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Liposome-Mediated Cancer Gene Therapy: Clinical Trials and their Lessons to Stem Cell Therapy

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The promise of stem cell therapy for various clinical applications seems getting realistic. An increasing number of researchers, from virtually every discipline of natural sciences, are flocking into this new world. Only ten years ago, gene therapy was *the* medicine for the 21st century. The possibility was endless. Although the science itself underlying gene therapy was very young, the field was exploding under the optimism that this *new* medicine would revolutionize both the basic and clinical sciences. For many reasons, the initial target was cancer. Here, we will focus on the results of cancer gene therapy clinical trials using liposome or nonviral gene carrier, hoping that the lesson from here will be a guideline for the new generation of cell-based therapies.

Key Words : Gene therapy, Cancer, Clinical trial, Liposome

Introduction

The idea of gene therapy is originated from 1970s when techniques for genetic engineering were actively being developed. Various methods, either using virus or nonviral-based, have been developed to express transgene in cell and tissue, initially for experimental purposes. From the first gene therapy clinical trial in early 90's, most gene therapy's clinical applications have been aimed at cancer, based on the fact that many human cancers are the result of accumulated genetic lesions that culminate in a transformed malignant phenotype.[1] The gene therapy concept was quite elegant; by replacing an aberrant gene to a normal counterpart in specific cells, thus, the pathological cause of diseases could be treated at the molecular level. The public expectation and fame rose faster than growth of scientific information. Even when the first clinical trial of gene therapy was performed using adenovirus in 1990, the transduction/transfection mechanism was not clearly identified, and its biological consequence of transgene expression *in vivo* was overly simplified. In 1999, unfortunately a genetic disease patent treated with adenovirus-mediated gene therapy became the first victim of gene therapy. However, there was cancer that became a more attractive clinical target for gene therapy mainly because of two reasons: 1) the limitation of effective intervention methods which are still only surgery, radiation, and chemotherapy and 2) the economic outlook from the government and the pharmaceutical industry from the ever-growing number of cancer patients (Note that there were 1.2 million new cases of cancer in the US in 2001.[2] It grew to 1.6 million in 2011). Indeed, in the first decade of the twenty-first century, the largest number of the gene therapy clinical trials have been focused on cancer by 64.6% out of total 1714 clinical trials.[3]

Another important statistic fact is that most of the gene therapy clinical trials chose viral vectors, such as retrovirus, adenovirus, and adeno-associated virus, which may mediate highly efficient gene transfer into cells. Accordingly, a great majority of reviews and books on clinical gene therapy deal with viral vectors,[4-10] while only a few reports describe nonviral vectors.[11,12] That also implies that, although there have been great attempts to improve nonviral vectors since Felgner and colleagues' remarkable finding of cationic liposome-mediated gene delivery,[13] one might suspect that nonviral strategies have their intrinsic limitations for human application, which must be the poor efficiency of gene delivery, cytotoxicity and lack of specific targeting. However, there was more safety concerning of viral vectors after the death of the patent by a severe immune response, and there were great efforts to achieve a breakthrough in developing efficient nonviral gene carriers. Although there were many hurdles for nonviral vectors to face a clinical application for cancer treatment, the advances in understanding the chemical and biological principles in nonviral vector-mediated gene delivery will allow liposome/DNA complex to be the most promising tool for cancer gene therapy. The purpose of this article is to review clinical applications, the strategies, and advance of cationic liposomes and other nonviral carriers for the cancer treatment. We would like to compare them with the current development of stem cell therapy to understand promise and limitation of latter, which will be a good guideline for future stem cell therapy.

Clinical Trials

Although numerous liposome systems have been developed for gene therapy, the major type of liposomes used in clinical trials are the complex mixture of a prototype cationic lipid, 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) or dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE), and dioleoyl phosphatidylethanolamine (DOPE), a zwitterionic helper lipid that

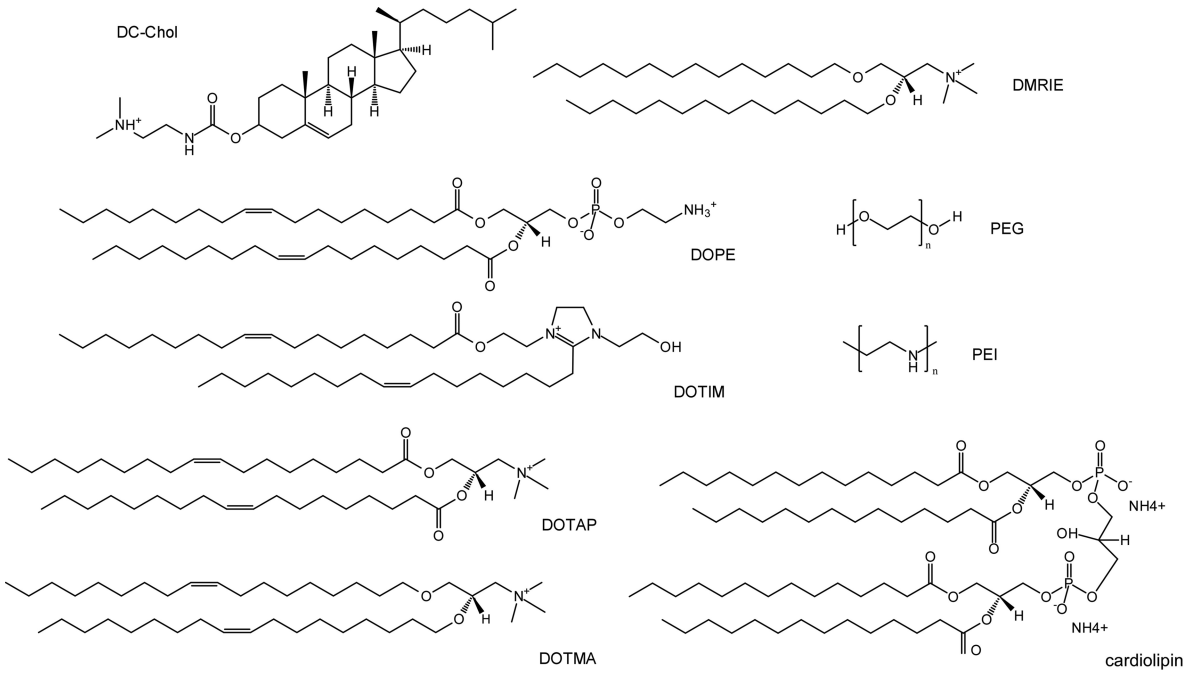

Figure 1. Structure of lipids used in cancer gene therapy clinical trials and related molecules.

increases fusogenic properties of lipid[14] (Figure 1 and Table 1[15-30]). It was thought that the most medically applicable non-viral vectors are cationic liposomes. Since the appearance of these first-generation cationic lipids, enormous efforts to find an optimal transfection condition both *in vivo* and *in vitro* have been made; for examples, lipid/DNA ratio, transgene expression time, and additional factors which may influence the expression of introduced gene. However, the generalization of transfection efficiency, depending on cell type, chemical structure of lipid, charge ratio between liposome and DNA, are still futile. Nonetheless, some biotech companies have commercialized the prototype cationic liposomes/therapeutic gene complexes such as Allovectin-7, HLA-B7 gene with β_2-microglobulin formulated with DMRIE/DOPE; Leuvectin, human IL-2 gene formulated with DMRIE/DOPE (Vical, San Diego, CA); and tgDCC-E1A, pE1A-K2 plasmid complexed to DC-Chol/DOPE (Targeted Genetics, Seattle, WA), and these products have applied to upper phase II and phase III clinical trials (Table 1). Allovectin-7 from Vical Incorporation has been studied for metastatic melanoma in phase III clinical trials for 5 years. Its efficacy was compared with chemical therapy using dacarbazine (DTIC) and temozolomide (TMZ). The phase III study was initially expected to be finished in 2006, but currently it is postponed to February 2012. Another pipeline product of Vical, Leuvectin, uses interleukin-2 (IL-2) and has been tested for the treatment of recurrent prostate cancer. A multi-institutional phase II study showed relatively good responses with minor toxicity when applied to head and neck cancer and breast cancer. Despite their success and failure stories in the clinical trials, it should be noted here that cancer gene therapy using nonviral vector is still in its early stage. Considering continuous invention and development of liposomal gene delivery system,[31-34] the authors believe that there is greater possibility in its clinical application in the near future.

Not much information is available on the interaction between biological environment and cationic liposome/DNA complex when they are injected intravenously. Therefore, the general approach to cationic lipid-mediated gene delivery system as well as viral vector system has been direct intratumoral injection, intraprostatical, intrapleural and intraperitoneal injection, with or without using ultrasound- or CT-guidance, or catheter (Table 1). Consequently, solid cancers, such as metastatic melanoma, colorectal adenocarcinoma, renal carcinoma, sarcoma, head and neck, breast, ovary and prostate cancer, have been major target diseases. However, local administration is not an attractive method to most cancer therapy because the identification and physical operation of tumor location is not always achievable and tumorous cells often disperse throughout body due to its metastatic nature. Many studies to improve gene delivery efficiency *via* systemic administration will be discussed later.

Following the choice of delivery system and vector to use, another important factor is to decide therapeutic gene. It might be true that, although the efficiency of cationic liposome-mediated gene transfer is not usually as high as viral vectors and the systemic administration into human is still in safety concern, immunotherapy would be the most feasible therapeutic strategy using cationic liposome-mediated gene transfer. The local injection in this strategy can bypass a major side effect of systemic administration, which is non-selective recognition of host immune system. For example, using IL-2 or HLA-B7 gene, which encodes human interleukin (IL)-2, one of the most effective antitumor cytokine,[35,36]

Table 1. Liposome-Mediated Cancer Gene Therapy Clinical Trials

Cancer	Major Carrier	Gene	Administration Route	Phase (Start Year)	Note
Stage IV melanoma	DC-Chol	HLA-B7	Intratumoral, Intrapulmonaty	Phase I (1993)	
Head and Neck cancer	DC-Chol	EGFR antisense	Intratumoral	Phase I (1999)	
Head and neck cancer, Breast cancer	DC-Chol	E1A	Intratumoral with catheter	Phase I (1999)	
Breast cancer, Ovarian cancer	DC-Chol	E1A	Intrapleural, Intraperitoneal	Phase I (1999)	
Ovarian cancer	DC-chol	E1A	intraperitoneal	Phase I/II (2004)	tgDCC-E1A in combination with paclitaxel
Head and neck cancer	DC-chol	E1A	intratumoral	Phase II (2002)	tgDCC-E1A
Metastatic melanoma	DMRIE	HLA-B7/β_2-microglobulin	Intratumoral	Phase I (1997)	
Metastatic melanoma	DMRIE	HLA-B7/β_2-microglobulin	Intratumoral	Phase II (2002)	Allovectin-7 alone
Stage 3 or Stage 4 melanoma	DMRIE	HLA-B7/β_2-microglobulin	Intratumoral	Phase III (2006)	Allovectin-7 alone compared with chemotherapy
Head and neck cancer	DMRIE	HLA-B7	Intratumoral	Phase I (2001) Phase II 2002)	Allovectin-7
Prostate cancer	DMRIE	IL-2	Intraprostatiscal	Phase I/II (2000)	Leuvectin
Leukemia	DOTIM	Noncoding plasmid DNA	Vaccination	Phase I (2009)	As an adjuvant (JVRS-100)
Advanced solid tumor, advanced malignancy	Cationic cardiolipin	c-raf antisense	Intravenous	Phase I (2004)	LErafAON-ETU
Refractory or Relapsed Acute Myeloid Leukemia, Acute Lymphoblastic Leukemia, and Myelodysplastic Syndrome	Unknown	L-Grb-2 antisense	Intravenous	Phase I (2010)	

and a foreign major histocompatibility complex (MHC) protein,[37,38] respectively, cationic liposomal delivery has been shown to enhance the response of the immune defense against to cancers. Meanwhile, apart from the immunotherapic strategy, E1A gene has been used as another important therapeutic gene in clinical trials for oncogene inactivation.[39-41] This gene is known to inhibit expression of HER-2/neu oncogene. More strategies in the therapeutic gene level will be discussed in the *Strategy in Therapeutic Gene Levels*. The pioneering works were conducted by Nabel and his colleagues at the University of Michigan and Howard Hughes Medical Institute.[16,22] They reported the first and the second results of liposome-mediated gene therapy clinical trials. HLA-B7 gene/lipid complex was administrated directly into timorous tissues of melanoma patients after complexed with either DC-Chol or DMRIE. The transferred gene was locally expressed and detected near the site of injection, and no apparent toxicity or *anti*-DNA antibody was associated with those treatments. Similar protocols were performed by independent investigators to patients with melanoma, hepatic metastasis from colorectal carcinoma, renal carcinoma, and head and neck cancer.[23-25,27,29] These early studies showed promising results on the use of HLA-B7-mediated immunotherapy using DMRIE for cancer therapy, which warranted phase II clinical trials.

Stopeck and her colleague at Arizona Cancer Center conducted the first phase II clinical trial using DMRIE/HLA-B7 gene complex to treat patient with metastatic melanoma.[21] They concluded that this approach was safe, accessible to tumor nodules, and well tolerated as well. The treatment induced both local and overall disease responses in melanoma. The regression of the injected lesion was observed in 18% of patients. In another phase II trial using DC-Chol/E1A complex, Villaret *et al.* reported 4.2% of complete response, 8.3% of minor response, 29.2% tumor stabilization in patients with head and neck cancer.[18]

In summary, many phase I and phase II clinical trials have addressed the safety, low toxicity, simplicity, and feasibility of liposome-mediated gene transfer, which yields further clinical trials. Since it gave no significant or meaningful indication of antitumor activity, they may not be called a success story. However, efficient and safe gene delivery were became a not formidable challenge any more. The latter portion of this article will review the strategies in therapeutic gene level and vector system level for that purpose.

Strategies in Therapeutic Gene Level

Regardless of diverse causal factors of cancers that could be chemical, physical (e.g. radiation), and infectious carcinogens, most carcinogenesis emanates from loss of expression of tumor suppressor genes and/or aberrant expression of oncogenes. Indeed current gene therapy strategies have been focusing on replacing a defective tumor suppressor gene and inactivating an oncogene expression. In addition to direct restoration of a genetic disorder, indirect methods, including delivering a drug sensitivity gene, inhibiting angiogenesis/neovasculation, and increasing preexisting immune system,

have been general approaches for cancer gene therapy as well.

Tumor Suppressor Gene Therapy. p53, a tumor-suppressor gene product, is one of key regulators of cell cycle, signal transduction, and apoptosis. It is also the most common mutation seen in various cancers.[42] A rationale, that insertion and expression of a normal copy of a tumor-suppressor gene would induce either cell-cycle arrest or apoptosis, makes it an important target for the treatment of cancer resulted from genetic lesions. Despite the initial skepticism that the expression of one gene can correct multiple genetic abnormalities in cancer cell, the restoration of one tumor suppressor gene, such as *p*53, has been shown to exhibit sufficient *anti*-tumor effects because the "bystander effect" to adjacent tumor cells induces cellular apoptosis and arrests tumor growth, even if they are not directly transfected.[43] Not confined in p53 gene replacement, this bystander effect can be applied to the use of herpes simplex virus thymidine kinase (HSV-TK) and ganciclovir (GCV). The underlying mechanism must be multifactorial, involving simple transmembrane diffusion of toxic metabolites, direct metabolic cooperation *via* gap junctions, immune cell recruitment, and/or antiangiogenesis[6].

Phase I clinical trials using viral vectors containing wild-type p53 demonstrated significant transgene expression, low toxicity, and indication of antitumor activity.[44-47] *In vivo* gene transfer efficiency varies but is usually between < 0.01% and 4% with retroviruses and between < 0.01 and 11% with adenoviruses.[48] Not too surprisingly, p53 gene replacement has its high potential when cooperated with traditional treatments such as chemotherapy and radiation. That is in part because various mutations that deactivates *p53* in tumor cells often confers resistance to chemotherapy or radiation-induced apoptosis. Conversely, p53-induced apoptosis may provide one of major mechanisms involving therapeutic interventions. Related with this rationale, some gene therapy approaches are to overcome acquired drug resistance by restoring the p53 pathway. It has been shown that reconstitution of p53 by gene transfer sensitized tumor cells more to chemotherapy, leading to significant increase in antitumor activity.[49]

More than 20 tumor suppressors, including retinoblastoma gene (Rb), anaphase promoting complex (APC), and Von Hippel-Lindau (VHL) gene, function as potential *anti*-tumor therapeutic genes for breast cancer, colorectal cancer, renal cell carcinoma, and so on. Breast cancer type 1 susceptibility protein (BRCA1) was also utilized in retrovirus-mediated gene delivery. They were used in phase I and phase II clinical trials resulting in no immune response, no disease stabilization, and little or no vector stabilization.[50] The selection of an appropriate tumor suppressor gene for cancer therapy may depend on whether the specific gene is over-expressed or inactivated in tumor cells. For example, p21 may be a more desirable target in tumor cells which over-expresses MDM2, an E3 ligase targeting p53 ubiquitination, because they can bypass the inactivation of p53.[7] Some attention has been given to the potential for a modified adenovirus, ONYX-015 from ONYX Pharmaceuticals, for its selective replication in p53-deficient cancer cells. The results of phase II clinical trial using ONYX-015 with chemotherapeutic agents cisplatin and 5-fluorouracil reported that 60% of patients with head and neck cancers experienced decrease in some degree of tumor size.[51,52] However, the viral gene therapy method showed too limited therapeutic windows to be clinically used.

Different from the replacement approach or expression of intact gene, several investigators have developed direct gene repair methods such as triplex-forming oligonucleotides (TFO)[53,54] and chimeric RNA/DNA oligonucleotides (RDO).[55-57] TFOs are designed either to correct mutations or to induce mutation for inhibiting overexpression of oncogenes. Although only a few of these methods have been tested in animals, they offer potential means to achieve the ultimate goal of gene therapist.[12]

Oncogene Inactivation. Oncogenes encoding oncoproteins such as Ras have a key role in various key signal transduction and transcription. For inhibiting the expression of oncogene, antisense oligonucleotides and siRNA, which of both can block downstream protein synthesis, have been widely applied. Exogeneous antisense oligodeoxynucleotides, which are single stranded DNA sequences specifically designed to bind the promoter regions of oncogenes or their mRNAs, can block transcription or translation of oncogenes. It has also been shown that antisensing growth factor such as insulin-like grow factor (IGF) can be applied to inhibit growth and development of IGF-overexpressing tumor cells.[58] Ribozymes, having cleavage function for mRNA, also have shown great promising applications for cancer therapy. Endogeneous delivery of genes which encodes specifically designed ribozymes to cleave only oncogenes such as Ras, HER2/neu, c-raf, Grb-2, and Bcl2, enhances tumor regression.[59-62] Antibody with specificity against the oncoprotein can prevent transportation of oncoprotein in the protein level.

Another frequent approach for oncogene suppression is the transfer of a gene that is known to block a specific activated oncogene. For example, Her-2/neu-mediated malignant transformation, observed in many cancers, can be blocked by E1A gene, leading to reduction of their metastatic potentials and angiogenesis.[17,19,39,63] In phase I clinical trial using E1A/DC-Chol complex, Yoo *et al.* confirmed E1A expression and apoptosis induction.[17] More interestingly, E1A is known to increase Her-2/neu-overexpressing tumor cell's sensitivity to the chemotherapy such as Taxol and irradiation.[64,65] This could be another good instance to show a possibility of combination of traditional treatment and gene therapy.

Initially, this antisense technique was thought to have many hurdles to overcome such as stability of oligonucleotide, selective targeting, and cost of manufacturing.[66] In addition, because oncogene inactivation may not strong enough to lead tumor cell death, sustaining transgene expression is more needed than other strategies for successful clinical application. However, the synthesis of chimeric or

mixed-backbone oligonucleotides (MBOs) has circumvented several problems of the first-generation antisense molecules. The MBO structures are nuclease stable and well-hybridized with target mRNA and maintain sensitivity to cleavage by RNaseH, thereby improving bioavailability and pharmacokinetic properties, with a reduction in the toxicity profile.[6] Moreover, antisense technique can be applied not only to oncogene inactivation, but also to *anti*-angiogenesis for cancer gene therapy. So far, most *anti*-angiogenic gene therapies have targeted on vascular endothelial growth factor (VEGF), which plays a central role in tumor development. Antisense mRNA, complementary to the target VEGF, theoretically prevents its translation, and inhibits vascularization, following suppression of tumor growth.

Immunotherapy. Recognition of tumor cells seldom leads to enough immune response because they often secrete immunosuppressive cytokine such as transforming growth factor β (TGF-β). The aim of immunotherapy is either to enhance or to augment the response of the immune system of patients with cancer. Activating host T cell-mediated immunity involving cytotoxic T lymphocytes and T-helper cells may mediate tumor regression and antimetastatic effects with the hypothesis that these molecules can act as general immune adjuvants. Major advantage of this strategy is that transient transgene expression is enough to stimulate the immune response. Moreover, cytotoxic T-cell activation can enhance the antitumor immunity in many tumor sites, such that the immunological memory will be induced, offering its possible use for cancer prevention as 'cancer vaccine'. These advantages allow immunotherapy to be the major strategy for cancer gene therapy clinical trial, which is more than 40% based on protocols by gene types.[3]

Studied have shown that genes encoding following relevant cytokines, such as tumor necrosis factor-α (TNF-α), glanulocyte-macrophage colony stimulating factor (GM-CSF), INF-γ, IL-1, IL-2, IL-4, IL-6, IL-7, IL-12, and IL-18, promote antitumor immune response. Although cytokine activity varies depending on specific tumor types, IL-2 and IL-12 have been studied among these cytokines mostly because of their high possibility from their specific T cell-mediated immunity.[67-69] Genes encoding co-stimulatory molecules, such as CD40, CD80, and CD86, and allogenic MHC class 1 molecules, such as HLA-B7, are also intensively studied for immunotherapeutic strategy. Current noticeable subject of immunotherapy is to utilize dendritic cells as tumor antigen-pulsed antigen presenting cells (APCs),[70,71] different from initial trials to transfer cytokine gene directly into tumor cell. Nonviral vectors can fix the problems of low efficiency in delivering peptide form of antigen to dendritic cells. As advances have occurred in immunology and molecular biology, more effective and safe strategies in immunotherapy have been devised. For example, tagging cancer cells with specific genes that target a selective tumor cell would allow systemic administration of immunotherapy.

One thing to remember is that the efficiency of immunotherapy will vary depending on the severity of tumor developed. To date, patients enrolled in immunogenetherapy clinical trials are mostly on the late stage of cancer. Therefore, the real evaluation of immunotherapy should be made only after accordingly designed clinical trials and optimal immunization model.

Suicide Gene Therapy/Molecular Chemotherapy. Alternative strategy is to transfer gene encoding an enzyme which can convert prodrugs into toxic metabolites. Therefore, it also called molecular chemotherapy. Probably the most used 'suicide' gene is HSV-TK, which mediates phosphorylation of the prodrug GCV, consequently inducing cell death. Intratumoral injection of the suicide gene and following systemic GCV administration is one of the firstly employed methods for clinical trials. Many studies have shown that the bystander effect on neighboring nontransfected tumor cells increases the antitumor effect of molecular chemotherapy. From the knowledge of the authors, nonviral clinical trial using this strategy has not been published at the moment. However, various suicide gene trials mediated by viral vectors have shown their feasibility and safety for the treatment of cancer patients.[72-75] It seems still far from accomplishing for significant clinical benefit. A recent phase III clinical trial report showed no marked *anti*-tumor activity after retroviral delivery of the HSV-TK gene as an adjuvant to patients with glioblastoma.[76] Other strategies include the transfection/transduction of malignant cells with cytosine deaminase, p450-2B1, and nitroreductase genes, followed by fluorocytosine, cyclophosphamide, and CB1954 prodrugs, respectively.

Major advantage of the suicide gene therapy, which is that transient gene expression might be enough to lead tumor cell death, could also be a disadvantage for medical applications because of the possibility that normal cells can be collaterally destroyed by the transfected/transduced suicide gene. To overcome the limit of local administration, developing specific targeting methods to tumor cells is an urgent question.

Strategies in Vector System Level

Although liposome and other nonviral gene carriers have several advantages over viral vectors including simplicity of use, less restriction on the amount of transferred DNA, ease of large-scale production, and lack of specific immune response,[31-34] there are still more hurdles to accomplish effective cancer therapy. Among various levels of therapeutic strategies based on nonviral gene delivery mechanism, many scientists have tried to develop the liposome which is good enough for successful cancer therapy.

Efficiency. The Achilles heel of nonviral vectors has been their low transfection efficiency. Despite enormous effort, not a single nonviral vector showed comparable transfection efficiency with viral vectors. Electroporation has been emerged as effective and promising nonviral technique with 2- to 4-log fold increase in transfection efficiency than other nonviral methods. But it was still lower than viral vectors.[77] This comes from the intrinsically different mechanism of *nucleus* uptake, not much from *cellular* uptake, and stable

expression between viral vectors and nonviral vectors. Recently, many approaches have been developed to overcome these obstacles and achieve therapeutically effective gene transfer.

Stability in Circulation System: In the situation that local administration is not attractive for certain target organs, for repetitive administration, or metastatic cancer, one of the fundamental problems of existing nonviral vectors is their limitation in systemic gene delivery. The efficiency of normal cationic liposome-mediated gene expression often decreases with the addition of serum.[78] It is thought that the positive charge on the lipoplex are neutralized by negatively charged components of serum, resulting in an immediate bound form and hence shielding electrostatic interaction between lipoplex and cell surface.[79] Moreover, increased size of lipoplex can be a major factor determining the tissue distribution and endocytosis-mediated uptake into cytoplasm. One possible and classical method for long circulation of liposome is to modify the liposomal surface with hydrophilic and biocompatible polymers, such as polyethylene glycol (PEG).[80,81] Neutral liposome also can survive in the circulation system relatively longer. However, it also decreases the ability of liposome to bind the target cells, which results in the reduction of the transfection activity. It has been reported that cholesterol-based lipid formulation could be better to maintain a small size than DOPE-based methods when they are used in intravenous gene delivery.[82] To achieve a high level transfection efficiency in lung endothelial cells, a higher charge ratio of cationic lipid and DNA appears to be essential.[83,84] Liu *et al.* and other researchers have suggested a relationship between cationic liposome structures and their *in vivo* transfection activities.[85] There must be the most suitable transfection methods depending on target cells for delivery, which yet has to be identified for the first place to achieve the practical application of gene delivery.

Endosomal Release: Even though the number of DNA molecules internalized per cell by endocytosis is approximately 19,000,[86] most DNA in the endosome is destined to be degraded or inactivated.[87] The cellular uptake of plasmid DNA can be augmented either by buffering capacity of DNA delivery system or by the addition of other agents capable of mediating endosomolysis. The endosome disruptive function, also called "endosome buffering" or "proton sponge" effect, of a tertiary amine is one of the main approaches for effective DNA release into cytoplasm.[88,89] Amine functional groups, which are in the form of free bases at physiological pH, disrupt the endosomal membrane by osmotic swelling after becoming protonated at the acidic pH in endosomes. The endosomal release of DNA can also be achieved by destabilizing the endosomal membrane. Several methods to destabilize the endosomal membrane are the addition of a helper lipid such as DOPE, a endosomolytic drug such as chloroquine, and a fusogenic peptide[90] from a virus capable of mediating endosomolysis. Anionic lipids on the cellular membrane can be displaced by cationic liposome, thereby releasing the DNA or DNA-lipid complexes into the cytoplasm.[91]

Transport to Nucleus: Dissociating DNA from the DNA/liposome complex and importing it into the nucleus is thought to be the major rate-determining step of the nonviral gene transfer mechanism. That is simply because the process of DNA transport into the nucleus is very inefficient. DNA in the cytoplasm is easily degraded by cytoplasmic nuclease,[92] and the direct injection of lipoplex into the nucleus cannot achieve a significant gene expression.[93] It has been well-known that proliferating cells such as cancer cells can be transfected much more efficiently than normal cells, while non-dividing or post-mitotic cells are particularly resistant to transfection.[94] It has been proposed that plasmids can enter the nucleus more efficiently when the nuclear membrane is dissolved during the M phase of the cell cycle.

Modification of plasmid DNA to replicate itself in the cytoplasmic space was an attempt to bypass this hurdle.[95] More directly and efficiently, covalent or noncovalent conjugates of nuclear localization signal (NLS) sequences to liposome vector improved nuclear importation of plasmid DNA and consequent effective gene expression.[96,97] A synthetic peptide with nuclear targeting signal can also facilitate the transgene translocation to the nucleus and enhance the liposome-mediated transfection.[98] Polyethylenimine (PEI)/plasmid DNA complex has been reported to show enhanced nuclear uptake by condensing and protecting DNA.[99]

Targeting. In principle, cellular uptake of a lipoplex is a nonspecific process. A positively-charged complex binds to a negatively charged cell membrane with electrostatic interactions. Along with the effort for better transfection efficiency and for less cytotoxicity, various methods to target the specific cells have been extensively studied to overcome this nonspecific interaction. For viral vector targeting, peptide sequences targeting specific receptors are often genetically introduced and modified. For nonviral vector, binding a ligand to liposome or polymer, usually in a covalent-bond manner, is the most common method of delivery to a selective site. The delivery efficiency of exogenous DNA *via* surface receptor is dependent on several factors, including the presence and the number of specific receptors on the target cell surface, the receptor-ligand interaction affinity, the stability of the conjugate-DNA complex, and endocytosis of the complex.[100]

Various ligands such as proteins, peptides, carbohydrates, vitamins, or antibodies have been used for receptor-targeting gene transfer.[101] Transferrin, folate, and asialoglycoprotein receptor have been demonstrated to be a promising target receptor for each ligand *in vitro*. However, targeting after systemic administration still requires substantial development. For systemic gene delivery targeting tumor cells, angiogenic endothelial cells could be a good target. A specific *anti*-tumor therapy was attempted by blocking activation of many receptors on endothelial cell related with neovasculature.[102] Tissue- or cell-specific targeting can be enhanced by modification of plasmid DNA itself. Several tissue-specific enhancer or promoter elements have been isolated and used for both viral and nonviral vectors.[103] Chimeric vectors have been investigated to utilize both viral vector's tissue-specific and

nonviral vector's receptor-mediated targeting ability.

Toxicity. Although nonimmunogenecity has been known to be one of the major advantages of nonviral vectors, recent reports have shown that systemic administration of lipoplex can induce cytokines such as IFN-γ, TNF-α, IL-1, and IL-12, causing inflammations.[104,105] These cytokines reduce the expression of transgene as well as cause severe toxicity in the treated animals.[106] The main responsibility of this immune response seems to be related with the DNA sequences containing unmethylated CpG motifs, which resembles its prokaryotic origin.[106,107] One thing to note is that cationic liposome in the complexes has a role to amplify the immune response stimulated by CpGs.[108] Consistent with this, reducing the number of CpG motifs of transfected plasmid DNA or usage of short DNA fragment was proved to be a useful method to reduce immune response.[107-109] Li *et al.* have recently shown that the use of cytokine neutralizing antibodies can significantly prolong high level cytokine induction after tail vein injection of cationic lipid-protamine-DNA, LPD.[110] Recently Tan *et al.* showed sequential injection of cationic liposome and then naked DNA reduces cytokine induction of lipoplex and increased transgene expression.[111]

Transient Gene Expression. Due to the intrinsic inability for genomic integration, it has been thought to be inevitable for nonviral vector's short-term expression. The inactivation mechanism of transgene expression has not been fully understood. It is hypothesized that the transgene inactivation involves histone deacetylation and chromatin condensation, therefore, the use of whose inhibitors may induce longer-lasting and higher gene expression.[112] Several methods to prolong transgene activity were reported, including the uses of transposon[113] and linear DNA fragment.[114] Increasing blood pressure called hydrodynamics can also get long-term and relatively high gene expression either with[115,116] or without[117] clamping vessels.

Most transferred genes do not have their natural regulatory elements including promoter, exon and intron. Consequently, regulating spatiotemporal gene expression in gene therapy seems to be hardly possible. Perhaps more genetic information from the Human Genome Project and subsequent proteomic information can provide better understanding of what sequences such as promoter, enhancer, intron, polyadenylation, and transcriptional termination have to be delivered.

Naked DNA. Applying naked DNA without using any vector has been not only the simplest and safest but also one of the most successful methods for gene transfer. Intravenous administration of plasmid DNA showed only limited transfection efficiency. However, a notable exception is hydrodynamic injection, which facilitates hydrostatic pressure or rapid injection in excess volume through systemic administration.[117] This protocol is more useful than initially accepted to find candidates for therapeutic gene quickly. Various modified applications of hydrodynamics have been applied to clinical trials.[115,116]

Other efforts to improve naked DNA-mediated gene transfer are mainly based on the physical principle: gene gun and electroporation. The general mechanism of electroporation lies in an increase in membrane permeability following treatment with electrical pulses and is then followed by influx of DNA through the permeabilized membrane defect.[118] Recent reports have demonstrated optimized electroporation conditions including voltage, pulse duration, number of pulse, frequency, DNA concentration, and the amount of injected reporter gene.[77,119,120] In particular, gene delivery to skeletal muscle mediated by this technology is a promising strategy for the systemic secretion of therapeutic proteins, especially for genetic vaccination.[121-123]

Summary and Perspectives

In this review, we extensively discuss historic progress of human cancer gene therapy and its underlying mechanism. While there have been significant development in the nonviral gene delivery system during past several decades, researchers are confronted with more technical limitations, pathological complexity in diseases, and clinical regulations. This nonviral gene delivery field is not in its infancy any more. The use of DOTMA for gene delivery by Felgner and his colleagues was in 1987[13] and the first clinical trial using DC-Chol by Nabel and his colleagues was in 1993.[16] The death of a patient with ornithine transcarbamylase (OTC) deficiency treated by non-replicating OTC gene-containing adenovirus in 1999 has attracted some negative outlook on nonviral gene delivery methods. However, as mentioned above, there is enormous flexibility in chemical and biological modifications for the improvement of nonviral vector system. In addition, lower risk of toxicity and immunogenicity may possess great promise on a breakthrough in successful cancer gene therapy.

Cancer and other disease treatments using therapeutic stem cells are gaining attention. Besides their public inspiration, there are many similarities between the approaches. Both are pathotropic, i.e., rapidly proliferating cells for nonviral gene delivery and tumoritropic properties of stem cells, and more specificity and targeting can be achieved through chemical modification and molecular biological engineering. Since various stem cell reprogramming methods are developed, the source problem, as relatively easy production for nonviral vector, seems to be solved. However, the safety issue still poses the most challenging problem to both methods. A small chance of stem cells, which are able to grow to tumor cells, after grafting should not be allowed for their clinical application. Moreover, it will be very difficult to turn-off the therapeutic gene expression from the stem cells once they are incorporated with and differentiated into the adult cells.

We have already observed a successful phase III gene therapy clinical trial using adenovirus expressing p53. The fact that restoring normal p53 is beneficial enough to stop or to reverse tumorigenesis is substantial encouragement in the field and, at the same time, a long-anticipating proof-of-concept of gene therapy. In 21st century, cancer gene therapy

will be more common treatment as combinatorial therapeutic strategy with conventional methods such as chemotherapy and radiation therapy. Recently, stem cell therapy is gaining attention for its potential clinical application. Society follows the hype of stem cell therapy and requires higher level of bioethics, which is reminiscence of gene therapy. Authors believe that still more biological understanding of stem cells is needed. The clinical trial using stem cell should be more cautious not to repeat the hasty gene therapy in nineties. Fortunately, the history of success and failure of gene therapy has left good lessons to stem cell therapists, indeed which will be the impetus for successful clinical stem cancer therapy.

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