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Gene Transfer: How Can the Biological Barriers Be Overcome?

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Abstract Physical methods represent a promising approach for the safe delivery of therapeutic plasmid DNA in genetic and acquired human diseases. However, their development in clinics is limited by their low efficacy. At the cellular level, efficient gene transfer is dependent on several factors including extracellular matrix, plasmid DNA uptake and nucleocytoplasmic transport. We review the main barriers that plasmid DNA encounters from the extracellular environment toward the interior of the cell and the different strategies developed to overcome these biological barriers. Diffusional and metabolic fences of the extracellular matrix and the cytoplasm affect plasmid DNA uptake. These barriers reduce the number of intact plasmids that reach the nucleus. Nuclear uptake of plasmid DNA further requires either an increase of nuclear permeability or an active nuclear transport via the nuclear pore. A better understanding of the cellular and molecular bases of the physical gene-transfer process may provide strategies to overcome those obstacles that highly limit the efficiency and use of gene-delivery methods.

Keywords Gene therapy · Physical methods · Extracellular matrix · Plasma membrane · Cytoplasm · Nucleus

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Introduction

The clinical development of gene therapy requires the use of safe and efficient methods that deliver a therapeutic gene to target cells where gene expression can be achieved. A gene-delivery method has to overcome several obstacles (Fig. 1): (1) it should first protect the transgene against degradation by the nucleases present in the extracellular matrix (ECM) and increase transgene diffusion through the matrix (McMahon et al. 2001; Bureau et al. 2004; Mesojednik et al. 2007); (2) it should bring the transgene across the plasma membrane of target cells (Stephens and Pepperkok 2001); (3) it has to facilitate the intracellular migration of transgene to the nucleus and limit transgene degradation by intracellular nucleases (Lukacs et al. 2000); and (4) finally, it should bring the transgene across the nuclear envelope (Vaughan et al. 2006).

In general, recombinant viral vectors have been used preferentially in clinical trials due to their high gene-delivery efficiency and their ability to induce high-level and long-lasting gene expression in various tissues (Gardlik et al. 2005; Waehler et al. 2007). The efficiency of these vectors lies in the infectious properties of virus that are controlled by viral proteins. Nevertheless, these same proteins could induce specific immune responses that would limit the ability to readminister the viral vector and inhibit the efficiency of gene transfer (Lehrman 1999; Marshall 1999). Moreover, retrovirus- or lentivirus-derived vectors could evoke insertional mutations during their integration into the host genome (Hacein-Bey-Abina et al. 2003a, b). In addition, previous works showed that recombination events could lead to the formation of replication-competent viruses (Broeke and Burny 2003).

In contrast, plasmid DNA molecules are just covalent closed circles of double-stranded DNA with no associated

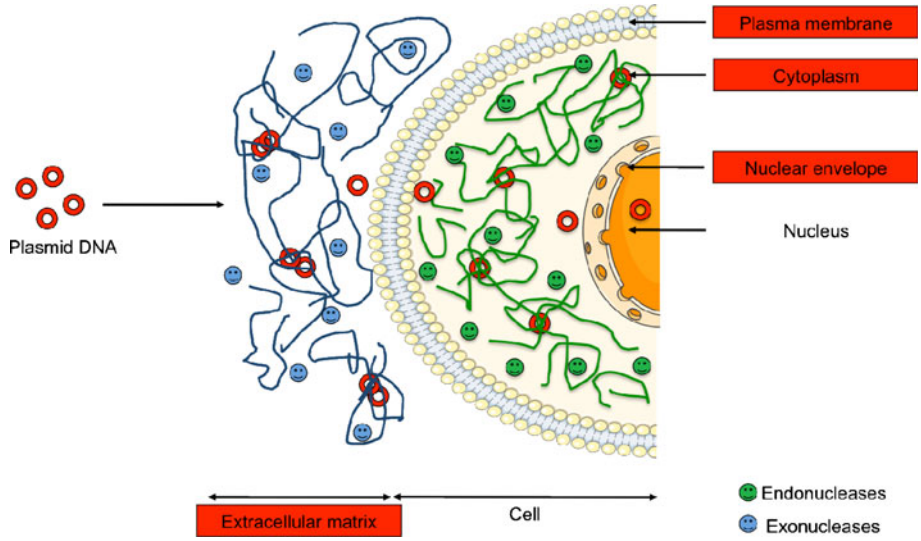

Fig. 1 After its injection into the target tissue, plasmid DNA encounters biological barriers from extracellular environment toward the interior of the target cell. In the extracellular environment, plasmid DNA encounters the exogenous nucleases, which degradate it and the extracellular matrix which prevent it from diffusing into the tissue. Then, plasmid DNA has to across the plasma membrane of target cells. Into the cytoplasm, its diffusion is limited by the intensive molecular crowding of cytoplasm and by the presence of endogenous nucleases. Finally, plasmid DNA has to cross the nuclear envelope. These barriers reduce the number of useful plasmid that reach the nucleus where the gene expression takes place. This figure has been drawn by using the Servier Medical Art free access software

proteins. Plasmid DNA molecules are simpler, easier to mass-produce and potentially safer than viral vectors (Wells 2004). Low immunogenicity and lack of integration of plasmid DNA make it a highly attractive molecule for gene therapy provided that an efficient, safe and targeted delivery can be achieved (Wells 2004).

In this review, we will describe the different barriers encountered by plasmid DNA during gene delivery to cells and tissues and we will propose various strategies to overcome these physicochemical barriers. Then, we will discuss the mechanisms, advantages and limitations of the physical approaches that are shown to be active for ex vivo and in vivo gene delivery.

Extracellular Matrix

The space which surrounds the cells, called the "extracellular space," contains a set of macromolecules, polysaccharides or glycosaminoglycans, fibrous proteins, salts and water, known as the "extracellular matrix" (ECM) (Berrier and Yamada 2007). The glycosaminoglycans are negatively charged and include hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparan, heparin and keratin. The main proteins of structure are collagen and elastin as well as the proteins of adhesion, fibronectin and laminin (Berrier and Yamada 2007; Kass et al. 2007). According to its composition, the ECM can take various aspects, liquid like interstitial liquid or the synovial liquid rich in polysaccharide, gelatinous like the tendons rich in fibrous proteins or solid like the bone rich in calcium phosphate (Berrier and Yamada 2007). The basal membrane, which separates epithelial tissue from conjunctive tissue, constitutes a kind of ECM. The components of the ECM are synthesized and secreted by cells such as fibroblasts and chondroblasts and broken up by enzymes called "matrix metalloproteinases" (Stamenkovic 2003; Berrier and Yamada 2007).

To reach the plasma membrane of target cells, the plasmid DNA must diffuse through the ECM without being degraded by the extracellular nucleases (Bureau et al. 2004). Compared to the small molecules, diffusion, distribution, degradation and uptake of plasmid DNA into cells have been investigated to a much smaller extent. Previous works showed that the delivery of macromolecules such as plasmid DNA to cells in target tissues such as skeletal muscles (Bureau et al. 2004), tumors (Netti et al. 2000; Pluen et al. 2001; Alexandrakis et al. 2004; Walther et al. 2005a, b) and skin (Vandermeulen et al. 2007) is affected by the amount of nucleases, which degrade plasmid DNA, and by the amount of ECM components, such as collagen (Netti et al. 2000; Pluen et al. 2001) and hyaluronic acid (Alexandrakis et al. 2004). Consequently, in order to increase the efficiency of gene transfer, some strategies have been developed to increase the diffusion of plasmid DNA and to limit its degradation into the target tissues. The main strategy to increase the diffusion and distribution of plasmid DNA into the tissue consists of controlled and partial degradation of ECM using enzymes such as hyaluronidase and collagenase. This approach is

efficient in skeletal muscle (Favre et al. 2000; McMahon et al. 2001; Mennuni et al. 2002; Molnar et al. 2004; Schertzer et al. 2006; Evans et al. 2008), liver (Dubensky et al. 1984) and tumors (Kuriyama et al. 2001) but inefficient in skin (Vandermeulen et al. 2007). Indeed, some works showed that pretreatment with hyaluronidase induced a 10- to 25-fold increase of gene expression in mice and rabbit skeletal muscle after i.m. injection and electroporation compared to no pretreatment (McMahon et al. 2001; Mennuni et al. 2002; Molnar et al. 2004; Schertzer et al. 2006). Dubensky et al. (1984) combined hyaluronidase with collagenase in liver and spleen by injecting calcium phosphate-precipitated plasmid DNA. Their results showed that these organs were more homogeneously transfected than without enzymatic treatment.

To limit the degradation of plasmid DNA, the main approach was to protect the plasmid DNA using a chemical formulation or to inhibit the exogenous DNAses. Nicol et al. (2002) showed that plasmid DNA formulated with poly-L-glutamate induced a 4- to 12-fold increase of gene expression in skeletal muscle after i.m. injection and electroporation in comparison to saline injection alone. Other works showed that the plasmid DNA formulated with poloxamers such as SP1017 increased gene expression by about 10-fold and maintained higher gene expression in skeletal muscle after i.m. injection compared with naked DNA (Riera et al. 2004). These data suggested that chemical formulation might enhance plasmid DNA uptake by protecting the plasmid DNA from degradation by nucleases (Lemieux et al. 2000). The second approach uses nuclease inhibitors such as aurintricarboxylic acid (ATA) (Walther et al. 2005a). Previous works showed that the in vitro and in vivo coadministration of ATA with naked plasmid DNA was able to improve transfer efficiency in respiratory tissues and skin of mice, pigs and macaques (Glasspool-Malone and Malone, 1999; Glasspool-Malone et al. 2000, 2002). For example, Walther et al. (2005a) demonstrated that jet injection of plasmid containing ATA led to a threefold increase in gene expression in human colon tumors compared to jet injection without ATA.

Plasma Membrane

The cell membrane is a physical barrier that separates the intracellular components from the extracellular environment. It contains two main classes of molecules, proteins and lipids. The cell membrane hinders the free diffusion of plasmid DNA between the cell cytoplasm and external medium (Stephens and Pepperkok 2001).

Direct injection of naked plasmid DNA into a tissue is a simple and safe method available to achieve gene delivery and expression. Previous works suggested that the mechanism involved in gene transfer could be based on the interaction between plasmid DNA and specific or nonspecific receptors on the plasma membrane and their internalization into target cells (Budker et al. 2000).

The proof of concept has been reported by Wolff et al. (1990), who obtained gene expression in mouse muscle for 60 days. Local injection of plasmid DNA has also been shown to facilitate gene delivery in tumor xenografts (Kawase 2003), liver (Andre et al. 2006), brain (Schwartz et al. 1996), skin (Choate and Khavari 1997), heart (Ardehali et al. 1995), thyroid (Sikes et al. 1994) and tendons (Jayankura et al. 2003).

The main limitations of this delivery method are low levels of transgene expression, interindividual heterogeneities and limited distribution. These limitations could reflect the low diffusion and degradation of plasmid DNA in the ECM and cytoplasm of cells. In order to improve the efficiency of plasmid DNA delivery, physical methods have been developed (Stephens and Pepperkok 2001). The general principle of physical methods is based on the transient disruption of cell membrane in order to facilitate DNA uptake (Table 1).

It is difficult to draw up a comparative table of physical methods in terms of their transfection efficiencies and innocuity. The preferential use of one technique over another is presently determined by the nature of the compound to be transferred, of the target tissue and of the desired biological or therapeutic effects.

Electroporation

The application of controlled electric field pulses induces transient and localized cell membrane permeabilization (Neumann and Rosenheck 1972; Escoffre et al. 2007), a process that can be used for the enhancement of naked plasmid DNA uptake into cells (Neumann et al. 1982; Golzio et al. 2004; Escoffre et al. 2009). The transfection efficiency depends on electric field parameters such as the number (up to 10), duration ($\sim$ms) and strength ($\sim$V/cm) of electric pulses. These parameters are controlled and can be adjusted according to the cells and tissues used (Golzio et al. 2004). Theoretical models have been proposed to explain the mechanism of this reversible membrane electropermeabilization and its potential to allow the access of nonpermeant molecules inside the cells. Nevertheless, the molecular definition of the transient permeable structures is not yet known (Teissié et al. 2005). Indeed, two hypotheses are defended. The first hypothesis suggests that plasmid DNA moves through large and hydrophilic pores without interaction between plasmid DNA and plasma membrane. The size of these pores was estimated at 1–10 nm. Plasmid DNA has direct access to the cytosol (Krassowka and Filev 2007; Smith et al. 2004; Tieleman

Table 1 Characteristics of physical gene-delivery systems

Methods	DNA injection	Tissue	Mechanism	Advantages	Limitations	Future clinical applications
Local injection	Intratissue	Skin, muscle, tumor, liver, brain, heart, thyroid, tendons	Pore	Simplicity, safety	Low efficiency, access to internal organs	Genetic vaccination
Electroporation	Topical, intratissue	Skin, muscle, kidney, lung, liver, joints, heart, spleen, brain, eyes, tumor	Pore	High efficiency, safety	Access to internal organs	Genetic vaccination, cancer gene therapy
Sonoporation	Topical, systemic	Muscle, liver, heart, tumor, bone	Pore	Good efficiency, safety	Transfection efficiency	Cardiovascular diseases, cancer gene therapy
Hydrodynamic injection	Systemic,	intravascular	Liver, muscle	Pore	High efficiency, safety	Translation to human body
Genetic vaccination, cancer gene therapy						
Gene gun	Topical	Skin, mucosa	Membrane disruption	Good efficiency, safety	Weak tissue penetration, fate of particle	Genetic vaccination
Laser beam gene transduction	Topical	Skin, muscle	Pore	Good efficiency	Weak tissue penetration	Genetic vaccination
Magnetofection	Topical	Gastrointestinal tract, vasculature of ear, airway epithelium	Endocytosis, membrane disruption	High efficiency in vitro	Low efficiency in vivo, fate of particle, safety	Cancer gene therapy
Jet injection	Topical, intratissue	Skin, muscle, tumor	Membrane disruption	High efficiency, safety	Access to internal organs	Genetic vaccination, cancer gene therapy
Plasma	Topical	Skin	Membrane disruption	Low efficiency	Weak tissue penetration	Genetic vaccination

2004; Neumann et al. 1982). The second hypothesis proposes a plasmid DNA/plasma membrane interaction (Golzio et al. 2002; Sukharev et al. 1992; Tarek 2005). This interaction is the result of electrophoretic accumulation of plasmid DNA with permeabilized membrane. Membrane translocation and intracellular migration occur after the electric field application (Golzio et al. 2002).

A wide range of tissues have been transfected by electroporation including skin (Titomirov et al. 1991; Vandermeulen et al. 2007), kidney (Isaka et al. 2005), lung (Zhou et al. 2007), liver (Heller et al. 1996; Liu and Huang 2002), muscle (Aihara and Miyazaki 1998; Mir et al. 1999; Golzio et al. 2005), joints (Bloquel et al. 2007), heart (Dean 2005), spleen (Tupin et al. 2003), brain (Saito and Nakatsuji 2001), eyes (Bloquel et al. 2006) and tumors (Rols et al. 1998; Goto et al. 1999). As we will see in the following paragraphs, the other physical methods are generally compared with electroporation. This approach consists of local or systemic injection of plasmid DNA solution, followed by controlled application of electric field. Only the tissue placed between the electrodes is transfected (Escoffre et al. 2008). In most works, electroporation increases gene expression by 100- to 1,000-fold compared to the simple injection of plasmid DNA. Moreover, electrotransfection decreases the interindividual variability compared to direct injection of plasmid DNA (Mir et al. 1999). In addition, electrotransfection can achieve long-lasting expression in tissues. Indeed, gene expression was detected from 9 months to 1 year in skeletal muscle (Mir et al. 1999). To improve transfection efficiency by electrotransfection, factors such as the size and concentration of plasmid DNA, electrode shape, number, orientation and duration of the electric field pulses have been optimized (Mir et al. 1999; Faurie et al. 2003, 2004). Previous works showed that the in vivo biodistribution of plasmid DNA was slowed down by the complex organization of tissues like skeletal muscle or tumors (Bureau et al. 2004; Mesojednik et al. 2007). Nevertheless, treatment of tissues with hyaluronidase and/or collagenase prior to injection of plasmid DNA induced partial ECM degradation (McMahon et al. 2001). This treatment improved plasmid DNA diffusion into the tissues and limited the nuclease degradation of plasmid DNA. Consequently, gene transfection was enhanced (McMahon et al. 2001). In addition, the

application of electric pulses with different polarities and orientations allowed an increase in gene transfection by enhancing the plasmid DNA/membrane interaction around the cells (Faurie et al. 2003, 2004). Preclinical studies showed the therapeutic potential of this gene-transfer method in the treatment of monogenic diseases such as muscular atrophy (Lesbordes et al. 2002), β-thalassemia (Payen et al. 2001) and ischemic limbs (Nishikage et al. 2004). Moreover, recent works showed that electrotransfection of vaccine DNA into muscle or skin increased the immune response compared to direct injection of vaccine plasmid (Dupuis et al. 2000; Widera et al. 2000; Babiuk et al. 2002; Dayball et al. 2003). Potent immune responses against bacterial (Zhang et al. 2007) and viral (Hirao et al. 2008) infections and cancer (Kalat et al. 2002; Buchan et al. 2005; Curcio et al. 2008) were obtained by electrotransfection of vaccine plasmid into muscle or skin tissues. Then, the expression of suicide genes, cytokines, antiangiogenic factors and antitumor suppressors such as thymidine kinase/ganciclovir (Shibata et al. 2002), IL-12 (Heller et al. 2006) and p53 (Grosel et al. 2006) are under investigation for cancer gene therapy.

Sonoporation

Sonoporation is a physical method of gene delivery into cells and tissues using ultrasound exposure in the presence of microbubbles (Greenleaf et al. 1998; Newmann and Bettinger 2007; Karshafian et al. 2009). Ultrasound frequencies used for gene delivery are near the range of ultrasound frequencies. Therefore, the microbubbles are the same as those used for ultrasound contrast imaging. The transfection efficiency depends on the acoustic pressure, the duty cycle and the time of exposure of cells to ultrasound (Duvhani-Eshet et al. 2006). Ultrasound exposure is necessary but not sufficient to deliver genes into cells. In addition, the nature of microbubbles, their lifetime and their concentration are involved in transfection efficiency (Li et al. 2003). The mechanism of DNA uptake into cells is not very clear, but some work suggests that sonoporation induced the creation of pores in the plasma membrane. Imaging studies showed that the microbubbles interacted with the plasma membrane (van Wamel et al. 2004). Under ultrasound exposure, the microbubbles induced mechanical constraints on the plasma membrane and increased the membrane permeability by the creation of pores (Tran et al. 2006; Postema et al. 2004). Meiher-Humber et al. (2005a) demonstrated that the size of these pores is $\leq$75 nm for rat mammary carcinoma cells. The kinetics of plasmid internalization and gene expression showed that sonoporation allowed a rapid and direct transfer of naked DNA into the cell cytoplasm, probably via ultrasound-induced pores in the membrane. The kinetics of protein expression was significantly faster for sonoporation than lipofection, the mechanism of which requires endocytosis (Meiher-Humber et al. 2005b).

Sonoporation is a simple and efficient method to transfer genes into skin (Yoon et al. 2009), skeletal muscle (Li et al. 2003), cardiac muscle (Newmann and Bettinger 2007; Kondo et al. 2004), liver (Miao et al. 2005) and tumor (Suzuki et al. 2008). In addition, Hynynen et al. (2003) showed that intracranial sonoporation induced a transient permeabilization of the blood–brain barrier of rabbit. Sonoporation is a noninvasive method to deliver therapeutic genes in the treatment of neurodegenerative diseases and brain cancer. This method has a promising future in the treatment of cardiac diseases. For example, Kondo et al. (2004) demonstrated that sonoporation may enable myocardial hepatocyte growth factor gene transfer with systemic administration of naked plasmid, which enhances angiogenesis, limits infarction size and prevents left ventricular remodeling after myocardial infarction in a rat model. In addition, Hauff et al. (2005) led the proof of concept to transfer therapeutic gene into tumors. The delivery of suppressor gene *p16* into human pancreatic adenocarcinoma by sonoporation induced a twofold decrease of growth tumor after three treatments.

Hydrodynamic Injection

Hydrodynamic injection is based on rapid injection of a large volume of plasmid DNA solution via the tail vein (e.g., 5 μg plasmid DNA in 5–8 s in 1.6–1.8 ml saline solution for a 20-g mouse). The transfection efficiency of hydrodynamic injection is determined by the combined effect of a large volume and high injection speed. This procedure induced gene transfer in highly perfused internal organs, especially the liver (Herweijer and Wolff 2007).

Previous work has attempted to explain the mechanisms involved in hydrodynamic gene transfer. Budker et al. (2000) suggested that plasmid DNA crossed the plasma membrane of hepatocytes by a receptor-mediated pathway. Zhang et al. (2004) demonstrated that hydrodynamic injection induced a transient irregularity of heart function and a rapid increase in vena cava pressure. Hydrodynamic injection enlarged liver fenestrae and enhanced the membrane permeability of hepatocytes. At the cell level, their data suggested that hydrodynamic gene delivery is accomplished by the generation of membrane pores in the hepatocytes. This mechanism could allow direct entry of plasmid DNA into the cytoplasm without endocytosis (Zhang et al. 2004; Andre et al. 2006).

Such a simple and efficient method for gene delivery has been used to express therapeutic proteins such as fetal liver kinase-1 (Yazawa et al. 2006) and IFN-γ (Takehara et al. 2007) in mouse and rat models of liver disease. Moreover, hydrodynamic injection is used to deliver genes into

muscle tissues in the temporarily isolated limbs of primates and isolated limb perfusion (Zhang et al. 2001). This procedure is performed via the arterial and venous routes. Transfection efficiency is higher than a simple intramuscular injection and only muscles of the perfused limb are transfected (Herweijer and Wolff 2007). Previous work demonstrated high-efficiency delivery of a dystrophin plasmid to a dystrophic murine model (Liang et al. 2004).

The translation of hydrodynamic injection for gene therapy in humans proposed injection of up to 8 l (i.e., 10% of body weight) of saline solution at a high rate. The human body would not tolerate this high volume. Nevertheless, to overcome this obstacle, balloon catheter-based (Eastman et al. 2002) and occlusion-assisted infusion (Alino et al. 2007) could be used for gene delivery by hydrodynamic injection in future clinical applications.

Gene Gun

The gene gun is a nonviral method whose principle is based on the bombardment of small gold particles coated with plasmid DNA accelerated using pressurized gas on tissues. Ballistic gene delivery allows direct penetration through the plasma membrane into the cytoplasm and even the nucleus, bypassing the endosomal pathway (Cheng et al. 1993).

The gene gun is a simple and efficient method to deliver genes into skin (Gaffal et al. 2007), mucosa (Wang et al. 2003) and internal organs such as the liver after surgery (Chang et al. 2008). Various works have reported use of the gene gun to introduce plasmid DNA-encoding antigens such as $A\beta_{42}$ (Ghochikyan et al. 2003) or cytokines such as IL-4 (Ghochikyan et al. 2003) and IL-12 (Dietrich et al. 2006) for genetic vaccination and immunotherapy against infectious diseases and cancer.

The main limitation of the gene gun is the shallow penetration of plasmid DNA into the tissue (i.e., a few millimeters). Nevertheless, Dileo et al. (2003) adjusted a new gene gun that allowed shooting microparticles under high pressure. Thus, subcutaneous tissues such as muscle and tumors could be transfected and long-lasting expression could be achieved. Another limitation is that there is little knowledge of the becoming and clearance of these gold particles in vivo.

Laser Beam Gene Transduction

In 1986, Kurata et al. demonstrated that the application of a laser beam to culture cells incubated with plasmid DNA solution increased transfection efficiency. The mechanism involved in the plasmid DNA uptake could be membrane pores that allow direct transfer into the cytoplasm without endocytosis.

In vitro, Tao et al. (1987) showed that comparable transfection was achieved by laser irradiation and microinjection. Femtosecond laser beam transduction is a safe and efficient method of intradermal (Zeira et al. 2007; Tsen et al. 2009), intratumoral (Tsen et al. 2009) and intramuscular (Zeira et al. 2003) nonviral gene delivery in mice. Indeed, Zeira et al. (2003) reported that gene delivery into muscle could be enhanced by application of a femtosecond infrared laser (5 s at 30 mW). The gene transfer was more efficient than a simple intramuscular injection, and gene expression was detected for 100 days without tissue damage. The same group showed the use of laser beam gene transfer in genetic vaccination against hepatitis B virus (HBV) surface antigen (HBsAg). High titers of HBsAg-specific antibodies were detected lasting 210 days, and activation of Th1 and Th2 immune responses was induced (Zeira et al. 2007).

The main limitation of this method is the focus depth of the laser beam. Indeed, the laser was focused to 2 mm under the mouse skin (Zeira et al. 2003). The translation of this method to the human clinic requires a high power of penetration of the laser beam. The use of laser beams in gene therapy seems to be limited to the muscle and skin tissues.

Magnetofection

Magnetofection rests on the delivery of paramagnetic nanoparticles coated with plasmid DNA into cells or tissues under the effect of strong magnetic fields. There was negligible transfection with paramagnetic nanoparticles in the absence of magnetic fields (Scherer et al. 2002; Kamau et al. 2006). Previous evidence suggested that paramagnetic nanoparticles entered via the endocytosis pathway. Nevertheless, Chorny et al. (2007) demonstrated that the use of large paramagnetic nanoparticles (i.e., 375 nm diameter) resulted in higher transfection rates than small nanoparticles (i.e., 185 and 240 mm diameter). This result can be explained by the fact that the internalization of large paramagnetic nanoparticles escaped the lysosomal pathway and released DNA next to the nucleus.

Application of magnetic fields following local injection of magnetic nanoparticles coated with DNA enhanced in vivo reporter gene transfer to the gastrointestinal tract and vasculature of the ear (Scherer et al. 2002). In a therapeutic strategy for the treatment of cystic fibrosis, Xenariou et al. (2006) showed that magnetofection can enhance reporter gene transfer into the airway epithelium in vivo. Recently, Huttinger et al. (2008) demonstrated that feGM-CSF applied by magnetofection is a safe and feasible approach for the treatment of feline fibrosarcomas in veterinary oncology. Nevertheless, it would seem that the improvement of transfection efficiency induced by magnetofection is not as high as the improvement induced by the other physical methods.

Jet Injection

In 1992, Furth et al. developed jet injection, which allows gene transfer into different tissue types with deeper penetration of the applied naked DNA. The physical method is based on the use of jets with high velocity exerting force to penetrate the skin and underlying tissues, leading to efficient transfection of the jet-injected areas (Furth et al. 1995). Indeed, low-volume jet injection uses compressed air to inject solutions of 3–10 μl containing naked or formulated DNA at high speed (>300 m/s) into the desired tissue. The energy of this high-speed liquid allows the jet to precisely penetrate the tissue associated with a spread distribution of the liquid allowing more effective penetration of the targeted area (Walther et al. 2001). The variation in pressure setting has an influence on the penetration depths and transfer efficiencies and therefore allows adaptation of the jet injection to the specific application (Walther et al. 2001). The mechanism involved in the plasmid DNA uptake is unknown, but probably a membrane disruption took place (Walther et al. 2001).

Jet injection increased the transfection efficiency into muscle (Furth et al. 1995; Cartier et al. 2000), skin (Furth et al. 1995; Sawamura et al. 1999; Heansler et al. 1999) and tumors (Walther et al. 2001, 2002, 2005a, b, 2006, 2008; Stein et al. 2007; Cartier et al. 2000). The transgene expression produced by jet injection covers broad areas associated with deep penetration of 5–10 mm within the jet-injected tissue (Walther et al. 2001). In vivo application of jet injection is not associated with tissue damage or significant reactions at injection sites (Furth et al. 1995). This method can achieve transfer efficiencies that are comparable to the gene gun and electroporation (Furth et al. 1992, 1995). The main applications are cancer gene therapy and DNA vaccination. Indeed, Stein et al. (2007) led the proof of concept to complete in vivo reversal of multidrug resistance phenotype by jet injection of anti-MDR1 short hairpin RNA-encoding plasmid DNA. Two jet injections of anti-MDR1 shRNA vectors into tumors combined with two intravenous administrations of doxorubicin were sufficient to induce a decrease of tumor growth. Recently, Walther et al. (2008) published the first results of a phase I clinical trial, which evaluated the safety, feasibility and efficiency of nonviral intratumoral jet injection gene transfer in patients with skin metastases from melanoma and breast cancer. These results showed that intratumoral jet injection of plasmid DNA led to efficient reporter gene expression in all patients. No side effects were experienced, supporting the safety and applicability of this novel physical method.

Plasma

Recently, Jaroszeski and Connolly's group have developed plasma-mediated gene delivery. This approach rests on the ion deposit on the cell and tissue surface by using direct current plasma. The results showed that in vitro plasma-mediated delivery of tracer molecules occurred in a dose-dependent manner, with the optimal condition increasing delivery by up to 60% relative to control. Plasma exposure caused no adverse affects on cell viability (Connolly et al. 2009). The same team investigated the use of plasma-mediated gene delivery into murine skin in vivo. This delivery method included injecting the dermis with plasmid DNA and then exposing the outer skin surface to direct current helium plasma to increase DNA plasmid uptake into cells. This method did not require physical contact between the skin and an electrical source. No involuntary muscle contractions were observed during plasma treatment, and no skin damage was observed posttreatment. Gene expression was dependent upon plasma exposure time, polarity and DNA dose. Maximum expression levels were 20-fold higher than control samples that received DNA injections alone. Thus, Jaroszeski et al. (2009) demonstrated the feasibility of plasma-based delivery and that it could potentially compete with other DNA delivery methods for the skin. Plasma-mediated gene delivery has a future in the human clinic for genetic vaccination.

Combination of Physical Methods

The prospects of nonviral delivery lie in the combination of various methods to improve transfection efficiency and the development of strategies to overcome physical obstacles such as ECM and nuclear uptake.

Indeed, Yamashita et al. (2002) investigated the efficiency of a combination of electroporation and ultrasound (called "electrosonoporation") as a new potent nonviral gene-transfer method into mouse skeletal muscle using naked plasmid DNA encoding luciferase gene as the marker of local protein expression and mouse IL-12 gene as the marker of systemic secretion of therapeutic protein. This work demonstrated that luciferase activity and serum mIL-12 in mice that had undergone electrosonoporation were twofold higher after gene transfer than were those in mice having undergone electrotransfection alone. Electrosonoporation of IL-12 plasmid DNA into skeletal muscle could be used to stimulate or induce an antitumoral immune response. Moreover, Habib et al. (2004) proposed the same approach to transfer human thrombopoietin plasmid DNA into skeletal muscle to treat thrombocytopenia, which is one clinical consequence frequently encountered in patients with chronic liver failure or cancer following

chemotherapy. This combination induced 2.5- and fivefold increases of thrombopoietin compared to sonoporation or electroporation alone, respectively. This increase is correlated with platelet production.

Then, Holzbach et al. (2010) investigated the potential of magnetofection of magnetic liposphères (called "magnetobubbles") containing VEGF-165 encoding plasmid DNA and sonoporation on survival and perfusion of ischemic skin flaps. The magnetic field maintained the magnetobubbles at the ischemic skin flap level. Then, the ultrasound induced gene transfer. The results showed an increased flap survival of 50% and a significant increase of flap perfusion compared to no ultrasound or magnetic field. The safe combination of magnetofection–sonoporation is equally efficient as adenoviral transduction.

Cytoplasm and Nuclear Envelope

Cytoplasm

Enclosed within the plasma membrane, the cytoplasm contains organelles, which are filled with liquid that is kept separate from the rest of the cytoplasm by other intracellular membranes. The part of the cytoplasm that is not held within organelles is called the "cytosol." This cytosol is a complex mixture of cytoskeletal filaments (e.g., actin microfilaments and microtubules), dissolved molecules and water that fills much of the cell volume. The cytosol can be considered a gel, with a fiber network dispersed through water (Lechardeur and Lukacs 2006). The mesh-like structure of the cytoskeleton, the presence of organelles and the high protein concentration impose an intensive molecular crowding of the cytoplasm, which limits the diffusion of large macromolecules (Lechardeur and Lukacs 2002). The translational mobility of macromolecules smaller than 500–750 kDa is only three- to fourfold slower than in water, but it is markedly impeded for larger molecules (Seksek et al. 1997). The mobility of plasmid DNA is negligible in the cytoplasm of microinjected myotubes (Dowty et al. 1995) and HeLa cells (Lukacs et al. 2000). Thus, the DNA becomes sensitive to degradation by the intracellular enzymes. Previous work revealed that 50% of plasmid DNA is eliminated in 12 h from HeLa and COS cells and in 4 h from myotubes (Lechardeur et al. 1999; Pollard et al. 2001). In tissue, radiolabeled plasmid DNA indeed progressively leaves muscles and is degraded as soon as 5 min after plasmid injection (Bureau et al. 2004).

Nuclear Envelope

Besides cytoplasm, the nuclear envelope represents the last physical barrier to gene expression (Miller and Dean 2009). While molecules smaller than 40 kDa can diffuse through the nuclear pore complexes, larger molecules must carry a specific targeting signal, the nuclear localization sequence (NLS) to cross the nuclear envelope (Miller and Dean 2009). The significant size of plasmid DNA (2–10 kDa) makes it unlikely that nuclear entry occurs by passive diffusion (Lukacs et al. 2000). Dividing cells are more transfectable than quiescent ones, suggesting that plasmid DNA enters the nucleus after disassembly of nuclear envelope during mitosis (Miller and Dean 2009). Indeed, cell synchronization affects gene delivery by physical methods, reinforcing the statement that the disappearance of the nuclear envelope facilitates direct access of plasmid DNA to the expression machinery (Chida et al. 1998; Golzio et al. 2002).

Strategies

New challenges are to overcome the limitations represented by cytoplasm crowding and transfer through the nuclear envelope. One possibility is that plasmid DNA becomes cargo on cytoskeletal motors, much like viruses do, and move to the nucleus in a directed fashion (Vaughan et al. 2006). Indeed, the presence of a DNA nuclear targeting sequence (DTS) in the plasmid DNA is specifically recognized by cytosolic proteins carrying a NLS. Thus, it increased the intracellular migration mediated by the microtubule network and nuclear uptake using active transport mechanisms of target cells (Vaughan et al. 2006). Transfection efficiency is improved when plasmid DNA carrying DTS are transferred in dividing and quiescent cells and tissues by microinjection (Vaughan et al. 2006) and electroporation (Vaughan et al. 2006; Zhou and Dean 2007). Moreover, the same team showed that given DTSs can be used to restrict DNA nuclear import to specific cell types, providing a novel means of cell targeting for gene therapy (Miller and Dean 2008). Indeed, they demonstrated that the smooth muscle cell-specific DTS from the smooth muscle γ-actin promoter drives nuclear accumulation of plasmids and subsequent gene expression exclusively in the smooth muscle cell layer of the vessel wall in the intact vasculature of rats using electropulsation-mediated delivery (Miller and Dean 2008; Young et al. 2008). In the same idea, an approach consisted in complexing plasmid DNA with NLS peptides by electrostatic interactions, in attaching these peptides in a random and covalent way to the plasmid DNA and in attaching NLS peptides at a specific site to the plasmid using peptide nucleic acids (Dean 2005). Indeed, NLS plasmid DNA complexes enhance transfection efficiency mediated by electroporation (Mir and Piedrahita 2004; Collins et al. 2007) and sonoporation (Duvhani-Eshet et al. 2006).

Alternatively, it has been shown that nuclear uptake of macromolecules such as plasmid DNA can be enhanced significantly by addition of the amphiphilic molecule *trans*-cyclohexane-1,2-diol (TCHD) (Ribbeck and Gorlich 2002; Lentacker et al. 2008). The mechanism by which TCHD causes nuclear localization of macromolecules can be explained based on the recent finding that the inner channel of the nuclear pore complex is filled with a hydrogel. This hydrogel is a buildup by cross-linked nucleoporins. The cross-links are formed between hydrophobic phenylalanine-glycine repeats present in the nucleoporins. TCHD causes a temporary, nonselective gating of the pore by inducing a disruption of the hydrophobic interactions between the hydrophobic repeats of the nucleoporins. Such a disruption is expected to increase the mesh size of the hydrogel present in the nuclear pore complex and, hence, nuclear entry of macromolecules that are otherwise excluded from the nucleus. It is important to mention that the effect of TCHD is reversible and does not cause damage to the nuclear pores (Ribbeck and Gorlich 2002). To improve gene transfer mediated by sonoporation, Lentacker et al. (2008) proposed treating the target cells with TCHD. Thus, they induced a twofold increase of transfection efficiency (Vandenbroucke et al. 2007; Lentacker et al. 2008).

Another alternative could come from nanosecond pulsed electric fields (nsPEFs). Recent work suggested the benefit of nsPEFs, called "supraelectroporation," in gene transfer (Gowrishankar et al. 2006). This approach consists of applying intense and short electric pulses, i.e., 10–300 kV/cm lasting tens of nanoseconds, in order to induce the permeabilization of intracellular organelles such as the nucleus (Tekle et al. 2005). The authors reported that when nsPEFs (i.e., 10 ns, 150 kV/cm) were applied after classical electroporation pulses (i.e., 3.5 ms, 0.3 kV/cm), transfection efficiency was increased by threefold compared to classical electroporation only (Beebe et al. 2003).

Conclusions

Adverse effects associated with virus-mediated gene therapy trials have stimulated efforts to improve the efficiency of physical vectors. The path taken by the DNA plasmid during gene transfer is not a straight and direct one but one fraught with pitfalls. The additive effect of extracellular and cellular obstacles, accounting for the poor gene-transfer efficiency of physical delivery systems, has been largely determined. At the cellular level, the trafficking of plasmid DNA between the extracellular environment and the nucleus and gene expression is prevented by both physicochemical and metabolic barriers. Thus, improvements in gene-transfer protocols to overcome these obstacles will allow us to achieve safe and efficient therapeutic transgene expression for future clinical applications such as genetic vaccination and cancer gene therapy.

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