Gene electrotransfer: from biophysical mechanisms to in vivo applications

Part 2 - In vivo developments and present clinical applications

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Abstract Gene electrotransfer can be obtained not just on single cells in diluted suspension. For more than 10 years, this is a quasi routine strategy in tissue on the living animal and a few clinical trials have now been approved. New problems have been brought by the close contacts of cells in tissue both on the local field distribution and on the access of DNA to target cells. They need to be solved to provide a further improvement in the efficacy and safety of protein expression. There is a competition between gene transfer and cell destruction. Nevertheless, present results are indicative that electrotransfer is a promising approach for gene therapy. High level and long-lived expression of proteins can be obtained in muscles. This is used for a successful method of electrovaccination.

Keywords Electroporabilization · Electroporation · Gene electrotransfer · In vivo applications

Introduction

Although the applications of tissue electroporabilization are compelling, success has been limited by a poor understanding of the differences between electroporabilization

of single cells and intact tissues. In simple systems, such as isolated cells in suspension, plasmid transport into cells has been shown to be under control of electrical parameters. More than two decades of studies have provided theoretical models of electroporabilization at the membrane level and largely phenomenological understanding at the cellular level. This was summarized in the companion paper. But relatively little basic approach has been done at the tissue level. Because there are different physical barriers and heterogeneous geometries within tissue, in vitro pseudo-tissue models such as dense cell suspensions (Pucihar et al. 2007) and multi-cellular tumor spheroid (Wasungu et al. 2009; Canatella et al. 2004) have been developed to understand the biophysical processes of electroporabilization and gene transfer in tissues. These studies showed a perturbation of local electrical field on dense cell suspensions (Pucihar et al. 2007) and a limitation of gene delivery (Wasungu et al. 2009) related to the self-organization of cells in pseudo-tissues. Indeed, close contacts between cells and extracellular matrix may: (1) modify the electric field distribution, and (2) act as physical barriers that limit the diffusion of DNA plasmid (steric hindrance) and therefore its access to cells present in the core of the tissue. The systematic comparison of biophysical studies from isolated cells to 3D spheroid model allows the development and the optimization of in vivo gene electrotransfer procedures. In vivo biophysical mechanisms and applications of gene electrotransfer can now be addressed (Gehl 2008).

In vivo biophysical considerations

Plasmids are injected in the tissue and electric field pulses are generated in situ by an electrode system (so-called

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applicators) (Luxembourg et al. 2007). Expression is obtained when suitable electrical parameters are applied. The *in vivo* mechanism is still to be elucidated. However, most results bring evidence that *in vivo* gene electrotransfer is a multi-step process where plasmid distribution, cell permeabilization and plasmid electrophoresis act synergically as predicted from experiments at the single cell level (Golzio et al. 2002).

In vitro studies showed that electroporation and electrophoretic forces are involved in the process of gene electrotransfer. As a consequence, Mir and collaborators proposed the combination of short intense pulses (termed HV pulses for high-voltage pulses) and low long pulses (termed LV for low-voltage pulses) for gene delivery (Satkauskas et al. 2002, 2005; Andre et al. 2008). The current data suggest that the HV+LV pulse combination does not bring significant improvement to gene delivery *in vitro* (Kanduser et al. 2009), whereas it is able to boost gene transfer into skeletal muscle (Satkauskas et al. 2005). Indeed, they showed that, under appropriate pulses conditions, the HV permeabilize the cells and the LV, which cannot permeabilize the muscle fibers, are supporting the migration of plasmid towards or across the permeabilized membrane of muscle fibers. The HV+LV combination was extended to other tissues such as liver and skin in rodent models (André et al. 2008). Striking differences between the different tissues were found, likely related to cell size and tissue organization (Hojman et al. 2008). This study has revealed differences in the electrical parameters needed to ensure efficient gene electrotransfer in various tissues. In each tissue, transfection efficiency depends on the plasmid distribution and electric field distribution within the tissue. In particular, plasmid distribution is different in different types of tissue (Andre et al. 2006; Mesojednik et al. 2007). Plasmids distribute easily in muscle; in small rodents, plasmids are dispersed all across the whole muscle. In liver, on the other hand, they found it necessary to perform intravenous injection in order to avoid the hydrodynamic effect of the injection of fluid directly in the tissue (Liu et al. 1999). Moreover, in agreement with the theory of electroporation, higher HV field strengths are required to permeabilize small cells like tumor and skin cells compared to large cells like muscle fibers (Valic et al. 2003). However, no HV is necessary to obtain efficient gene transfer in the liver. Hepatocytes are electrically connected through gap junctions. These junctions permit viewing a cluster of hepatocytes as one large cell, such that the electric potential drop induced by the external field involves a large amplification factor. In consequence, small external field strengths might become effective in electroporating the hepatocyte membrane. Nevertheless, LV parameters must be adapted in each case to the specific tissue. Optimal gene expression with the HV and LV pulse

combination in muscle and skin is obtained with a rather large range of field intensities for the HV pulses. Because the transfection efficiency appears to be due largely to the electrophoretic displacement during LV pulse, this pulse combination is less susceptible to the lack of homogeneity in the field distribution. In fact, in a large part of the tissue, the local electric field intensity of the HV pulse will remain within the window of efficiency over a wide range of field strengths. Thus, using the HV and LV pulse combination allows manipulation over a range of therapeutic field strengths. Moreover, Hojman and collaborators showed that slight cell disturbances occur with the HV+LV combination used for gene transfer. This is highly important, as minimal perturbation of cell physiology is essential for efficient transgene expression (Hojman et al. 2008).

A key parameter of gene electrotransfer is the local electric field strength. As the field results from a voltage applied between two electrodes, the electrode configuration is obviously controlling the field distribution and transfection efficiency (Gehl and Mir 1999). Electrode configurations for therapeutic purposes are parallel plates and wire and contact plate electrodes as well as needle electrodes and arrays (Jaroszeski et al. 1997; Ramirez et al. 1998; Gilbert et al. 1997; Mazères et al. 2009). Electrode configuration controls electric field distribution in tissue. However, due to its anatomy and its electrical properties, tissue reacts to the applied external electric field. If the applied external electric field is high enough, local permeabilization of the tissue occurs, i.e., electric field distribution strongly controls permeabilization (Miklavcic et al. 1998). If the local electric field is too high, an irreversible alteration in cell membrane occurs. This may result in local burns. In gene therapy, it is very important to obtain a large volume of permeabilized tissue, covering whole tissue being subjected to electroporation and preserving cell viability. Therefore, it is necessary to choose optimal electrode configuration and pulse parameters for particular target tissue. A safe approach is to compute in advance the electric field distribution in tissues by means of numerical modeling. Modeling of electric field distribution in tissue is difficult due to heterogeneous material properties of tissue and its shape (Mossop et al. 2006). Numerical modeling has been successfully used and is also validated by comparison of computed and measured consequences of electric field distribution (Miklavcic et al. 1998, 2000). Tissue electrical heterogeneity was never taken into account in the simulation, since tissues were always considered as an amorphous (ohmic) conducting gel (Gowrishankar and Weaver 2003). Electroporation induces a membrane conductance change as previously described (Kinosita and Tsong 1979; Abidor et al. 1994) and observed in tissue (Miklavcic et al. 2000). Due to the swelling, the volume fraction is affected (Deng et al. 2003). Such a geometrical change should affect

the field distribution in tissue and the value of the field at the cell level in the tissue (Pavlin et al. 2002). A precise simulation of the time dependent field distribution in the tissue is clearly needed for different electrode geometries to optimize this electro-technical aspect of the biological treatment (Pucihar et al. 2009).

Plate parallel electrodes and needle arrays are the most popular electrodes to in vivo gene electrotransfer. Plate electrodes offer the advantages that electric pulses can be applied transcutaneously and that electric field between the electrodes is quite homogeneous (Gehl et al. 1999). The two main limitations are (1) the small gap between the electrodes, which is limited by the electrical power of electropulsators, and (2) high field at the contact of the electrode with the skin, which can induce burns. The needle electrodes enable deeper penetration of the electric field into the tissue. However, the electric field distribution is not as homogenous resulting in higher field intensity around the needles. This may lead to local tissue necrosis. The heterogeneous field distribution is under the control of the diameter of each electrode. As a consequence, new electrodes have been designed and tested to minimize tissue damages (Dona et al. 2003; Babiuk et al. 2003).

The various biophysical studies of in vitro and in vivo gene electrotransfer processes allowed the development and the optimization of protocols for routine clinical applications.

In vivo gene electrotransfer

The use of electropulsation for introducing naked DNA plasmid in vivo is a growing field. Several works report the gene electrotransfer into the skeletal (Aihara and Miyazaki 1998; Mir et al. 1999) and cardiac muscle (Dean 2005; Harrison et al. 1998), liver (Heller et al. 1996; Liu and Huang 2002), skin (Titomirov et al. 1991; Vandermeulen et al. 2007), tumors (Rols et al. 1998), spleen (Tupin et al. 2003), kidney (Isaka et al. 2005), lung (Zhou et al. 2007), brain (Saito and Nakatsuji 2001), and joints (Khoury et al. 2006). The feasibility of electropulsation as a nucleic acid delivery method was demonstrated using reporter or therapeutic genes into specific tissues in vivo (Mir et al. 2005).

Gene electrotransfer procedure

Endotoxin-free plasmid solutions are injected in the target tissue localized between the electrodes, where electric pulses are applied. Gene expression depends on the amount of injected plasmid (range of plasmid amount: 20–100 µg) (Mir et al. 1999; Mathiesen 1999) and on the volume of injection (Dupuis et al. 2000). For clinical applications,

different routes of injection are defined on the target: intramuscular (i.m.), intradermal (i.d.), intratumoral (i.t.), and intravenous (i.v.) (Lucas et al. 2002). The volume of injection is limited by the size of the target to avoid a dramatic and damaging swelling. The injection speed is seldom taken into account. But a recent work suggested that the injection speed is a key parameter to gene delivery into skeletal muscle and liver (André et al. 2006). In case of mice skeletal muscle, injection speed of 1.5 µL/s is associated with a high level expression (Golzio et al. 2004).

The delay between injection and electropulsation depends on the tissue. In the case of murine B16 melanoma tumors, a short delay (<1 min) leads to a high level of transfection (Rols et al. 1998). Whereas, in murine skeletal muscle, a delay between a few seconds and 4 h does not change the transfection efficiency (Satkauskas et al. 2001).

The electric pulse parameter used in gene electrotransfer varied between the studies (Mir et al. 1999; Cemazar et al. 2009). Gene expression is detected only when an overcritical voltage to electrode distance is applied bringing about electroporabilization. This threshold is dependent on the target tissues (Heller et al. 1996; Rols et al. 1998). In the case of skeletal muscle, lowest values are found. As explained, the geometry of electrodes technically controls this threshold. It is higher with contact electrodes than with needle electrodes (Gehl et al. 1999). Electric pulse duration must be adjusted with the origin of the tissue. Indeed, an efficient transfection into skeletal muscles and tumors required the application of limited number of long pulses (several ms) at a low frequency (1 Hz). But, a very high number of repetitive short but stronger pulses (several µs) at a high frequency (kHz) give a high level of gene expression into the same tissues (Lucas et al. 2002; Vicat et al. 2000; Rizzuto et al. 1999; Mir et al. 1999; Durieux et al. 2002). A suitable protocol using a short high voltage pulse (kV/cm, µs) followed by several longer low voltage pulses (V/cm, ms) at a low frequency (1 Hz) has been proposed to aid gene delivery into skeletal muscle (Bureau et al. 2000). The first pulses permeabilize the tissue while the other ones act on plasmid electrophoresis (Satkauskas et al. 2002). In the case of liver, low numbers of short (several µs) stronger pulses at a low frequency (1 Hz) efficiently transfect this tissue (Heller et al. 1996).

Electropulsation on live animals may cause side effects such as reversible inflammation (Hartikka et al. 2001), burns of the skin (Lee et al. 2000), vasoconstrictory reflex (Gehl et al. 2002), or tissue destruction depending on the type of electrodes and electric field parameters.

Optimization of the experimental procedures is clearly needed. An empirical approach may be used (Molnar et al. 2004). A more systematic investigation of physical as well as biochemical parameters may bring a more rational evaluation.

In vivo applications

Secreted therapeutic proteins

The skeletal muscle is the most transfected tissue by gene electrotransfer. Indeed, the skeletal muscle has interesting physiological properties such as multi-nucleated (i.e., high number of expression machineries) and long-lived fibers (i.e., long-lasting gene expression), which open several applications in gene therapy (Aihara and Miyazaki 1998; Mir et al. 1999). This organ is composed of long-lived fibers, which allow long-lasting gene expression. Moreover, its rich vascularization makes it a secreting organ of therapeutic proteins (Trollet et al. 2008). Indeed, the i.m. electrotransfer of plasmid-encoding erythropoietin (EPO) induces the production of EPO therapeutic doses in order to treat the anemia related to kidney diseases (Rizzuto et al. 1999; Hojman et al. 2007) or β -thalassemia (Payen et al. 2001). In the same way, TGF β 2 receptor and VEGF-164 factor expressions treat respectively, the lung injury and fibrosis (Yamada et al. 2007) and diabetic neuropathy (Murakami et al. 2006).

Gene electrotransfer allowed the development of anti-tumoral immunotherapies. Indeed, electrotransfer of plasmid-encoding suicide gene (e.g., ePNP/fludarabine) (Deharvenge et al. 2007), cytokines (e.g., IL-12) (Daud et al. 2008), anti-angiogenesis factors (e.g., vasostatin) (Jazowiecka-Rakus et al. 2006), tumor suppressors (e.g., p53) (Kusumanto et al. 2007) into skeletal muscle or tumors stimulated or activated the immune responses against the tumours. This immunotherapy induced a decrease of tumor growth and limited the tumor recovery and metastasis progression in the case of melanoma, hepato-carcinoma, and colon and mammary carcinoma.

Electrovaccination

Gene electrotransfer allowed the development of genetic electrovaccination. Genetic vaccination rests on the principle of direct injection of plasmid encoding vaccinal protein into the muscle or the skin (Wolff et al. 1990; Rice et al. 2008). This protein induces host response and activates the immune system. Compared to the gene therapy, genetic vaccination requires only low and transient gene expressions in few cells (Rice et al. 2008). Several works showed that the gene electrotransfer increase the immune response against the antigen compared to the injection alone (Dupuis et al. 2000; Widera et al. 2000; Babiuk et al. 2002; Dayball et al. 2003). The electrovaccination allowed the development of genetic vaccines against bacterial infections such as *Mycobacterium tuberculosis* (Zhang et al. 2007) and viral infections such as HIV (Hirao et al. 2008). Recently, electrovaccination

showed its efficiency to induce immune response against tumors such as melanoma, and colon and mammary carcinoma (Kalat et al. 2002; Buchan et al. 2005; Curcio et al. 2008).

RNA interference

In the last 10 years, RNA interference (RNAi) has rapidly become an important tool for studying gene functions and holds promise for the development of therapeutic gene silencing (Cheng et al. 2003). RNAi is a post-transcriptional process triggered by the delivery of plasmid encoding short hairpin RNA (shRNA) that induces genes silencing in a sequence-specific manner (Takahashi et al. 2009). Recently, Escoffre et al. (2008) demonstrated that the electrotransfer of plasmid encoding shRNA induces a long-lasting reporter gene silencing into mice muscle. Moreover, this strategy induces an efficient silencing of endogenous genes such as myostatin and TLR-4 involved in the myopathies and inflammatory diseases (Magee et al. 2006; Eefting et al. 2007).

Conclusions

Electropulsation, a biophysical approach, is one of the non-viral methods successfully used to transfer plasmid DNA into living cells in vitro and in vivo. It has the main advantages of being easy to perform, fast, reproducible and safe (Golzio et al. 2004). Currently, it is described by an empirical model supported by direct experimental evidences in which gene electrotransfer appears as a multistep process (see the companion paper). Its further development need a better understanding of the basic effects induced at the membrane, cellular and tissue levels by electrical events and the plasmid entry in the cell. Gene electrotransfer appears promising for gene and cell therapies (Daud et al. 2008). But if the effects of the electric field parameters are under control (electric pulse strength higher than a threshold value, millisecond pulse duration for efficient gene expression), the associated membrane destabilization, which is a stress for the cells and may affect the cell viability, has still to be clearly described (see the companion paper). Moreover, it becomes evident that extracellular barriers, e.g., extracellular matrix and exogenous nucleases, and intracellular barriers, e.g., cytoplasm crowding, endogenous nucleases, and nuclear envelope, compromise the transfection efficiency. Studies will also be necessary to understand the cascade of events triggered by electroporation at the tissue levels where new constraints coming from tissue organizations are present, such as the inhomogeneity of the electric field strength and the intercellular distribution of plasmid DNA.

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