liver, systemic injection has the advantage of delivering genes more evenly to the liver.

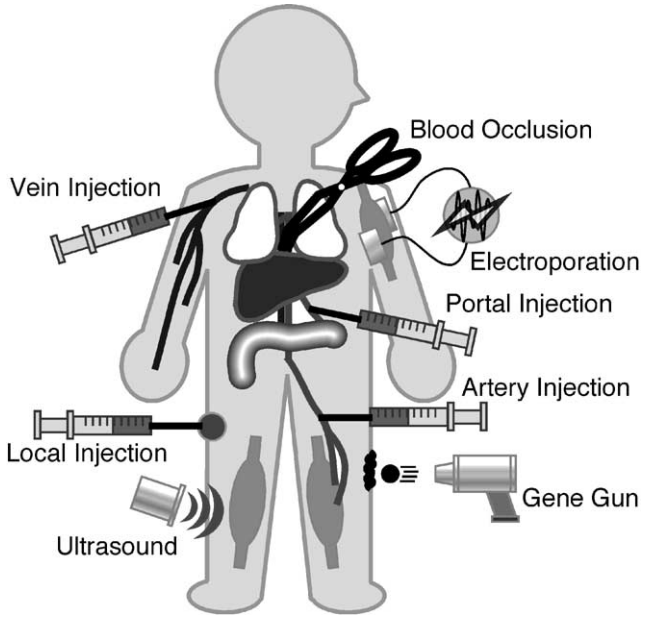

Figure 1 *Overview of nonviral gene delivery technologies. Different injection routes of naked DNA and enhancement strategies are outlined.*

Gene gun can achieve direct gene delivery into tissues or cells. Shooting gold particles coated with DNA allows direct penetration through the cell membrane into the cytoplasm and even the nucleus, bypassing the endosomal compartment. Majority of the efforts reported in the last 2 years are to introduce genes for antigen or cytokines such as IL-12 into skin[15,16] or liver[17–19] for vaccination and immunotherapy, respectively. However, a disadvantage of this method is the shallow penetration of DNA into the tissue.

Ultrasound can increase the permeability of cell membrane to macromolecules such as plasmid DNA. Indeed, enhancement of gene expression was observed by irradiating ultrasonic wave to the tissue after injection of DNA.[20,21] Since ultrasound application is flexible and safe, its use in gene delivery has a great advantage in clinical use. Recently, it was reported that combination of microbubble with ultrasound could further increase the gene expression level. Microbubbles, or ultrasound contrast agents, lower the threshold for cavitation by ultrasound energy. In most cases, perfluoropropane-filled albumin microbubbles or Optison (Mallinckrodt, San Diego, USA) were used as microbubbles. It was modified with plasmid DNA before injection, followed by irradiation of ultrasound. At present, this technique is used for gene delivery to vascular cells,[22–26] muscle[26,27] and fetal mouse.[28]

Hydrodynamic injection, a rapid injection of a large volume of naked DNA solution (eg 5 μg plasmid DNA injected in 5–8 s in 1.6 ml saline solution for a 20 g mouse) via the tail vein, can induce potent gene transfer in internal organs, especially the liver. Budker *et al* hypothesized that naked plasmid DNA is taken up by receptor-mediated pathway by hepatocytes.[29] Certain DNA receptors have been found in various tissues;[30] however, their function has not been elucidated. It has been proposed that the injected DNA solution accumulates mainly in the liver because of its flexible structure,

which can accommodate large volume of solution, and the hydrostatic pressure forces DNA into the liver cells before it is mixed with blood. Furthermore, breaking of the endothelial barrier by the pressure has been proposed as the major mechanism responsible for the highly efficient expression in the liver. Recently, our group reported that external massage of the abdomen after small-volume injection of DNA via the tail vein can enhance gene expression in the liver.[31] The observation suggests that mechanical stretching of the endothelial barrier may affect uptake of DNA into the hepatocytes. This pressure-mediated transfection method can be applicable to other tissues. Wolff's group showed that large-volume injection with high speed via the portal vein of liver or the artery of limb muscles achieved high gene expression in the respective organ.[29,32]

Our group has demonstrated that significant gene expression can be achieved in the liver by transiently restricting blood flow through the liver immediately following peripheral intravenous injection of naked DNA.[33] Occlusion of blood flow either at vena cava or at hepatic artery and portal vein increased the expression level in the liver. Presumably, the injected DNA is internalized into the hepatic cells by receptor-mediated mechanism as proposed by Budker *et al*[29] or via a nonreceptor-mediated pathway. However, the binding of DNA to the surface of hepatic cells might be so weak that DNA could be easily dissociated and washed away by the blood flow in the normal physiological condition. Only when the blood flow is transiently stopped, the DNA can stably bind with the receptor and be internalized into cells. A similar uptake of DNA by the diaphragm muscle cells was achieved by a brief occlusion of the blood flow through the diaphragm immediately after peripheral intravenous injection of DNA.[34]

Gene delivery using a chemical carrier: to establish functional gene delivery in vivo

Novel carriers to achieve high-level gene expression and functional delivery have been designed. Gene carriers can be categorized into several groups: (1) those forming condensed complexes with the DNA to protect the DNA from nucleases and other blood components; (2) those designed to target delivery to specific cell types; (3) those designed to increase delivery of DNA to the cytosol or nucleus; (4) those designed to dissociate from DNA in the cytosol and (5) those designed to release DNA in the tissue to achieve a continuous or controlled expression. Lipids and polymers are mainly used for gene delivery.

Lipid-mediated gene delivery

Liposome-based gene delivery, first reported by Felgner in 1987, is still one of the major techniques for gene delivery into cells. In 1990s, a large number of cationic lipids, such as quaternary ammonium detergents, cationic derivatives of cholesterol and diacylglycerol, and lipid derivatives of polyamines, were reported. However, the development of novel types of lipid molecules appears to be saturated, and most of the efforts have shifted to improving efficacy by the modification listed above, as well as to specific *in vivo* applications. We will highlight a number of new concepts that have appeared in the last 2 years.

The reduction–oxidation (redox) sensitive character of thiol groups has been exploited to control DNA–lipid complex formation. Dauty *et al*[35] reported a dimerizable cationic detergent, which contains free thiol, amine and alkyl groups. This alkylated ornithinyl cysteine derivative forms a complex with plasmid DNA. Subsequent oxidation of the thiol groups to disulfides converts the complex into stable nanometric particles. The particle is made of a single molecule of condensed plasmid DNA with a uniform diameter of less than 40 nm and showed reasonable transfection activity *in vitro*. Practical advantages include the small size for *in vivo* gene delivery (improved particle diffusion) and that the disulfide bonds should be reduced to thiols in the cytosol because of the reductive environment provided by intracellular glutathione, thus resulting in DNA release.

Peptide-mediated gene delivery

Redox-sensitive thiols have also been incorporated into peptide gene carriers. McKenzie *et al*[36] developed peptides containing a cysteine residue and a continuous sequence of lysine residues, for example, Cys-Trp-Lys_{18}.[36] This peptide can also condense plasmid DNA, and the thiol group is spontaneously oxidized, resulting in a highly stable complex with potent transfection activity *in vitro*. Cross-linking the peptide caused elevated gene expression, without increasing DNA uptake by the cells, suggesting that intracellular release of the DNA triggered by disulfide bond reduction played a key role. Furthermore, Park *et al*[37] have also synthesized sulfhydryl cross-linking poly(ethylene glycol)-peptides (for stealth activity) and glycopeptides for targeted delivery of genes *in vivo*.

Polymer-mediated gene delivery

Wightman *et al*[38] systematically compared the ability of branched and linear PEI/DNA complexes to transfect cells *in vitro* and *in vivo* at various amine/phosphate ratios and salt concentrations. They showed that salt-free DNA complexes of linear PEI (22 kDa), which showed high transfection efficiency in the lung, were small, but subsequently aggregated when salt was added. In contrast, DNA complex of branched PEI (25 kDa), which showed low transfection efficiency in most of the organs, remained small even after salt was added. The greater efficiency of linear PEI *in vivo* might be because of a dynamic structure change of the complex under high salt concentrations as found in blood. Understanding of the interaction between linear PEI and DNA could help in designing future vectors.

Biodegradable polymers are known for their low toxicity and high biocompatibility. Recently, a biodegradable polymer, poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA), a derivative of poly-L-lysine, in which the ester link is substituted with amide, was designed by Kim's group.[39] This biodegradable and water-soluble polymer condenses DNA and subsequently releases DNA upon hydrolysis of the polymer. The complex showed higher *in vitro* gene transfection efficiency with lower cytotoxicity than poly-L-lysine. Significant expression of murine IL-10 was observed in the serum after tail-vein injection of PAGA/DNA complexes, and the systemic administration of murine IL-10 gene with PAGA into NOD mice markedly reduced insulitis.[40] The murine IL-12 gene was also injected with PAGA into subcutaneous tumors in

BALB/c mice. Significant level of the protein expression and reduction of tumor growth was observed.[41] Recently, other types of biodegradable polymers were reported by Kim's and Leong's groups, who have synthesized cationic copolymers derived from PEI and polyethylene glycol (PEG)[42] and cationic polyphosphoester,[43] respectively.

Thermosensitive polymers can control the release of encapsulated DNA in response to temperature changes that lead to swelling or de-swelling of the hydrated polymer. Kurisawa *et al*[44] synthesized a thermosensitive copolymer, poly(*N*-isopropylacrylamide (IPAAm)-co-2-(dimethylamino)ethyl methacrylate (DMAEMA)-co-butylmethacrylate (BMA), and investigated its thermosensitive character and transfection efficiency at different incubation temperatures.[44] A polymer containing 8 mol% DMAEMA and 11 mol% BMA had a low critical solution temperature of 21°C and complex formation/dissociation was modulated by temperature alteration. Transfection efficiency *in vitro* also depended on the incubation temperature. Kim's group have developed the biodegradable and thermosensitive polymer, PEG–poly(D,L-lactic acid-co-glycolic acid) (PLGA)–PEG triblock copolymer. This nonionic, hydrophilic polymer shows temperature-dependent solution-to-gel transitions[45,46] and can be loaded with plasmid DNA in aqueous phase at 4–20°C. At above 30–3°C (eg, at the body temperature), the solution-to-gel transition occurs. It is conceivable that DNA could be formulated and injected in the polymer solution at room temperature, and slowly released from the hydrogel for prolonged transfection at the injection site.

PEG-PLL block contains a hydrophilic part consisting of PEG and a DNA-binding moiety consisting of PLL and forms self-assembling particles with DNA in a core-shell structure with electrostatic interaction as the main driving force. These polyion complex micelles are water-soluble and nuclease-resistant nanoparticles, suitable for *in vivo* gene delivery. Thus, DNA in the complex remained intact in the blood stream for 30 min, although gene expression after injection via the tail vein of mice was only seen in the liver.[47]

Nonviral vector modifications with peptides to increase intracellular gene delivery

Many anionic pH-sensitive peptides[48] and cationic fusogenic peptides[49] show an enhancing effect on gene expression mediated by cationic liposome and PEI, respectively. These peptides show membrane disrupting activities in weakly acidic condition, which is similar to that in the endosome compartment, and could enhance the translocation of the DNA to cytosol. Rittner *et al*[50] reported that a bifunctional peptide with both DNA-binding and membrane-disrupting activities showed significant gene expression in the lung after tail-vein injection.[50]

Inefficient entry of DNA into the nucleus is a major limiting step in the development of nonviral gene delivery system. The problem is particularly serious in nondividing cells, where entry into the nucleus is thought to occur only through the nuclear pore complex. To achieve active transport to the nucleus, nucleus localizing signal (NLS) peptides have been widely used. Recent effort has been summarized in excellent reviews.[51–53] In most cases, NLS is conjugated with a gene carrier such as PEI, or with DNA directly.

Reduction of immune responses by modifying the administration protocol or the composition of the DNA

Although it is well known that nonviral gene delivery produces a less severe immune responses than virus-mediated delivery, problems still remain. The DNA/gene complex is recognized by macrophages, dendritic and other immune cells. For cationic liposomes, toxicity relates to the rapid induction of proinflammatory cytokines such as TNF-α, IL-6, IL-12 and IFN-γ.[54] This response stems from the stimulation of the immune cells by the unmethylated CpG motifs in the plasmid DNA. Various approaches have been taken to reduce this inflammatory toxicity, including elimination of CpG motifs in the plasmid DNA,[55] use of PCR fragments with reduced numbers of CpG motifs[56] and active targeting of the DNA to the endothelium, which minimizes interaction with immune cells.[57] Furthermore, sequential injection of cationic liposomes followed by naked plasmid DNA, first reported by Liu's group,[14] reduces the inflammatory response.[58] Thus, when plasmid DNA was injected into the tail vein of mice 2–5 min after the injection of cationic liposome, 50–80% lower levels of proinflammatory cytokines (compared to lipoplexes) were observed, without affecting gene expression level in the lung.

Design of tissue-specific, self-replicating and integrating plasmid expression systems to facilitate long-lasting gene expression

Producing sustained gene expression is also an important goal for nonviral gene therapy. Tissue-specific expression systems can produce stable expression by reducing the probability of inducing an immune response to the transgene. Thus, Kay's group constructed a plasmid DNA containing the apolipoprotein E locus control region, α1-antitrypsin promoter, human factor IX minigene sequence including a portion of the first intron, 3′-untranslated region, and the bovine growth hormone polyadenylation signal.[59] When the plasmid DNA was delivered to mouse liver by hydrodynamic injection, it produced not only increased gene expression of factor IX (in the therapeutic range), but also maintained these levels for at least 10 months. Furthermore, a linear DNA expression cassette originating from this plasmid showed 10- to 100-fold higher expression than the closed circular DNA for a period of 9 months.[60]

The Epstein–Barr virus (EBV)-based plasmid vector is known to self-replicate in cells. It carries two genetic elements from EBV, the EBV nuclear antigen 1 (EBNA1) gene and the oriP element. The EBNA1 protein binds to oriP, and facilitates the replication of the plasmid in synchrony with chromosomal DNA. Furthermore, the EBNA1 also facilitates nuclear localization of the plasmid DNA. This approach has been used for tumour suicide therapy[61] (coupled to a polyamidoamine den-

drimer), long-term expression of the ß2-adrenergic receptor in cardiomyocytes,[62] and efficient and long-lasting luciferase expression in murine liver after hydrodynamic injection.[63] Stoll *et al*[64] have also reported high-level and long-lasting expression of the α1-antitrypsin gene in mouse liver using the hydrodynamic injection protocol.

Controllable integration of plasmid DNA into the genome of mammalian cells would also provide long-lasting gene expression. Reconstitution of an ancient transposon, Sleeping Beauty, from sequence alignment of nonfunctional remnants of members in the *Tc1*/mariner superfamily of transposons within the genomes of salmonids, provided the first functional transposon for use in vertebrate species.[65] Sleeping Beauty has been used to accomplish stable chromosomal integration of functioning genes in somatic cells of adult mice.[66] In addition, several phage integrases and their corresponding recognition elements, which can mediate integration into mammalian chromosomes, were reported by Calos's group.[67–70] Although the integration efficiency of integration system is still low, this technology may one day enable site-specific and high-efficiency integration into the host chromosome without the potential for mutagenesis.

Summary

To establish efficient and safe gene delivery *in vivo*, a number of new techniques and concepts have been introduced in the last 2 years, with improvements in targeted or controlled delivery of genes. However, we are still far from the perfect gene carrier suitable for clinical use. We have come a long way in understanding the cellular barriers which prevent proper delivery of DNA, but still relatively ignorant about factors controlling the stability, pharmacokinetics and biodistribution of nonviral vectors. Much of the above effort has been carried out in rodents and whether the new improvements are applicable to larger animals remains to be seen. We are still far from the perfect gene carrier suitable for clinical use, and much more work is still ahead of us.

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