Ultrasound-based and Non-viral Technologies in Gene Therapy

Cheng-Huang Su[2,3], Hung-I Yeh[1,2,3], Charles Jia-Yin Hou[2], Yu-San Cheu[2], and Cheng-Ho Tsai[1,2,3]

[1]Taipei Medical University, Taipei, Taiwan;
[2]Division of Cardiology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan;
[3]Mackay Medicine, Nursing and Management College, Taipei, Taiwan

Abstract

Gene therapy is a technique for the purpose of correcting or preventing a disease by delivering genes into an individual's cells and tissues. Gene therapy is still in its infancy and at an experimental stage. Synthetic vectors are considered to be a prerequisite for gene deliveries, as viral vectors have fundamental problems in relation to safety issues, as well as large-scale production. Among the physical approaches, ultrasound with its bioeffects-acoustic cavitation, especially inertial cavitation, can increase the permeability of cell membrane to macromolecules such as plasmid DNA. Microbubbles, or ultrasound contrast agents, lower the threshold for cavitation by ultrasound energy. Furthermore, ultrasound-enhanced gene delivery using polymers or other non-viral vectors, though also in its preclinical stage may hold a lot of promise for the future. The aims of this brief review focus on understanding of the barriers to gene transfer and useful vectors or tools that are applied in gene delivery and on introducing the feasible models in terms of ultrasound-based gene delivery. (J Intern Med Taiwan 2007; 18: 167-180)

Key Words：**Gene therapy, Vector, Ultrasound, Cavitation, Microbubble, Transfection efficiency**

Introduction

Gene therapy is a term that can be applied to any clinical therapeutic procedure in which nucleic acids are introduced into cells for the purpose of altering the course of a medical condition or disease [1]. Most commonly, the nucleic acids are DNA molecules that encode wild type or modified gene products or pro-

Correspondence and requests for reprints : Dr. Cheng-Huang Su
Address : Division of Cadiology, Department of Internal Medicine, Mackay Memorial Hospital, 92, Section 2, Chung-San North Road, Taipei, 10449, Taiwan

teins. It is a novel approach by transfering nucleic acids to the cells or tissues and the subsequent overexpressions of the endoded proteins, results in a therapeutic effect.

Gene therapy can be targeted to somatic (body) or germ (egg and sperm) cells. In somatic gene therapy, alterations in the genetic makeup of individual somatic cells are not passed to the next generation. In germline gene therapy, the parent's eggs or sperms are changed with the goal of passing on the changes to their offspring. Germline gene therapy is not being actively investigated, at least not in large mammals or humans. Currently gene therapy is solely concerned with introducing genes into somatic cells and has nothing to do with the genetic modification of the human germline, as it is not acceptable in most countries. In Taiwan, the development of gene delivery technologies was still in its early stage. Applications of gene therapy require a Good Manufacturing Practice (GMP) certificate by experienced researchers in medical center, according to the guidelines from the Department of Health, (www.doh.gov.tw/EN2006/index_EN.aspx) and fulfill the guidelines in terms of ethical and safety issues. For instance, the applications of gene therapy are only allowed to apply in somatic cells concerning ethical problems. Furthermore, the manipulation of virus vectors (gene carriers), owing to their possible lethal responses in humans, needs to be performed in an appropriate lab, such as P1 laboratory (lab) (adeno-associated virus (AAV)), P2 lab (adenovirus (Ad)) and P3 lab (retrovirus (Rv)). However, those advanced labs are only located in medical centers or advanced research institutes in Taiwan. Therefore, in efforts to performing gene therapy in the future concerning safety in the local hospitals, gene therapists need to choose proper vectors for use such as non-viral vectors. The cost-benefit analyses of gene therapy, in other words, analyses of the possibility between causing adverse effects/expense and gaining positive clinical effects can not be overemphysized before each treatment. The focus of this brief review is upon the use of non-viral technologies and physical approaches, especially ultrasound (US)-assisted gene delivery, a potential tool for clinical gene therapy. Most of the basic technical principles regarding US was located in the section of "Ultrasound-based Technologies in Gene Delivery", therefore, clinicians can choose their own interests.

Overview of Applications in Gene Therapy

The first report of vascular gene transfer was demonstrated by Nabel et al.[2], who transfected porcine endothelial cells ex vivo with a Rv encoding the beta-galactosidase (β-gal) gene and reintroduced the cells onto the denuded iliofemoral artery of a syngeneic pig. Arterial segments isolated 2 to 4 weeks later demonstrated endothelial cells (ECs) expressing β-gal, thus indicating successful incorporation of the transgene into the transduced cells. In September 1990, the first federally approved clinical trial of somatic gene therapy for a genetic disorder was started in the United States. In this study, the adenosine deaminase (ADA) gene was transferred into the T-cells of two children with severe combined immunodeficiency[3]. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Since then, more than 1000 clinical trials have taken place worldwide. The diseases most often treated with gene therapy are cancer (67%), vascular diseases (8.9%), monogenic diseases (8.6%) and infectious diseases (6.5%) (data adapted from http://www.wiley.co.uk/genmed/clinical/, Journal of Gene Medicine Clinical Trials website 2006).

Candidate Diseases and Target Therapeutic Genes

There are several promising areas for gene therapy in genetic and acquired diseases. For monogenic diseases, haemophilia, cystic fibrosis, and familial hypercholesterolaemia are of importance. For acquired diseases, cancer and cardiovascular diseases (more specifically, therapeutic angiogenesis for myocardial ischaemia[4-6] and peripheral artery occlusive disease that is a group of diseases caused by the obstruction of peripheral arteries, mainly resulting from atherosclerosis[7-9], restenosis[10,11], in-stent restenosis[12,13] and bypass graft failure[14]) are the most explored. Further research to identify defective genes in individual conditions with a view to introducing the normal counterpart by gene therapy is a major area of ongoing research.

Vectors and General Approaches in Gene Therapy

Gene therapy is still in its early stages of development and remains mainly experimental. Many factors have prevented researchers from developing successful gene therapy techniques. The process of gene delivery into cells and expression is known as transfection. Strictly speaking, viral vectors deliver exogenous nucleic acids by transduction, but for ease of use the term transfection is used for all techniques. Successful transfection relies on achieving a balance between gaining adequate access of DNA into the cytoplasm/nucleus and causing excessive damage to the cell. The first issue to be addressed is the gene delivery tool. This is done via vehicles called vectors, which deliver therapeutic genes to the patients' cells. There are three main categories of methods that have been used to deliver the gene to the target cell or tissues in gene therapy protocols: viral vectors (69%), non-viral vectors (25%) and physical delivery systems (1%, data adapted from Journal of Gene Medicine Clinical Trials website 2005). Currently, the most common vectors are viruses[15], of which the three most common are Ad, Rv, and AAV. Due to their highly evolved and specialised components, viral systems are by far the most effective means of DNA delivery, achieving high efficiencies for both transfection efficiency (TE) (i.e., percentage of cells exposed to vector that expresses the transgene) and levels of expression in transfected cells. Scientists have tried to take advantage of virus biology and manipulate its genome so that they can replace nonessential genes, particularly those necessary for viral replication, with therapeutic genes. Viral vectors, whilst efficient, introduce other problems to the body-producing toxicity and immune and inflammatory responses[16]. Non-viral vectors have been developed to overcome some of these problems encountered with viral vectors, particularly their immunogenicity[17]. However, gene expression following non-viral transfection is often transient, falling rapidly within the first few days and disappearing within one week. To date, some important non-viral alternatives that have been considered are complexes of DNA with lipids or polymers for gene delivery. In terms of physical non-viral delivery systems, needle-free injection, electroporation and US are the three major technologies currently under evaluation.

Once a vector is designed, two general approaches are used for somatic gene transfer:

1) the ex vivo model, where cells are removed, genetically modified, and transplanted back into the same subject.

2) the in vivo model, where genes are administrated directly to target cells in the body[1].

Challenge in Gene therapy

The death of a 18-year old boy, Jesse Gelsinger, from a gene therapy clinical trial in 1999 raised critical questions concerning the safety of experimental gene therapy treatments[18]. Jesse, who suffered from a deficiency of ornithine transcarbamylase, a genetic defect that prevents the correct metabolism of ammonia, died of complications from an inflammatiory response shortly after receving a dose of Ad carrying a corrective gene. His death illustrates the challenge in gene therapy well and gives rise to a much-demanded discussion in using gene delivery vectors, especially viral vectors and evaluating possible adverse effects in animal models.

Gene-transfer Systems

A gene therapy vector needs to meet three important criteria: safety; adequate gene transfer efficiency as well as stable and reliable expression of the transgene (the gene of interest) for a duration appropriate for the disease being treated. There are at least five barriers that need to be overcome for successful gene delivery: in vitro and in vivo stability, cell entry, endosome escape, cytoplasmic transport and nuclear entry. Unfortunately, the ideal gene delivery systems are still under investigation. In this section, non-viral vectors and physical approaches are briefly introduced. It is well known that non-viral vectors give low transfection efficiency, especially in vivo and more transient expression in gene delivery. However, comparisons between them are not possible since no literature was published in this regard. The important non-viral vectors and physical approaches are summarised in table 1 in terms of their key mechanisms.

Non-viral Vectors

The safety concerns associated with viral vectors have encouraged the development of non-viral vectors. pDNA delivered by non-viral approaches is not integrated into the cellular genome and is maintained in an extrachromosomal site[19]. The most popular materials used in current non-viral applications include purified pDNA, lipids (usually a mixture of cationic and neutral lipids) and synthetic polymers.

(1) Naked DNA

The simplest non-viral gene delivery system currently in use in vivo is the direct injection of naked pDNA. The use of naked pDNA without any carrier vehicle is also the safest method. However, because of the rapid degradation by nucleases in the serum and the clearance by the mononuclear phagocyte system in the systemic system, expression levels after the injection of naked DNA are generally limited. Although this technique has a low delivery efficiency, it is simple and safe with a very low risk of insertional mutagenesis. One of the promising approaches in this field is the combined use of naked DNA and a physical approach (such as electroporation) to enhance plasmid-mediated gene expression in muscle[20-23].

(2) Lipid-based vectors

Lipid-based gene delivery, first reported by Felgner in 1987[24], is still one of the major systems for increasing the TE of naked DNA. Liposomes or lipoplexes are formed by DNA with positively charged lipids and detergents[25,26]. The positively charged lipid-DNA complexes are capable of condensing with negatively charged DNA. Zabner et al. reported that the condensed lipid-DNA complexes (see figure 1) appear to be at least 100 nanometre (nm) or larger, at least in one dimension[27]. Cationic lipids and cationic polymers both share this important property.

Furthermore, the resulting net positive charge of

Table 1.**A summary of mechanisms between important non-viral vectors and physical approaches in gene delivery**

Non-viral vector	Central mechanism
pDNA	Endocytosis
Lipid-based vectors	Endocytosis; condensation
Synthetic polymers	PLL: endocytosis; condensation
(PEI, PLL)	PEI: endocytosis; condensation; proton sponge
Physical approach	**Central mechanism**
Needle-free injection	Gene gun: high-pressure helium stream
	Jet gun: high-pressure solution
Electroporation	Electric pulse-induced promotion of cell membrane-permeability
Ultrasound	US energy-induced promotion of cell membrane-permeability (sonoporation)

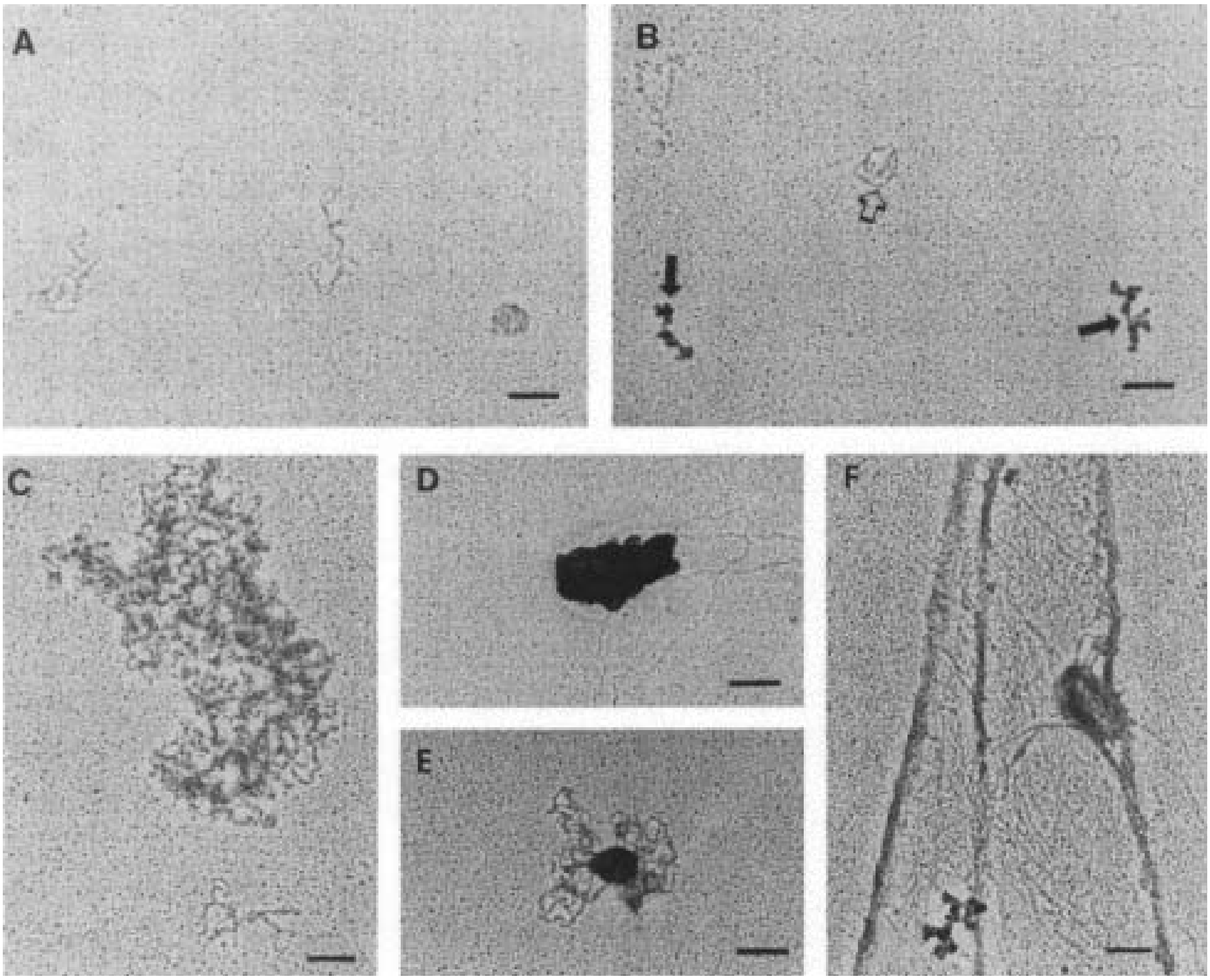

Fig.1.Electron photomicrographs of heterogenous forms of lipid-DNA complexes. Bar indicates 100 nm. (Zabner et al. 1995)
(A) pDNA with lipid
(B) uncomplexed plasmid (open arrow), plasmid complexed with lipid (solid arrow)
(C) large aggregates of lipid-DNA complexes
(D) completely condensed DNA
(E) looped structures of lipid-DNA complexes
(F) strands of complexes

lipid-DNA complexes may facilitate fusion with the negatively-charged cell membrane. Endocytosis is considered to be the major mechanism for liposomes to pass through the cell membrane[27-29]. Most of the liposome-DNA complexes are degraded by lysosomal enzymes, and only 1% of the DNA enters the nucleus where it remains extrachromosomal. Therefore, transgene expression using liposomes is transient. Liposomes are nonpathogenic, with no size limit for the transgene, and are cheap and easy to produce, relative to viral vectors anyway. Although the major limitation with its application is the poor efficiency at transfecting non-proliferating cells, there were several experiments showing high levels of transgene expression following direct administration or injection[30-32].

(3) Synthetic polymers

Synthetic polymers have also been evaluated as non-viral DNA vehicles. This principle is based on the concept of forming condensed DNA particles by complex formation with cationic polymers-polyplexes. The use of polycationic polymers leads to electrostatic neutralisation of anionic charges of DNA, and condense the polynucleotide structure of DNA, thereby protecting it from nuclease digestion[33,34]. Furthermore, due to reduced dimension of the molecule, the transport of the compact polymer-DNA particles is facilitated through the ECM. As a result, the cellular uptake through endocytosis is enhanced.

Many polycationic molecules are used, including poly-l-lysine (PLL), polymethacrylate dendrimers, polyamidoamine and polyethyleneimine (PEI). PEI and PLL are the commonest and most important ones used as non-viral vectors.

PLL is a well-known polycation. It has been used to deliver drugs for many years. It has been used to condense pDNA under various salt conditions[33,35-37]. The PLL-DNA particles have been shown to be protected against DNA degradation[38,39]. Electron microscopic studies have demonstrated that PLL-DNA complexes assumes a rod-like appearance with a diameter of 15 nm and a length of 109 ± 36 nm, much smaller than lipoplexes (see figure 2). The poor circulatory half-lives of PLL-DNA complexes, typically shorter than 3 min, also limit their use in vivo [40-43]. Generally, PLL or PLL-DNA complexes have been reported to have low immunogenicity[40,44].

Among cationic polymers, PEI has been the one most commonly used for gene delivery. The polycationic PEI is receiving much attention due to its characteristic of condensing DNA with an intrinsic endosomolytic activity[45]. Completely condensed PEI-DNA complexes are more homogenous and smaller in diameter than lipospermine (a cationic lipid)-DNA complexes (20-40 nm and 50-70 nm, respectively, see figure 2) [46].

The most prominent feature of PEI is its extremely high cationic charge density. Since every third atom of the PEI molecule is a nitrogen atom that can be protonated at endosomal pH range[47,48], PEI has the ability to capture protons that are pumped into endolysosomes-"proton sponge". It is, presumably, followed by a passive chloride influx into the endosomes and subsequent osmotic swelling and disruption of the endosomes. This permits the escape of endocytosed PEI-DNA complexes. However, it is highly cytotoxic. Factors influencing cytotoxicity include:

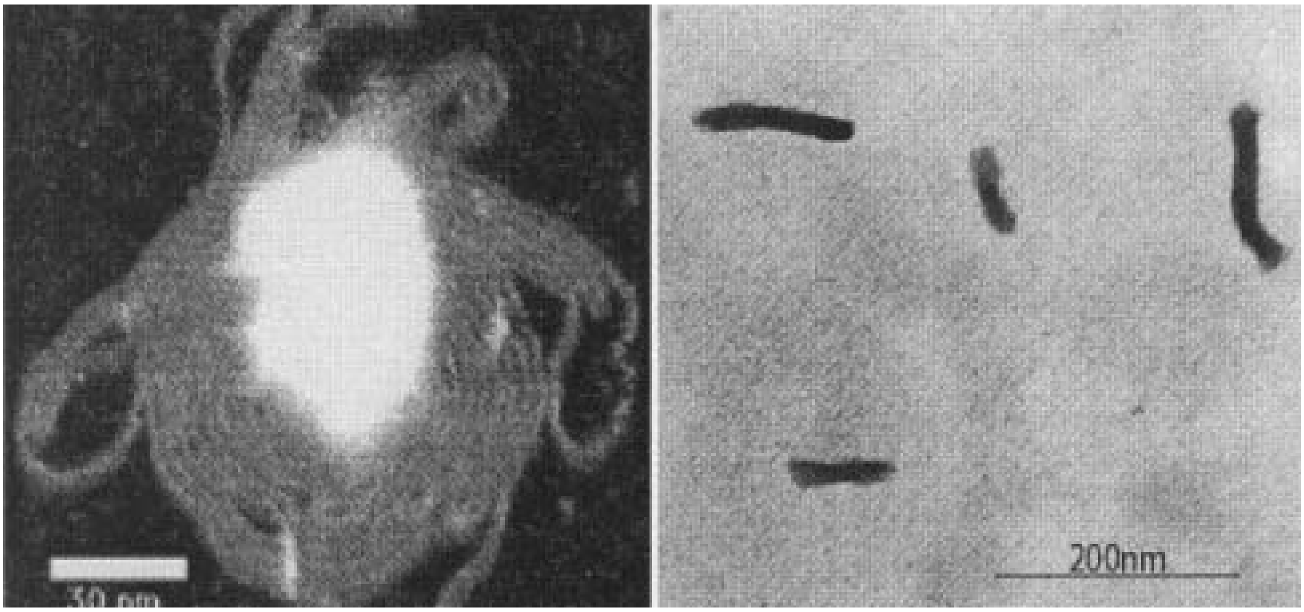

Fig.2.Electron micrographs of PLL-DNA (right) and partially condensed PEI-DNA (left) complexes. (Dunlap et al. 1997)

molecular weight, incubation time, concentration of cation, and density of the cationic group[49-51]. The toxic effect of PEI on cells can be reduced by conjugation with other polymers such as PEG[52] but it is insufficient for solving the cytotoxicity problem completely.

Physical Approaches

To date, there are three major physical approaches of gene delivery-"needle-free injection", electroporation and US.

(1) Needle-free injection

There are two devices developed that allow gene delivery by injection without needles. The first device, which is referred to as the "gene gun"[53], uses a high-pressure helium stream to deliver DNA, coated onto gold particles, directly into the cytoplasm. The efficiency of the gene gun is variable, and the duration of the expression is transient. The advantages of the gene gun, relative to some viral vectors, are that it can be used to transfer genes to nondividing cells and the DNA-gold beads are cheap and easy to prepare. The gene-gun delivery into the skin is a promising alternative to the injection of naked pDNA into muscle for genetic vaccinations[54].

The second device, called "jet gun", uses DNA-containing solution under high pressure for delivery into interstitial spaces. Jet injections of naked DNA may provide an option for keratinocyte gene therapy in the future[55].

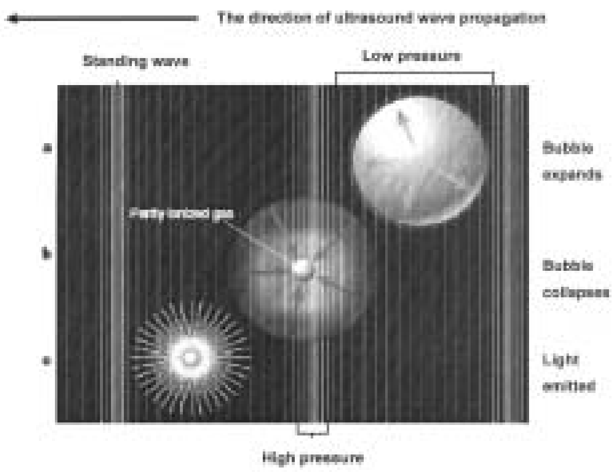

Fig.3.Pictorial representation of an inertial cavitation within a high intensity acoustic field. (Lohse 2005)

(2) Electroporation

Since 1982, the use of electric pulses for cell electroporation has been used to introduce foreign DNA into prokaryotic and eukaryotic cells in vitro[56]. Electroporation uses electrical fields to create transient pores in the cell membrane that allow the entry of normally impermeable macromolecules into the cytoplasm. To date, electroporation has been used in in vivo studies of gene transfer into skeletal muscle[20,57].

Ultrasound (US)-based Technologies in Gene Delivery

US waves are defined as mechanical sound waves that have a frequency above the audible sound of humans, generally about 20 kHz[58].

The principle of piezoelectricity is commonly applied to generate US waves. Piezoelectric materials can be used as ultrasonic transducers for medical purposes. The application of a rapidly alternating potential across a piezoelectric crystal induces corresponding alternating, dimensional changes, consequently converting electrical energy into sound waves. The direction of US wave propagation is the same as the direction of oscillation. The medium that the sound wave propagates through is alternately compressed ("compression" zone or "high pressure" zone, as shown in figure 3) and stretched ("rarefaction" zone or "low pressure" zone, as shown in figure 3), resulting in pressure variations in the medium.

(1) Bioeffects of ultrasound

The physical effects of US have been studied in vitro and in vivo. Its physical effects can be classified in two principal groups: thermal and mechanical. The mechanical group includes acoustic cavitation[59], acoustic microstreaming[60], and radiation pressure. Among these, acoustic cavitation is thought to be the most important bioeffect. Briefly, as the US waves propagate through the medium, the characteristic compression and rarefaction causes microscopic gas bubbles in the tissue fluid to contract and expand. Two types of cavitation are recognised. Gas body activation [61,62] or stable cavitation, is the term used to describe bubbles which oscillate in diameter with the passing pressure variations of the sound wave. Generally, in gas body activation, only a relatively low level of US intensity is demanded to activate a pre-existing gas body. Inertial cavitation[62] (figure 3) or transient cavitation, occurs when bubble oscillations are so large that the bubbles finally implode violently, producing pressure discontinuities (shock waves), free radicals, extremely high localised temperatures (at least 5000 K), pressures (up to 1200 bars) and light (sonoluminescence) (see figure 3).

(2) Fundamental parameters of ultrasound

The intensity of the US beam is one of the crucial parameters that determine the rate and extent of the thermal and non-thermal effects. Intensity (Watts/cm^2) refers to the amount of energy contained in a wave as it passes through any one point. More recently, the MI[63,64] has come into use as an indicator or predictor of possible biological responses to cavitation-related bioeffects.

The MI is defined as: $MI = P / \sqrt{f}$

Where f is the driving frequency in MHz and "P" is the peak rarefactional (negative) pressure (figure 4) in MPa. "P" is the amount of negative acoustic pressure within an US field and often used to describe the likelihood of causing a nucleus to undergo inertial cavitation in response to a series of US pulses.

(3) Applications of ultrasound in gene delivery

It is well known that USE can induce transient pore formation in the cell membranes[65-68]-sonoporation (see figure 5), allowing for access by proteins and other macromolecules. Sonoporation can be regarded to be the same as the promotion of membrane-permeability induced by US energy. Although researchers believe that non-thermal bioeffects (cavitation) play a crucial role in US-induced gene expression, the exact mechanism remains under investigation.

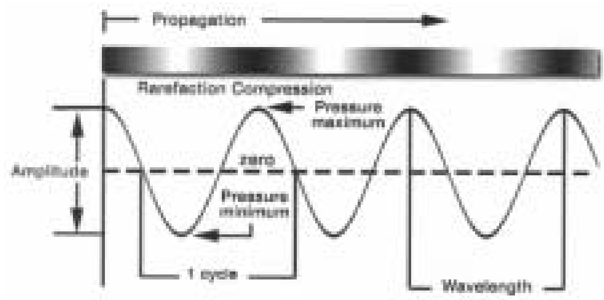

Fig.4. Schematic representation of a continuous ultrasound wave.
The peak US rarefaction pressure (pressure minimum) is proportional to MI.

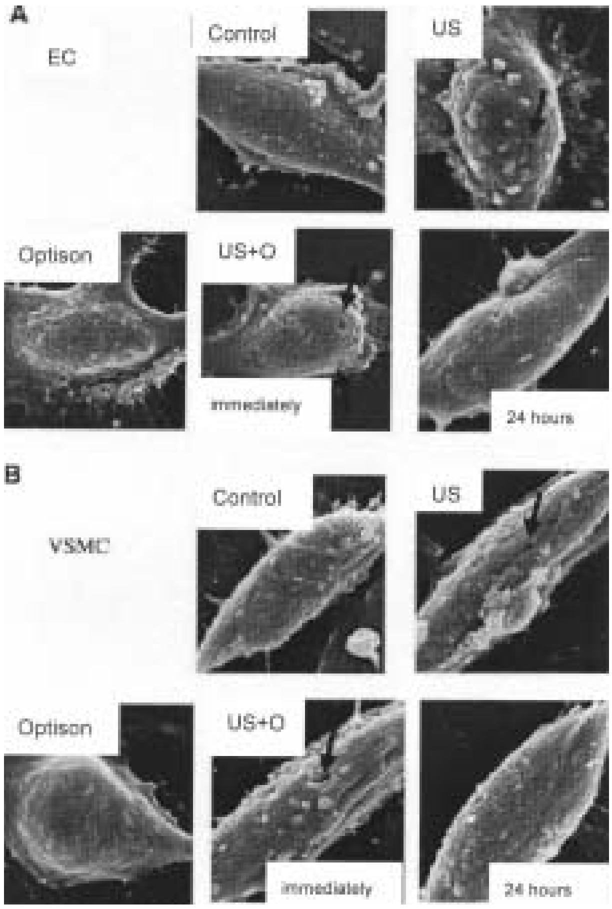

Fig.5. Electron microscopic views of ECs (A) and VSMCs (B) transfected with naked pDNA by means of ultrasound (US) and Optison (O). Arrows point out holes created by sonoporation at different time points. (Taniyama et al. 2002)

(4) In vitro applications of ultrasound in gene delivery

Naked pDNA is the simplest non-viral vector.

However, the phosphate group on the deoxyribose rings of DNA presents a net negative charge to the molecule, hampering its potential for electrostatic interaction with the anionic lipids in the cell membrane and causing a very low cellular uptake. Disadvantages in systemic gene delivery with naked DNA have also been found, since pDNA vector can be rapidly degraded and neutralised by endogenous DNases. Therefore, it is reasonable to combine naked pDNA delivery with another methods to improve the transgene efficiency. In 1987, Fechheimer et al. first demonstrated that US had potential as a tool of pDNA delivery into murine fibroblasts[69]. The first major investigation in this field came in 1996. Kim et al. studied the potential use of USE as a novel transfection method for laboratory use[70]. The maximal transfection rate was 2.4% of surviving primary chondrocytes when cell killing was ～50% of exposed cells. Lawrie et al. used a custom-built US transducer to expose cultures of porcine vascular smooth muscle cells (PVSMCs) and ECs to very low intensity 1 MHz US (0.1 MI, 0.4 Watts/cm^2)[71]. The result showed that USE for 1 minute (min) enhanced LUC transgene expression 48 h post transfection by 7.5 fold and 2.4 fold in PVSMCs, compared to naked plasmid transfection and lipofection respectively.

In 2005, Feril et al. investigated the effect of US (1 MHz) on liposome-mediated transfection, using three types of liposomes (L1, L2 and L3) containing DC-6-14, DOPE and cholesterol at varying ratios[72]. HeLa cells were treated with liposome (L1 or L2)-DNA complexes containing LUC plasmid for 2 h before USE (0.5 Watts/cm^2, 1 MHz for 1 min). LUC expression 24 h after USE were significantly increased by 2.4 fold with L1, and 1.7 fold with L2. The above important results suggested that US, even without adding MECA, could enhance gene delivery, possibly via cavitation.

(5) Microbubble echo contrast agents and their applications in gene delivery

The concept of US contrast imaging was introduced in the 1960s. It has significantly extended the use of US imaging during recent years thanks to a dramatic improvement in the stability, circulation time and echogenicity of microbubble echo contrast agents (MECAs). MECAs, due to their capability to increase the US backscatter signal from blood with minimal toxicity, have been applied in combination with conventional two-dimensional and Doppler imaging for diagnosing diseases and creating better images of the state of organs.

The ideal MECAs should be non-toxic, injectable intravenously, capable of crossing the pulmonary capillary bed after a peripheral injection, and stable enough to achieve enhancement for the duration of the examination. They are typically gas-encapsulated microbubbles around 1-10 μm in diameter[73,74]. Contrast agents have a gas core which is filled with air or a higher MW substance such as perfluoropropane with lower aqueous solubility. The surrounding shell can be stiff (e.g., denatured albumin) or more flexible (lipid or phospholipids), and the shell thickness can vary from 10-200 nm. Microbubbles have been shown to lower the energy threshold for cavitation by US energy and to have the potential of enhancing cavitation[75]. When US interacts with the MECAs leading to cavitation, pDNA and fragments of the microbubbles are driven across cell membranes into the target cells[76]. Therefore, acoustic cavitation is important in US-assisted gene delivery.

(6) In vivo applications of ultrasound in gene delivery

Recently, US gene delivery has been applied in several tumour cell lines, and in ECs and VSMCs[71,77]. In terms of transdermal delivery of various molecules in vitro and in vivo, US has shown an enhancing effect, including in vitro and in vivo delivery of insulin[78-80], glucose[81,82] and heparin[83].

Although these are promising in vitro findings, US-based gene delivery is still in its infancy. Since 1996, there have been several in vivo investigations concerning US-assisted gene delivery with or with-

Table 2.**In vivo (or ex vivo) ultrasound-assisted transfection.**

Model	Frequency/mode	Intensity (Watts/cm2)/MI	Enhancement	MECA	Reference
Mouse melanoma	-/lithotripter shock wave	-/-	8 fold compared to control	-	Miller et al. 1999[88]
MC38 murine colon cancer	1 MHz/CW	20/-	3 fold compared to control	-	Manome et al. 2000[89]
Rat prostate tumour	118 MHz/PW	0.3 - 833/0.01 - 0.46	10 fold - 15 fold compared to control	-	Huber et al. 2000[90]
Rat myocardium	1.3 MHz/PW	-/1.5	10 fold compared to control	Albumin-coated gas-filled MECA	Shohet et al. 2000[91]
Rabbit femoral artery	2 MHz/PW	50/-	12 fold compared to control	-	Amabile et al. 2001[92]
Porcine coronary artery (ex vivo)	2.2 - 4.4 MHz/CW	-/1.2	Significantly led to a increase in the expression of eNOS	DNA-loaded albumin MECA	Teupe et al. 2002[93]
Rabbit skeletal muscle	1 MHz/-	2.5/-	Significantly led to a increase in capillary density	Optison	Taniyama et al. 2002[67]
Rat carotic artery	1 MHz/-	-/-	Significantly led to a 50% reduction in intima/media ratio	Optison	Taniyama et al. 2002[68]
Rat kidney(ex vivo)	2 MHz/-	2.5/-	Significant prolongation of graft survival	Optison	Azuma et al.2003[94]
Mouse skeletal muscle	1 MHz/PW	3/-	50 fold compared to control	Optison	Lu et al. 2003[95]
Rat skeletal muscle	1.75 MHz/PW	-/1.9	200 fold compared to control	DNA-loaded Lipid-stabilised MECA	Christiansen et al. 2003[96]
Rat carotid artery	1 MHz/-	2.5/-	Significantly led to a 50% reduction in intima/media ratio	Optison	Hashiya et al. 2004[97]
Adult rat brain	1 MHz/CW	5/-	10 fold compared to control	Optison	Shimamura et al. 2004[98]
Rat myocardium	1.3 MHz/CW	-/1.5	6 fold compared to control	Lipid-stabilised MECA	Bekeredjian et al. 2005[99]
Porcine saphenous vein graft (ex vivo)	1 MHz/PW	-/1.8	Lumen and total vessel areas were significantly greater in the TIMI-3 group	BR14	Akowuah et al. 2005[100]
Rat myocardium	1.6 MHz/CW	-/1.6	Significantly increase capillary density	Lipid-stabilised MECA	Korpanty et al. 2005[101]
Mouse femoral artery	1 MHz/PW	1/-	Neointima/media areas were significantly reduced	MECA	Inagaki et al. 2006[102]

out microbubbles. The in vivo studies of US-assisted gene delivery are summarised in table 2.

Conclusion

There are two main reasons why gene therapy has not globally succeeded in the clinical setting: firstly, inefficient delivery of gene of interest to their correct sites of action, and secondly, safety concern of some viral-based vectors which are 1 - 3 orders of magnitude more efficient than conventional non-viral techniques in gene delivery in vivo. Many transfection methods are much less efficient in vivo than in vitro (such as liposome-mediated transfection). US has several potential advantages over other techniques, especially that it can be focused and in turn targeted to specific and, if necessary, to deep locations within the body. US gene delivery has been urged as an applicable tool through its bioeffects, especially cavitation.

Last but not least, in efforts to further improve the level of transgene expression, targeted gene delivery may be one of the promising methods that will work through US. In this regard, it would be feasible to design a targeted MECA that can selectively bind to the areas of interest in the tissue/body, either for a diagnostic or therapeutic purpose. These active targeting strategies can be achieved by the development of targeted microbubbles - by attaching antibodies or peptides to microbubble shells[84-87]. Therefore, the targeted microbubbles with a specific ligand to the target receptors that are expressed in the diseased area can be applied either for the purpose of attaining US imaging or for a potentially therapeutic purpose via US-induced cavitation. There is also the theoretical potential to load microbubbles with genetic material that is already condensed by polymers or liposomes, and also to modify the surface of the microbubbles. The important step required to develop this technique will be to load the ligand-modified MECA with polymer/liposome-condensed genetic material (such as pDNA) without compromising its stability and by eliminating the cytoxicity in vitro and in vivo. These "smart" microbubbles may be applied as specific contrast agents for US to improve diagnosis, and also as therapeutic agents in US-based gene delivery in clinical settings.

References

1. Russell SJ. Science, medicine, and the future. Gene therapy. BMJ 1997; 315: 1289-92.
2. Nabel EG, Plautz G, Boyce FM, Stanley JC, Nabel GJ. Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science 1989; 244: 1342-44.
3. Blaese RM, Culver KW, Miller AD, et al. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. Science 1995; 270: 475-80.
4. Lee M, Rentz J, Han SO, Bull DA, Kim SW. Water-soluble lipopolymer as an efficient carrier for gene delivery to myocardium. Gene Ther 2003; 10: 585-93.
5. Hamawy AH, Lee LY, Crystal RG, Rosengart TK. Cardiac angiogenesis and gene therapy: a strategy for myocardial revascularization. Curr Opin Cardiol 1999; 14: 515-22.
6. Davis HL, Whalen RG, Demeneix BA. Direct gene transfer into skeletal muscle in vivo: factors affecting efficiency of transfer and stability of expression. Hum Gene Ther 1993; 4: 151-9.
7. Rajagopalan S, Mohler E, 3rd, Lederman RJ, et al. Regional Angiogenesis with Vascular Endothelial Growth Factor (VEGF) in peripheral arterial disease: Design of the RAVE trial. Am Heart J 2003; 145: 1114-8.
8. Rissanen TT, Markkanen JE, Arve K, et al. Fibroblast growth factor 4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. Faseb J 2003; 17: 100-2.
9. Roy S, Rothschild JG, Chen A. Vascular complications and gene therapy. Expert Opin Biol Ther 2003; 3: 71-83.
10. Shyu KG, Chang H, Wang BW, Kuan P. Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia. Am J Med 2003; 114: 85-92.
11. Waehre T, Damas JK, Gullestad L, et al. Hydroxymethylglutaryl coenzyme a reductase inhibitors down-regulate chemokines and chemokine receptors in patients with coronary artery disease. J Am Coll Cardiol 2003; 41: 1460-7.
12. Garza L, Aude YW, Saucedo JF. Can we prevent in-stent restenosis? Curr Opin Cardiol 2002; 17: 518-25.
13. Klugherz BD, Song C, DeFelice S, et al. Gene delivery to pig coronary arteries from stents carrying antibody-tethered adenovirus. Hum Gene Ther 2002; 13: 443-54.
14. Autieri MV, Carbone C, Mu A. Expression of allograft inflammatory factor-1 is a marker of activated human vascular smooth muscle cells and arterial injury. Arterioscler Thromb Vasc Biol 2000; 20: 1737-44.
15. Kay MA, Liu D, Hoogerbrugge PM. Gene therapy. Proc Natl Acad Sci 1997; 94: 12744-6.
16. Niven R, Zhang Y, Smith J. Toward development of a non-viral gene therapeutic. Adv Drug Deliv Rev 1997; 26: 135-50.
17. Mountain A. Gene therapy: the first decade. Trends Biotechnol 2000;18:119-28.
18. Lehrman S. Virus treatment questioned after gene therapy death. Nature 1999; 401: 517-8.
19. Ledley FD. Non-viral gene therapy. Curr Opin Biotechnol 1994; 5: 626-36.
20. Mir LM, Bureau MF, Gehl J, et al. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci 1999; 96: 4262-7.
21. Rizzuto G, Cappelletti M, Maione D, et al. Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation. Proc Natl Acad Sci 1999; 96: 6417-22.
22. Scherman D, Bigey P, Bureau MF. Applications of plasmid electrotransfer. Technol Cancer Res Treat 2002; 1: 351-4.
23. Fattori E, La Monica N, Ciliberto G, Toniatti C. Electro-gene-transfer: a new approach for muscle gene delivery. Somat Cell Mol Genet 2002; 27: 75-83.
24. Felgner PL, Gadek TR, Holm M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci 1987; 84: 7413-7.
25. Lleres D, Dauty E, Behr JP, Mely Y, Duportail G. DNA condensation by an oxidizable cationic detergent. Interactions with lipid vesicles. Chem Phys Lipids 2001; 111: 59-71.
26. Dauty E, Remy JS, Blessing T, Behr JP. Dimerizable cationic detergents with a low cmc condense plasmid DNA into nanometric particles and transfect cells in culture. J Am Chem Soc

2001; 123: 9227-34.
27.Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem 1995; 270: 18997-9007.
28.Felgner JH, Kumar R, Sridhar CN, et al. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem 1994; 269: 2550-61.
29.Legendre JY, Szoka FC, Jr. Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. Pharm Res 1992; 9: 1235-42.
30.Turunen MP, Hiltunen MO, Ruponen M, et al. Efficient adventitial gene delivery to rabbit carotid artery with cationic polymer-plasmid complexes. Gene Ther 1999; 6: 6-11.
31.Horiguchi Y, Larchian WA, Kaplinsky R, Fair WR, Heston WD. Intravesical liposome-mediated interleukin-2 gene therapy in orthotopic murine bladder cancer model. Gene Ther 2000; 7: 844-51.
32.Domashenko A, Gupta S, Cotsarelis G. Efficient delivery of transgenes to human hair follicle progenitor cells using topical lipoplex. Nat Biotechnol 2000;18:420-3.
33.Kwoh DY, Coffin CC, Lollo CP, et al. Stabilization of poly-L-lysine/DNA polyplexes for in vivo gene delivery to the liver. Biochim Biophys Acta 1999; 1444: 171-90.
34.Richardson SC, Kolbe HV, Duncan R. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. Int J Pharm 1999; 178: 231-43.
35.Gonsho A, Irie K, Susaki H, Iwasawa H, Okuno S, Sugawara T. Tissue-targeting ability of saccharide-poly(L-lysine) conjugates. Biol Pharm Bull 1994; 17: 275-82.
36.Liu G, Molas M, Grossmann GA, et al. Biological properties of poly-L-lysine-DNA complexes generated by cooperative binding of the polycation. J Biol Chem 2001; 276: 34379-87.
37.Hansma HG, Golan R, Hsieh W, Lollo CP, Mullen-Ley P, Kwoh D. DNA condensation for gene therapy as monitored by atomic force microscopy. Nucleic Acids Res 1998; 26: 2481-7.
38.Gao X, Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry 1996; 35: 1027-36.
39.Baeza I, Gariglio P, Rangel LM, et al. Electron microscopy and biochemical properties of polyamine-compacted DNA. Biochemistry 1987; 26: 6387-92.
40.Stankovics J, Crane AM, Andrews E, Wu CH, Wu GY, Ledley FD. Overexpression of human methylmalonyl CoA mutase in mice after in vivo gene transfer with asialoglycoprotein/polylysine/DNA complexes. Hum Gene Ther 1994; 5: 1095-104.
41.Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc Natl Acad Sci 1992; 89: 7934-8.
42.Xu B, Wiehle S, Roth JA, Cristiano RJ. The contribution of poly-L-lysine, epidermal growth factor and streptavidin to EGF/PLL/DNA polyplex formation. Gene Ther 1998; 5: 1235-43.
43.Wagner E, Cotten M, Foisner R, Birnstiel ML. Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc Natl Acad Sci 1991; 88: 4255-9.
44.Ferkol T, Pellicena-Palle A, Eckman E, et al. Immunologic responses to gene transfer into mice via the polymeric immunoglobulin receptor. Gene Ther 1996; 3: 669-78.
45.Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. J Control Release 1999; 60: 149-60.
46.Dunlap DD, Maggi A, Soria MR, Monaco L. Nanoscopic structure of DNA condensed for gene delivery. Nucleic Acids Res 1997; 25: 3095-101.
47.Boussif O, Lezoualc'h F, Zanta MA, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci 1995; 92: 7297-301.
48.Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. J Biomed Mater Res 2000; 51: 321-8.
49.Ferrari S, Moro E, Pettenazzo A, Behr JP, Zacchello F, Scarpa M. ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. Gene Ther 1997; 4: 1100-6.
50.Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res 1999; 16: 1273-9.
51.Jeong JH, Song SH, Lim DW, Lee H, Park TG. DNA transfection using linear poly(ethylenimine) prepared by controlled acid hydrolysis of poly(2-ethyl-2-oxazoline). J Control Release 2001; 73: 391-9.
52.Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther 1999; 6: 595-605.
53.Yang NS, Burkholder J, Roberts B, Martinell B, McCabe D. In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. Proc Natl Acad Sci 1990; 87: 9568-72.
54.Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD, Jr. DNA-based immunization by in vivo transfection of dendritic cells. Nat Med 1996; 2: 1122-8.
55.Sawamura D, Ina S, Itai K, et al. In vivo gene introduction into keratinocytes using jet injection. Gene Ther 1999; 6: 1785-7.
56.Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. Embo J 1982; 1: 841-5.
57.Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. Gene Ther 1999; 6: 508-14.
58.Yu T, Wang Z, Mason TJ. A review of research into the uses of low level ultrasound in cancer therapy. Ultrason Sonochem 2004; 11: 95-103.
59.Apfel RE. Acoustic cavitation: a possible consequence of biomedical uses of ultrasound. Br J Cancer 1982; 45: 140-6.
60.Nyborg WL. Ultrasonic microstreaming and related phenomena. Br J Cancer 1982; 45: 156-60.

61. Church CC, Carstensen EL. "Stable" inertial cavitation. Ultrasound Med Biol 2001; 27: 1435-7.
62. Miller MW, Miller DL, Brayman AA. A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective. Ultrasound Med Biol 1996; 22: 1131-54.
63. Apfel RE, Holland CK. Gauging the likelihood of cavitation from short-pulse, low-duty cycle diagnostic ultrasound. Ultrasound Med Biol 1991; 17: 179-85.
64. Meltzer RS. Food and Drug Administration ultrasound device regulation: the output display standard, the "mechanical index," and ultrasound safety. J Am Soc Echocardiogr 1996; 9: 216-20.
65. McNeil PL. Incorporation of macromolecules into living cells. Methods Cell Biol 1989; 29: 153-73.
66. Tachibana K, Uchida T, Ogawa K, Yamashita N, Tamura K. Induction of cell-membrane porosity by ultrasound. Lancet 1999; 353: 1409.
67. Taniyama Y, Tachibana K, Hiraoka K, et al. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther 2002; 9: 372-80.
68. Taniyama Y, Tachibana K, Hiraoka K, et al. Local delivery of plasmid DNA into rat carotid artery using ultrasound. Circulation 2002; 105: 1233-9.
69. Fechheimer M, Boylan JF, Parker S, Sisken JE, Patel GL, Zimmer SG. Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading. Proc Natl Acad Sci 1987; 84: 8463-7.
70. Kim HJ, Greenleaf JF, Kinnick RR, Bronk JT, Bolander ME. Ultrasound-mediated transfection of mammalian cells. Hum Gene Ther 1996; 7: 1339-46.
71. Lawrie A, Brisken AF, Francis SE, et al. Ultrasound enhances reporter gene expression after transfection of vascular cells in vitro. Circulation 1999; 99: 2617-20.
72. Feril LB, Jr., Ogawa R, Kobayashi H, Kikuchi H, Kondo T. Ultrasound enhances liposome-mediated gene transfection. Ultrason Sonochem 2005; 12: 489-93.
73. Chen WS, Matula TJ, Crum LA. The disappearance of ultrasound contrast bubbles: observations of bubble dissolution and cavitation nucleation. Ultrasound Med Biol 2002; 28: 793-803.
74. Allen JS, May DJ, Ferrara KW. Dynamics of therapeutic ultrasound contrast agents. Ultrasound Med Biol 2002; 28: 805-16.
75. Feril LB, Jr., Kondo T, Zhao QL, et al. Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents. Ultrasound Med Biol 2003; 29: 331-7.
76. Vannan M, McCreery T, Li P, et al. Ultrasound-mediated transfection of canine myocardium by intravenous administration of cationic microbubble-linked plasmid DNA. J Am Soc Echocardiogr 2002; 15: 214-8.
77. Alter A, Rozenszajn LA, Miller HI, Rosenschein U. Ultrasound inhibits the adhesion and migration of smooth muscle cells in vitro. Ultrasound Med Biol 1998; 24: 711-21.
78. Boucaud A, Garrigue MA, Machet L, Vaillant L, Patat F. Effect of sonication parameters on transdermal delivery of insulin to hairless rats. J Control Release 2002; 81: 113-9.
79. Kost J. Ultrasound-assisted insulin delivery and noninvasive glucose sensing. Diabetes Technol Ther 2002; 4: 489-97.
80. Smith NB, Lee S, Shung KK. Ultrasound-mediated transdermal in vivo transport of insulin with low-profile cymbal arrays. Ultrasound Med Biol 2003; 29: 1205-10.
81. Merino G, Kalia YN, Delgado-Charro MB, Potts RO, Guy RH. Frequency and thermal effects on the enhancement of transdermal transport by sonophoresis. J Control Release 2003; 88: 85-94.
82. Mitragotri S, Kost J. Low-frequency sonophoresis: a noninvasive method of drug delivery and diagnostics. Biotechnol Prog 2000; 16: 488-92.
83. Mitragotri S, Kost J. Transdermal delivery of heparin and low-molecular weight heparin using low-frequency ultrasound. Pharm Res 2001; 18: 1151-6.
84. Villanueva FS, Jankowski RJ, Klibanov S, et al. Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells. Circulation 1998; 98: 1-5.
85. Lanza GM, Wallace KD, Scott MJ, et al. A novel site-targeted ultrasonic contrast agent with broad biomedical application. Circulation 1996; 94: 3334-40.
86. Klibanov AL. Targeted delivery of gas-filled microspheres, contrast agents for ultrasound imaging. Adv Drug Deliv Rev 1999; 37: 139-57.
87. Bian AN, Gao YH, Tan KB, et al. Preparation of human hepatocellular carcinoma-targeted liposome microbubbles and their immunological properties. World J Gastroenterol 2004; 10: 3424-7.
88. Miller DL, Bao S, Gies RA, Thrall BD. Ultrasonic enhancement of gene transfection in murine melanoma tumors. Ultrasound Med Biol 1999; 25: 1425-30.
89. Manome Y, Nakamura M, Ohno T, Furuhata H. Ultrasound facilitates transduction of naked plasmid DNA into colon carcinoma cells in vitro and in vivo. Hum Gene Ther 2000; 11: 1521-8.
90. Huber PE, Pfisterer P. In vitro and in vivo transfection of plasmid DNA in the Dunning prostate tumor R3327-AT1 is enhanced by focused ultrasound. Gene Ther 2000; 7: 1516-25.
91. Shohet RV, Chen S, Zhou YT, et al. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. Circulation 2000; 101: 2554-6.
92. Amabile PG, Waugh JM, Lewis TN, Elkins CJ, Janas W, Dake MD. High-efficiency endovascular gene delivery via therapeutic ultrasound. J Am Coll Cardiol 2001; 37: 1975-80.
93. Teupe C, Richter S, Fisslthaler B, et al. Vascular gene transfer of phosphomimetic endothelial nitric oxide synthase (S1177D) using ultrasound-enhanced destruction of plasmid-loaded microbubbles improves vasoreactivity. Circulation 2002; 105: 1104-9.
94. Azuma H, Tomita N, Kaneda Y, et al. Transfection of NFkappaB-decoy oligodeoxynucleotides using efficient ultrasound-mediated gene transfer into donor kidneys prolonged survival of rat renal allografts. Gene Ther 2003; 10: 415-25.
95. Lu QL, Liang HD, Partridge T, Blomley MJ. Microbubble ul-

trasound improves the efficiency of gene transduction in skeletal muscle in vivo with reduced tissue damage. Gene Ther 2003; 10: 396-405.

96. Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. Ultrasound Med Biol 2003; 29: 1759-67.

97. Hashiya N, Aoki M, Tachibana K, et al. Local delivery of E2F decoy oligodeoxynucleotides using ultrasound with microbubble agent (Optison) inhibits intimal hyperplasia after balloon injury in rat carotid artery model. Biochem Biophys Res Commun 2004; 317: 508-14.

98. Shimamura M, Sato N, Taniyama Y, et al. Development of efficient plasmid DNA transfer into adult rat central nervous system using microbubble-enhanced ultrasound. Gene Ther 2004; 11: 1532-9.

99. Bekeredjian R, Chen S, Grayburn PA, Shohet RV. Augmentation of cardiac protein delivery using ultrasound targeted microbubble destruction. Ultrasound Med Biol 2005; 31: 687-91.

100. Akowuah EF, Gray C, Lawrie A, et al. Ultrasound-mediated delivery of TIMP-3 plasmid DNA into saphenous vein leads to increased lumen size in a porcine interposition graft model. Gene Ther 2005; 12: 1154-57.

101. Korpanty G, Chen S, Shohet RV, et al. Targeting of VEGF-mediated angiogenesis to rat myocardium using ultrasonic destruction of microbubbles. Gene Ther 2005; 12: 1305-12.

102. Inagaki H, Suzuki J, Ogawa M, Taniyama Y, Morishita R, Isobe M. Ultrasound-microbubble-mediated NF-kappaB decoy transfection attenuates neointimal formation after arterial injury in mice. J Vasc Res 2006; 43: 12-8.

現代超音波及非病毒性基因治療科技

蘇正煌[2,3] 葉宏一[1,2,3] 侯嘉殷[2] 周友三[2] 蔡正河[1,2,3]

[1] 台北醫學大學

[2] 台北馬偕紀念醫院 心臟內科

[3] 馬偕醫護管理專科學校

摘 要

所謂的基因治療(gene therapy)是指利用分子生物學中DNA 重組(DNA recombination)以及轉殖(transgenetic)的技術，把重組後之DNA 分子傳送至體細胞內，以達成預防或治療疾病的現代醫療科技。由於病毒載體(viral vector)於生物體內不可預知的毒性及免疫反應，所以非病毒載體(non-viral vector)，如聚合物(polymer)，成爲另一種載體選擇。超音波由於其物理特性-超音波性空洞形成(acoustic cavitation)，尤其是瞬時超音波空洞現象(inertial cavitation)，可增強大分子如質體DNA(plasmid DNA)對細胞膜的通透性，並在細胞膜上形成顯微小孔。超音波用微氣泡(ultrasound microbubble contrast agent)更能進一步降低超音波輸出能量，減少細胞毒性。超音波基因傳送(gene delivery)合併應用微氣泡及非病毒載體，如聚合物，可進一步增強基因轉移效能。根據上述觀察，本文描述基因傳送過程中可能經歷之障礙及可運用之載體與傳送方法，並介紹目前超音波基因傳送的細胞及動物模式，提供臨床醫師及研究工作者另一種思考。