Nonviral gene therapy targeting cardiovascular system

Cheng-Huang Su,1,2 Yih-Jer Wu,1,3 Hsueh-Hsiao Wang,3 and Hung-I Yeh1,3

Departments of Internal Medicine and Medical Research, Mackay Memorial Hospital, New Taipei City, Taiwan; 2Mackay Medicine, Nursing and Management College, New Taipei City, Taiwan; 3Mackay Medical College, New Taipei City, Taiwan

Submitted 15 February 2012; accepted in final form 17 July 2012

Su C, Wu Y, Wang H, Yeh H. Nonviral gene therapy targeting cardiovascular system. Am J Physiol Heart Circ Physiol 303: H629–H638, 2012. First published July 20, 2012; doi:10.1152/ajpheart.00126.2012.—The goal of gene therapy is either to introduce a therapeutic gene into or replace a defective gene in an individual’s cells and tissues. Gene therapy has been urged as a potential method to induce therapeutic angiogenesis in ischemic myocardium and peripheral tissues after extensive investigation in recent preclinical and clinical studies. A successful gene therapy mainly relies on the development of the gene delivery vector. Developments in viral and nonviral vector technology including cell-based gene transfer will further improve transgene delivery and expression efficiency. Nonviral approaches as alternative gene delivery vehicles to viral vectors have received significant attention. Recently, a simple and safe approach of gene delivery into target cells using naked DNA has been improved by combining several techniques. Among the physical approaches, ultrasonic microbubble gene delivery, with its high safety profile, low costs, and repeatable applicability, can increase the permeability of cell membrane to macromolecules such as plasmid DNA by its bioeffects and can provide as a feasible tool in gene delivery. On the other hand, among the promising areas for gene therapy in acquired diseases, ischemic cardiovascular diseases have been widely studied. As a result, gene therapy using advanced technology may play an important role in this regard. The aims of this review focus on understanding the cellular and in vivo barriers in gene transfer and provide an overview of currently used chemical vectors and physical tools that are applied in nonviral cardiovascular gene transfer.

gene therapy; vector; ultrasound; cavitation; microbubble

Overview of Applications of Gene Therapy

Gene therapy is a novel approach that can be applied through a variety of clinical therapeutic procedures in which nucleic acids are introduced into human cells for the purpose of treating, curing, or ultimately preventing disease. The development of gene vectors for effectively carrying genes into cells has made a great deal of progress in recent years. Nonviral vectors should have a better chance to circumvent some of the problems possibly occurring with viral vectors, such as unwanted immune response and oncogenesis. In general, although nonviral vectors, such as plasmid DNA, allow remarkable organ specificity, they are often limited by low transfection efficiency (TE). In contrast, virus-based vectors deliver the transgene more efficiently, but organ specificity may be reduced and immunogenetic properties can limit their practical applications. Both systems need dose optimization and development of strategies to improve vector technology. Thus far, the main categories of methods that have been used to deliver genes into cells or tissues in gene therapy protocols are as follows: viral vectors (68.8%), nonviral chemical vectors (6.4%), naked/plasmid DNA (18.6%), and physical delivery systems (0.3%); data adapted from http://www.wiley.com/legacy/wileychi/genmed/clinical/, clinical trials website of The Journal of Gene Medicine, updated June 2011). In the present review, we summarized the progress made in the past few years regarding techniques developed in the following two areas: 1) chemical carrier-mediated gene delivery, such as cationic polymer and lipid, and 2) naked DNA transfection by a physical method, such as ultrasound (US) along with microbubbles. In addition, the nonviral carrier and technology of cardiovascular gene delivery are briefly overviewed, while the applications to the biomedical researches including regenerative medicine are introduced.

Candidate Diseases for Gene Therapy

There are several promising areas for gene therapy in genetic and acquired diseases. Thus far, more than 1,500 clinical trials have taken place worldwide. The diseases most commonly treated with gene therapy are cancer (64.6%), cardiovascular diseases (8.5%), monogenic diseases (8.3%), and infectious diseases (8.2%). Among all these areas, cancer is the most prominent one in clinical trials. Cancer was also the first disease in which a genetic therapy was approved by the US Food and Drug Administration (FDA) in 2000. Other areas that may be considered in future clinical trials include the treatment of degenerative diseases, cardiovascular diseases, and infectious diseases.
Cardiovascular disease is among the leading cause of morbidity and mortality in the developed world. The first investigation of vascular gene transfer was demonstrated in 1989 by Nabel et al. (73), who transfected porcine endothelial cells ex vivo with a retrovirus carrying the β-galactosidase gene and reintroduced the cells onto the denuded iliofemoral artery of a syngeneic pig. Since then, recombinant viruses have been used for gene transfer in most clinical studies. However, the death of a 18-year-old boy (54), who died of complications from a massive inflammatory response shortly after receiving a dose of adenovirus carrying a corrective gene in a phase I trial in 1999, illustrates the challenge in gene therapy. The mechanisms for this catastrophic reaction have never been completely understood, but a dose-specific adverse effect or a previous immunization due to a viral infection has been hypothesized as a potential cause. The future choice of viral or nonviral vectors will critically depend on specific purposes in gene therapy, potential adverse effects, and further improvements in vector design, though this review will focus on nonviral methods by which clinical or preclinical trials have shown feasibility into cardiovascular diseases.

Among those diseases related to aging process, gene therapy may provide an alternative treatment option in peripheral artery disease (PAD) and coronary artery disease (CAD). Neovascularization that plays a critical role in PAD and CAD involves complex processes that include the coordination of cytokines to induce new conduits of blood supply. Among these events, angiogenesis involves enhanced vascular permeability, vascular cell migration and proliferation, and collateral vessel formation (34). In patients with ischemic cardiovascular diseases, progressive occlusion of arteries often results in the formation of collateral vessels that supply the ischemic tissues. However, the natural compensatory development of neovascularization is often incomplete partly due to insufficient availability of angiogenic factors. Therapeutic angiogenesis is a method based on direct administration of angiogenic agents such as recombinant protein, indirect delivery of target genes that encode relevant proteins, or use of stem cells that have been shown to secret angiogenic factors and enhance angiogenesis in preclinical and clinical studies (26, 37). Clinical trials of the vascular endothelial growth factor (VEGF) gene was started in 1994 by Isner et al. (37). Since then, several angiogenic cytokines have been investigated in clinical trials. Administration of proper angiogenic proteins with controllable therapeutic levels and pharmacokinetics are required in this regard. Since the limited half-life of angiogenic proteins restricts the action duration, development of slow-release delivery systems to prolong the action may be more effective. In addition, multifactor therapy may be necessary to achieve adequate angiogenesis and provide functionally significant improvements in human tissue perfusion because of the sophisticated angiogenic process (103). Regarding angiogenic gene therapy, induction of inflammatory and immune responses, low efficiency of gene expression, and nonspecific gene transfer to other cell types with uncontrolled levels of growth factor are limiting factors (51).

Advantages of gene therapy in angiogenesis included persistent expression of the angiogenic factor with prolonged, local action. So far, several genes of growth factors and transcription factors have been tried to achieve therapeutic angiogenesis. Among the target genes that encode angiogenic factors used in clinical trials for CAD and PAD, VEGF (121 and 165 amino-acid isoforms of VEGF1 and VEGF2) and fibroblast growth factor (FGF, FGF1, and FGF4) families are relatively well investigated. VEGF and FGF in gene delivery have been shown to induce functionally significant angiogenesis in preclinical (19, 41) and clinical studies (53, 79, 91) for ischemic diseases. Although some studies showed no benefit or inconsistent results of growth factor gene therapy, designs may need to be further optimized with proper patient selection and growth factor delivery (40, 93). Another strategy investigated in angiogenic therapy is based on the transcription factor hypoxia-inducible factor-1α (82) by which the expression of several angiogenic genes, including VEGF and the VEGF receptors KDR/FLT-1, are regulated. Additionally, a factor associated with regulation of angiogenesis is the peptide PR-39, which is a proline- and arginine-rich peptide and increases the cellular levels of hypoxia-inducible factor-1α by inhibiting its proteosomal degradation (2). Although it is possible that nonspecific action is related to charge (55), PR-39 has been shown to enhance the expression of VEGF, its receptors KDR and FLT-1, and the FGF receptor 1.

For treatment of cardiovascular diseases, in addition to angiogenic genes, there are important nonangiogenic genes or nucleotides related to cell survival, such as genes associated with reduction-oxidation (redox) reactions and microRNAs (miRNAs). Redox regulation is an essential physiological process in the cell survival of almost all types of cells, including cardiomyocytes. Loss of cellular redox balance results in the development of oxidative stress in the cells, causing cellular dysfunction (12). There are critical antioxidant systems in cardiomyocytes to balance the oxidative stress: thioredoxin (Trx), glutaredoxin (Gx), and peroxiredoxin (Prx), all of which participate in redox regulation to protect the cells from oxidative stress and to antagonize apoptotic signaling (6). Overexpression of mitochondrial Prx-3 in mouse model showed resistance to ventricular remodeling and failure after myocardial infarction (68). Therapeutic strategies designed to attenuate mitochondrial oxidative stress such as enhanced expression of the antioxidant Prx-3 might be beneficial in preventing cardiac failure. Overexpression of both Gx-1 and Gx-2 rendered the heart resistant to ischemia-reperfusion injury and implicates a role in cardioprotection and redox signaling of the ischemic myocardium (66, 75). The above findings may suggest that Trx, Gx-, and Prx- are potential treatment targets for myocardial salvage in conditions associated with overwhelming oxidative stress. miRNAs consist of noncoding single-stranded RNAs of ~22 nucleotides that negatively regulate gene expression via degradation or translational inhibition of their target mRNAs. They have been implicated in the regulation of a diverse spectrum of cardiac functions, including cardiovascular cell differentiation, growth, proliferation, and apoptosis (50, 101). Among human miRNA genes, miRNA-21 was reported to be a mediator of extracellular signal-regulated kinase/mitogen-activated protein kinase signaling and is crucial for fibroblast survival and activation (43, 102). Further analysis showed that miRNA-21 is predominantly overexpressed in cardiac fibroblasts, especially those existing in the failing heart (83, 102). Moreover, in 2011, functional recovery and engraftment of transplanted cardiac progenitor cells that were transduced with lentivirus carrying a miRNA prosurvival cocktail (miRNA-21,
-24, and -221) can be improved in a murine model of myocardial infarction (36). Therefore, miRNAs may be a gene therapy target to promote cell survival in heart failure by regulating other genes.

Current Nonviral Gene-transfer Systems

Extra- and intracellular barriers for a successful gene delivery. A feasible gene therapy vector needs to meet three criteria: safety, adequate gene transfer efficiency, as well as stable expression of the transgene for a duration appropriate for treating the disease. Nonviral methods may only cause transient transfection and lack the ability to integrate into the genome that limit their use in conditions requiring long-term gene expression such as chronic heart failure. However, it is possible that transient expression of therapeutic genes can be favorable in certain applications. One such example is in bone healing, in which bone morphogenetic protein-2 just needs to express only for a few days because it triggers a cascade of activity that does not end even if it is withdrawn (56, 57). Moreover, nonviral vectors, due to anatomical and cellular barriers, limit the overall efficiency in gene therapy. There are biological obstacles impeding the nuclear accumulation of pDNA or DNA-vector complexes that need to be overcome: cell binding, cell entry/endocytosis, endosome escape, cytoplasmic transport, and nuclear entry (Fig. 1). Most of the chemical vectors compact DNA into particles by neutralizing the anionic DNA phosphate backbone and balancing charge repulsion force from the anionic cell surface (67). Considering a normal intracellular trafficking, after DNA-vector complexes interact with the cell membrane, they are endocytosed (97). This step may be nonspecific (mediated via electrostatic interactions) or may involve a specific target (using an antibody conjugates) (98). The vector should be designed to allow the DNA-vector complex to effectively escape from the endosome into the cytoplasm to protect it from the subsequent lysosomal degradation, and the vector should be capable of transporting its cargo to the nucleus. Additionally, there are many factors influencing the efficacy and safety of nonviral vector-mediated gene transfer, such as the charge of the vector, formulation of DNA/vector complexes (42), and interaction with serum and blood cells (85). Unfortunately, the ideal gene delivery systems are still under investigation. The potential nonviral vectors and physical approaches are summarized in Table 1 in terms of their key mechanisms, diseases/status, limitations, and advantages.

Nonviral vectors. The safety concerns associated with viral vectors have encouraged the development of nonviral vectors. pDNA delivered by nonviral methods is maintained in an extrachromosomal site, not integrated into the cellular genome. The most popular materials used in current nonviral applications include purified pDNA, chemical vectors such as lipids and synthetic polymers. Nonviral methods of gene delivery used in current clinical trials include pDNA and liposomal complexes.

DIRECT GENE TRANSFER USING PLASMID DNA. The simplest and safest nonviral gene delivery system currently in use in vivo is the direct gene transfer with naked pDNA. However, as the rapid degradation by nucleases and the clearance by the mononuclear phagocyte system in the systemic circulation, expression levels after the injection of naked DNA are generally limited. In 1990, Wolff et al. (108) first reported that intramuscularly injected naked pDNA can be expressed in myofibers. One of the promising approaches in this field is the combined use of naked DNA and physical approaches (such as electroporation) to enhance plasmid-mediated gene expression in muscle (20, 87). In the past 10 years, intramuscular injection of pDNA has been applied in a strategy of therapeutic angiogenesis (96). Isner and colleagues have used VEGF to enhance the formation of collateral blood vessels around occluded native arteries. Two catheter-based trials of myocardial gene delivery using pDNA encoding VEGF-2 have been reported (63, 105). In 2011, an open-label clinical study investigated the efficacy and safety of intramuscular injection of naked pDNA encoding the human hepatocyte growth factor (HGF) gene in Japanese patients with Buerger’s disease (89). The size of ischemic ulcers decreased in 6/9 (66.7%) patients, and the ulcers healed completely in 5/9 (55.6%) patients after gene therapy. The investigators concluded that HGF gene therapy is safe and effective for treating critical limb ischemia.

LIPID-BASED GENE DELIVERY SYSTEMS. First reported by Felgner et al. in 1987 (23), lipid-based gene delivery is still one of the major systems to increase the TE of naked DNA (15). Liposomes or lipoplexes are formed by DNA with positively charged lipids and detergents (16, 60). In lipoplex structure, DNA backbones are surrounded with positively charged lipids to form condensed complexes that possibly protect them...
against extracellular or intracellular nucleases. The resulting net-positive charge of lipid-DNA complexes may facilitate fusion with the negatively charged molecules of the cell membrane (glycoproteins and proteoglycans) that may subsequently facilitate their cellular uptake by endocytosis (112). Many cationic molecules cannot form liposomes alone and are normally accompanied by a neutral lipid (colipid or helper lipids), such as dioleoylphosphatidylethanolamine (DOPE) (22). DOPE promotes hexagonal-phase lipid polymorphism that is related to membrane fusion and increase in TE in vitro (47). In addition, most of the cationic lipids are more or less toxic to cells, and the presence of DOPE could lead to reduced charge ratio and less toxicity. Cationic lipids generally have the advantages of being inexpensive and can be engineered to have targeted specificity. However, because of the excessive surface charge, the half-life of cationic lipids in circulation is very short. Elimination of cationic lipids in systemic circulation occurs upon the formation of larger aggregates because of their interactions with the negatively charged serum molecules. Lipoplexes tend to initially accumulate in the pulmonary vasculature because of the first passage effect (58). As a result, cells that are transfected are mainly pulmonary vascular and endothelial cells. The drawback of a fast clearance of cationic lipids from the circulation limits their use to transfer gene to cells located beyond vascular endothelial cells. Surface shielding or modification of DNA-vector complex through the use of hydrophilic and charge neutral polymers such as polyethylene glycol (PEG) to reduce excessive charge-charge interaction appears very effective in prolonging the circulation half-life of lipoplexes (32). However, it is noted that the presence of the PEG moiety on the surface of lipoplexes reduces an interaction between lipoplexes and cell membrane and, as a result, reduces the TE. Several strategies have been used to make the PEG shielding conditional and nonpermanent, such as PEG-lipid conjugates. Other drawbacks of lipoplex-mediated gene delivery are short duration of gene expression and acute toxicity. When combined with unmethylated cytosine-phosphate guanosine-containing DNA, cationic lipids can stimulate potent inflammatory response in the hosts. Surface shielding with PEG-lipid and systematic deletions and mutations of cytosine-phosphate-guanosine-containing DNA sequences from plasmid sequences have resulted in some promising data in suppressing cytokine production (8). Delivery of growth factors by liposomes has been reported to be effective in animal models of angiogenesis (3, 65). In 2009, Hedman et al. (33) studied the long-term effects (a total follow-up time of 8.1 years) and safety of the local VEGF-A catheter-based gene transfer in 103 patients with CAD between three groups (adenoviral vector, liposome vector, or control) (33). The results showed that local intracoronary VEGF gene transfer is safe and does not cause an increased risk of major adverse cardiovascular events, cancer, or diabetes. More recently, in 2011, Muona et al. (71) examined the effects of VEGF on 25 patients having received VEGF-A gene transfer for the treatment of symptomatic PAD (71). There were no significant differences between study groups in the causes of death or in the incidence of cancer or diabetic retinopathy.

### Polymers

Polymer-based gene delivery systems. The principle of cationic polymers as nonviral DNA vehicles is based on the concept of forming condensed DNA particles by complex formation with cationic polymers-poloplexes. The use of polycationic polymers leads to electrostatic neutralization of anionic charges of DNA and condenses the polynucleotide structure of DNA into nanosized complexes, thereby protecting it from nuclease digestion and facilitating the cellular uptake through endocytosis.

Many polycationic molecules are used, including poly-L-lysine (PLL), dendrimers, polornithine, and polyethyleneimine (PEI). PEI and PLL are the commonest and most important ones used as nonviral vectors. PLL has been used to condense pDNA under various salt conditions (59). The PLL-DNA particles have been shown to be protected against DNA degradation (28). Electron microscopic studies had demonstrated that PLL-DNA complexes assumes a rod-like appearance with a diameter of 15 nm and a length of 109 ± 36 nm.

---

**Table 1. A summary of nonviral gene delivery systems for treatment of cardiovascular diseases**

<table>
<thead>
<tr>
<th>Delivery Methods</th>
<th>Central Mechanism</th>
<th>Diseases/Status</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked plasmid (VEGF, HGF, FGF)</td>
<td>Endocytosis</td>
<td>PAD and CAD (phase I/II)*</td>
<td>Safety and simplicity</td>
<td>Low transfection efficiency</td>
</tr>
<tr>
<td>Chemical vectors</td>
<td>Endocytosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomes</td>
<td>PAD and CAD (phase I/II)*</td>
<td>Safety</td>
<td>Low to medium high-efficiency, immunogenicity</td>
<td></td>
</tr>
<tr>
<td>Polymers</td>
<td>In vitro and in vivo</td>
<td>Low cytotoxicity</td>
<td>Complement activation, low efficiency</td>
<td></td>
</tr>
<tr>
<td>Dendrimers</td>
<td></td>
<td>Low immunogenicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrasound ± microbubble</td>
<td>Enhancement of cell membrane permeability</td>
<td>Safety and flexibility</td>
<td>Low efficiency</td>
<td></td>
</tr>
<tr>
<td>Electroporation</td>
<td>Enhancement of cell membrane permeability</td>
<td>Good efficiency and reproducibility</td>
<td>Tissue damage, limited accessibility of electrodes to internal organs</td>
<td></td>
</tr>
<tr>
<td>Gene gun</td>
<td>High-pressure helium stream</td>
<td>Good efficiency, transfer genes to nondividing cells</td>
<td>Shallow penetration of DNA into target tissues</td>
<td></td>
</tr>
<tr>
<td>Magnetofection</td>
<td>Magnetic force plus endocytosis</td>
<td>Flexibility and low cytotoxicity</td>
<td>Transient transfection</td>
<td></td>
</tr>
</tbody>
</table>

See text for abbreviations. *Clinical trials completed or currently conducted.
much smaller than lipoplexes. The poor circulatory half-lives of PLL-DNA complexes, typically shorter than 3 min, also limit their use in vivo (110). In the recent years, PLL-PEG conjugates with defined chemical composition have been shown to improve delivery to different organs (113) and have been applied in phase I/II clinical trials for treatment of ocular degenerative diseases and cystic fibrosis (48). Generally, PLL or PLL-DNA complexes were reported to have low immunogenicity (92).

Among cationic polymers, PEI, which is used in both linear and branched forms with molecular masses (MMs) between 0.7 and 800 kDa (29), has been one of the most effective polymer-based transfection agents. The polycationic PEI is receiving much attention because of its characteristic of condensing DNA with an intrinsic endosomolytic activity and has the ability to capture protons that are pumped into endolysosomes—“proton sponge” effect (9). This permits the escape of endocytosed PEI-DNA complexes. However, it is highly cytotoxic. Factors influencing cytotoxicity, TE, and the use for in vivo transfection include MM, incubation time, and the charge ratio of polymer to DNA used (25, 39). In general, high MM PEI (>25 kDa) is toxic to cells, whereas polymers with medium to low MM (5–25 kDa) are more efficient and less toxic (25). The toxic effect of PEI on cells can be partially reduced by conjugation with other polymers such as PEG (80). Upon systemic administration, these polyplexes of small particle size tend to aggregate to form larger complexes and accumulate in major tissues including lung and liver. So far, there have been no major clinical trials of cardiovascular diseases conducted by using cationic polymers alone.

**Physical approaches.** To date, there are three major physical approaches of gene delivery-gene gun, electroporation and US by creating transient membrane holes using mechanical forces. Additionally, magnetofection, which uses magnetic force to concentrate particles containing nucleic acid into target cells, has been recently developed as a feasible nonviral method to combine current chemical and physical methods in gene delivery (35, 76, 81).

**DIRECT GENE TRANSFER BY GENE GUN.** Gene gun (111) uses a high-pressure helium stream to deliver DNA, coated onto gold particles of varying sizes (1–3 μm), directly into the cytoplasm. The efficiency of the gene gun is variable, and the duration of the expression is transient. A drawback of this tool is the shallow penetration of DNA into target tissue. 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 (13). Several animal studies have reported that gene gun transfection of skin tissues can elicit immune responses to protect the animals from infection (27, 64). These immune responses have also been employed as a therapeutic approach for the treatment of cancer in human clinical trials (107). As a method to treat other disorders, Goudy et al. (30) have shown that vaccination in an animal model of type 1 diabetes with glutamic acid decarboxylase gene has induced type 2 immunity that resulted in blocking β-cell autoimmunity (30).

**ELECTROPORATION.** The application of controlled electric fields to facilitate cell permeabilization (electroporation) was first described in the 1960s (14). Extracellular molecules can pass through the cell membrane via the pores transiently generated by the electrical pulses. Since 1982, the use of electric pulses for cell electroporation has been used to introduce foreign DNA into cells in vitro and in vivo (77, 104). Skeletal muscle is a good candidate for electroporation (7). In general, DNA injection followed by electroporation showed 10- to 1,000-fold enhancements of expression compared with naked DNA injection alone but are accompanied by an elevation of serum creatine kinase levels (31). Indeed, one key limitation of electroporation is that a substantial procedure-related damage may occur and, as a result, may limit the efficiency of transfection and its use in cardiovascular tissues (18, 69). In addition to electroporation followed by local injection, in vivo electroporation performed in a localized manner after a systemic injection of pDNA has been reported (84). Although in vivo electroporation technique is generally efficient when parameters are optimized and can produce good reproducibility, the key limitation is the accessibility of the electrodes to the internal organs and its clinical applications need further optimization.

**US-BASED TECHNOLOGY 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.

**Bioeffects of US.** Among the physical effects of US, acoustic cavitation (4) is thought to be the most important bioeffect. Briefly, as the US waves propagate through the medium, the characteristic compression (Fig. 2) and rarefaction (Fig. 2) cause microscopic gas bubbles in the tissue fluid to contract and expand. Two types of cavitation are recognized. Gas body activation (61) or stable cavitation is the term used to describe bubbles that oscillate in diameter with the passing pressure
variations of the sound wave. As the ultrasonic intensity increases, inertial cavitation (70) (Fig. 2) occurs when the amplitude of bubble oscillations enhances to a point where the inward moving wall of fluid has sufficient inertia that it cannot reverse direction when the acoustic pressure reverses and subsequently persists to compress the gas in the bubble. Finally, the bubble implodes violently, producing pressure discontinuities (shock waves), free radicals, microjets, extremely high-localized temperatures (at least 5,000 K), pressures (up to 1,200 bars), and light (Fig. 2). Formation of shock waves, bubble wall motion, and microjets may affect membrane permeability (62). Both stable and inertial cavitation may induce membrane permeabilization (100).

Applications of US in gene delivery. US-mediated transient pore formation in the cell membranes (sonoporation) can be regarded to be the same as the promotion of membrane permeability induced by acoustic cavitation. Generally, the efficiency of sonoporation-mediated gene delivery depends on DNA concentration, the frequency and intensity of US exposure, the duration of exposure, and the presence of contrast agent. Most gene delivery studies use the therapeutic US at driving frequency of 1–3 MHz with an intensity of 0.5–2.5 W/cm² (44).

Microbubble echo contrast agents and their applications in gene delivery. The concept of US contrast imaging was introduced in the 1960s, and gene delivery using microbubbles has been intensively studied since 2000 (90). The ideal microbubble contrast agents should be nontoxic, 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 (1). Contrast agents have a gas core that is filled with air or a higher MM 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 to 200 nm. A critical improvement in US-assisted gene delivery was the combination of US irradiation with microbubbles. Most importantly, microbubbles have been shown to lower the energy threshold for cavitation by US energy and to have the potential of enhancing cavitation (24). When US interacts with the microbubbles leading to cavitation, pDNA are driven across cell membranes into the target cells (106).

US-assisted gene delivery in vitro and in vivo. Naked pDNA is the simplest nonviral vector. However, disadvantages in systemic gene delivery with naked DNA have also been found, since pDNA vector can be rapidly degraded and neutralized by endogenous DNases. Therefore, it is reasonable to combine naked pDNA delivery with another method to improve the transgene efficiency. In 1987, Fechheimer et al. (21) first demonstrated that US had potential as a tool of pDNA delivery into murine fibroblasts. The first major research in this field came in 1996. Kim et al. (44) investigated the potential use of US exposure as a novel transfection method for experimental use. In 2000, Lawrie et al. (52), sonicating vascular smooth muscle cells in the presence of Optison and naked luciferase pGL3 plasmid (52), achieved transgene expression levels 300-fold higher than with naked pDNA alone. In 2007, Deshpande and Pausnitz (17) performed US-assisted in vitro reporter gene transfection and found that US sonication alone increased transfection up to 18-fold and the combination of US and PEI synergistically increased transfection up to 200-fold, resulting in TF 34%. In 2010, our results showed that ultrasonic microbubble transfection causes phenotypic changes of primary vascular endothelial cells by enhancing proliferation and up-regulating kinase insert domain-containing receptor and fins-like tyrosine kinase-1, while possesses no obvious adverse effect on viable transfected cells (94).

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. Most importantly, in 2002, Taniyama et al. (100), by using intramuscular injection of rat hindlimbs, reported a 10-fold increase in luciferase activity after injection of plasmid and Optison, followed by topical external US compared with plasmid alone. Subsequently, using HGF plasmid in a rabbit ischemic hindlimb model, they demonstrated that transfection with plasmid and Optison followed by US resulted in increased capillary formation in the treatment group after 5 wk. Therefore, ultrasonic microbubble-mediated HGF plasmid delivery could be feasible for safe clinical gene therapy to treat PAD without a viral vector system. Using intravenously infusion of microbubbles loaded with plasmids encoding the human VEGF165 gene into rat myocardial microcirculation, Korpanty et al. (49) performed US exposure along with microbubbles (49). The data showed that the capillary density increased to 18% at 5 days and 33% at 10 days before returning to control levels at 30 days. Theoretically, it is feasible to apply the US energy to deliver the growth factor gene into the ischemic tissue in clinical setting. The molecular mechanism of US-assisted gene delivery in therapeutic angiogenesis remains to be determined. In 2011, Chen et al. (10), by using tail vein injections in mice, investigated the efficiency of in vivo gene transfer to the myocardium mediated by US-targeted liposome microbubble destruction accompanied by PEI. The significantly enhanced transgene expression was noted related to US-targeted microbubble destruction in the presence of PEI, and the results showed the feasibility of US-based method for primary myocardial disease.

At present, there are two main hurdles as to why gene therapy has not globally succeeded in the clinical setting: first, inefficient delivery of gene of interest to their correct sites of action and, second, safety concern of some viral-based vectors that are one to three orders of magnitude more efficient than nonviral techniques in gene delivery in vivo. Many gene delivery methods are much less efficient in vivo than in vitro (such as liposome-mediated transfection). US is a versatile modality and 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 along with its high safety profile, low costs, and repeatable applicability. 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 target-specific microbubble that can selectively bind to the areas of interest in the tissue/body. These active targeting strategies can be achieved by the development of targeted microbubbles by attaching antibodies or peptides to microbubble shells (45, 95). 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. Furthermore, since most of the major progress in US-based gene delivery involves microbubble cavitation by which a synergistic effect in enhancing US-assisted gene transfer occurs, further understanding of cavitation physics and US-microbubble-cell interactions cannot be overemphasized.

**Integrated Nonviral Gene Delivery into Cell Therapy: Cell-Based Gene Delivery**

Gene transfer can be performed either by isolating stem cells from the patient, delivering the gene(s) into the cells in vitro and then transplanting the genetically modified stem cells back into the patient, or by directly introducing the delivery vector into the target sites in vivo. The in vivo approach is limited by nonspecific cellular targeting and inefficient gene delivery. This may explain that several clinical trials failed to show the benefit of in vivo growth factor gene therapy. With the recent rapid progress in molecular biology together with the steady advance of genome projects, gene and cell therapy has become an exciting new hope for the treatment of cardiovascular diseases. Cell-based gene delivery is a novel ex vivo strategy that uses autologous cells (such as endogenous stem cells and progenitor cells) as natural carrier vehicles of gene to the target site after in vitro transfection with a functional gene of interest (11) (Fig. 3). The use of adult progenitor cells, for example, endothelial progenitor cells (EPCs), as angiogenic substrate that is capable of synthesizing multiple cytokines is an attractive strategy for neovascularization of ischemic cardiovascular diseases (86, 99, 109). Genetic manipulation of EPCs to overexpress angiogenic genes may enhance their biological functions, such as migration, proliferation, and secretion of angiogenic growth factors (5, 46). The advantage of the ex vivo approach is to provide specific genetically modified cells that may directly participate in tissue regeneration and, most importantly, allow for both paracrine (mainly) and autocrine effects from the expressed cytokine (72, 88). In cell-based gene delivery, the EPCs serve not only as a vehicle for gene delivery to target sites but also as a tissue-engineering tool in restoring normal tissues (74). By means of delivering autologous cells, cell-based gene delivery is capable of circumventing the unwanted inflammatory response, eliminating the possibility of insertional mutagenesis of other genes caused by viral vectors, and possibly achieving prolonged expression by transfection concomitantly using various nonviral methods including ultrasonic microbubble transfection, electroporation, and, in vitro chemical vector transfection. However, in the long term, this approach needs to develop further steps to avoid contamination, to isolate, manipulate, and expand cells, as well as to develop less invasive strategies to enhance endogenous stem cell function and survival ex vivo. In the recent years, insights have been focused on gene transfer by means of cellular transplantation to induce neovascularization in ischemic tissue (38, 46). Genetically manipulated stem cells, in which a VEGF gene can be transferred by US or other physical methods ex vivo, may yield a promising future in therapeutic angiogenesis.

**Future Perspectives**

Gene therapy by growth factor gene delivery in therapeutic angiogenesis or nonangiogenic gene delivery for myocardial salvage is emerging as a potential strategy for the treatment of cardiovascular diseases. Viral-based gene delivery is efficient but safety aspects have usually limited its clinical applications (78). Various nonviral transfer approaches were developed to facilitate the delivery of transgenes into target cells without concern of insertion mutation, though dose optimization of nonviral vectors needs to be performed because of potential cytotoxicity. In the past few years, the work continued in improving delivery modalities with increased local distribution and reduced systemic circulation of chemical vectors. Naked DNA remains the preferred method of DNA transfer to cardiovascular ischemic tissues and has been explored extensively in clinical trials because of its simplicity and safety. However, insufficient gene delivery is still the major hurdle of plasmid-mediated gene therapy. Improving plasmid-mediated gene transfer either by physical methods or chemical carriers to enhance cellular uptake and reduce susceptibility to circulating nucleases has shown successful applications for in vivo gene delivery. Further improvements to increase the efficiency and reduce the cytotoxicity of nonviral vectors are required before their clinical implication. The potential interest will rely on developing new vector system that are more target-specific and a better understanding of the limiting steps that nonviral vector must overcome. Last but not least, although only short- and mid-term follow-up data are available, the methods and tech-
nology that merge nonviral gene and cell therapy such as delivery of proangiogenic plasmids with EPCs may give rise to several attractive opportunities to achieve more efficient and nontoxic strategies for gene therapy in regenerative medicine.

GRANTS

This study was supported by National Science Council (Taiwan) Grant 99-2314-B-195-010-MY2 and Medical Research Department of the Mackay Memorial Hospital (Taiwan) Grant MMH-E 100003.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


64. Newell KD, Dunn PF, Owens JW, Schulick AH, Virmani R, Sukhova G, Libby P, Dichek DA. Adenovirus-mediated gene transfer into normal rabbit arteries results in prolonged vascular cell activation,


