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Electroporation for targeted gene transfer

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The utilisation of nonviral gene delivery methods has been increasing steadily, however, a drawback has been the relative low efficiency of gene transfer with naked DNA compared with viral delivery methods. *In vivo* electroporation, which has previously been used clinically to deliver chemotherapeutic agents, also enhances the delivery of plasmid DNA and has been used to deliver plasmids to several tissue types, particularly muscle and tumour. Recently, a large number of preclinical studies for a variety of therapeutic modalities have demonstrated the potential of electrically mediated gene transfer. Although clinical trials using gene transfer with *in vivo* electroporation have not as yet been realised, the tremendous growth of this technology suggests that the first trials will soon be initiated.

Keywords: cancer, cytokines, electroporation, erythropoietin, gene therapy, vaccines

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1. Introduction

The utilisation of nonviral gene delivery methods has been increasing steadily, partially due to the potential decrease in adverse effects. One of the major drawbacks of this method has been the relative low efficiency of gene transfer with DNA plasmid compared with viral delivery methods. Electroporation (EP) has been used historically to deliver genes *in vitro* for the purpose of transfection and has been an important tool for molecular biology. More recently this technique has been successfully applied to *in vivo* systems for the delivery of small therapeutic molecules. These studies provided the foundation for the utilisation of EP for the effective delivery of plasmid DNA *in vivo* for the purposes of vaccination or gene delivery.

In vivo EP significantly enhances the delivery of molecules and has been used successfully to deliver chemotherapeutic agents in preclinical and clinical studies [1], and more recently in preclinical studies to deliver plasmid DNA to a variety of tissues. During the past few years, many excellent reviews have been written focusing on diverse aspects of electroporation including the theory of EP and the delivery of plasmids encoding for reporter or therapeutic proteins [2-6].

Titomirov *et al.* first demonstrated the delivery of plasmid DNA *in vivo* using newborn mouse skin in 1991 [7]. This was later followed by the measurement and confirmation of expression in skin and other tissues. Subsequent studies in rodents and larger animals have demonstrated delivery into muscle, liver, brain, testes as well as other organs. In addition, delivery for therapeutic purposes has been demonstrated in tumour tissue such as melanoma, squamous cell carcinoma and hepatocellular carcinoma. Delivery of genes by EP into muscle and skin has also been shown to be useful for vaccination purposes most likely due to the enhancement of expression.

The versatility of EP has been established through its effective delivery into muscle [5]: an organ that because of its size and accessibility has become an attractive target for nonviral gene transfer studies. The application of intramuscular delivery of genes through EP has been particularly important for vaccination purposes because of the ability of muscle to provide relatively long-term expression of protein as well as

appropriate presentation of the expressed protein to the immune system. Muscle has also been demonstrated to be an excellent depot for gene-based protein replacement applications. It has also been shown that tumour tissue is a target for the delivery of therapeutic genes through EP [4] based on the previously demonstrated ability to effectively deliver anticancer drugs by this method.

Other gene delivery applications of *in vivo* EP have been directed to skin and other organs including the liver. Much of the previous work on the delivery of DNA, including that with therapeutic potential, has recently been reviewed [4]. A wealth of excellent studies describing delivery of plasmid DNA to a variety of tissues is available in the literature. However, this review is limited in scope to those studies describing *in vivo* electrically mediated delivery of DNA with a demonstrated therapeutic response and potential for use in treating or preventing disease in humans. Most studies involving therapeutic gene delivery are directed to eventual progression to clinical trials; therefore, a demonstration of therapeutic value is of fundamental importance.

2. Cancer therapeutics

Intratumour *in vivo* electroporation of plasmid DNA delivery using reporter genes has been demonstrated in many experimental tumour models [4,5]. Following these studies, plasmid delivery with the assessment of therapeutic response has been performed (**Table 1**). Cutaneous tumours such as melanoma or squamous cell carcinoma lend themselves to electrically mediated delivery. With one significant exception [8], nonsurface tumour models have also been assessed subcutaneously. Although the body site may be anomalous, the antitumour effects observed encourage future orthotopic studies once appropriate electrodes or techniques have been established.

In these initial therapeutic studies, delivery of plasmids encoding well characterised potential antitumour proteins resulted in both tumour growth inhibition and tumour regression. Theoretically, toxins should kill the tumour cells directly. Massive death of tumour tissue is observed in a colon adenocarcinoma model after delivery of a plasmid encoding diphtheria toxin A [9]. In other models, tumour growth inhibition with increased survival is observed in response to electrically mediated delivery of plasmids encoding diphtheria toxin A [10] and herpes simplex virus thymidine kinase (HSV-TK) [10,11]. The direct induction of apoptosis [12-14] or the inhibition of tumour angiogenesis [15-17] also induces tumour growth inhibition. A direct antitumour effect is also induced by interfering with cell signal transduction [18]. Interestingly, growth hormone releasing hormone (GHRH) gene transfer reduced the growth rate of lung carcinomas significantly in males, but not significantly in females [19].

The augmentation of host antitumour immunity by the localised or systemic expression of cytokines appears to be the most successful therapeutic approach in affecting growth of all types of tumours. Several T helper cell type 1 (T_H1) cytokines including IL-2 [20-22] and -12 [21,23-25], and IL-12/IL-18 [25], as well as granulocyte macrophage colony stimulating factor [20,22] induce significant tumour growth inhibition, increase survival time and inhibit metastases. Complete tumour regression followed by long-term antitumour immunity, which is the ultimate goal of cancer therapeutics, has been demonstrated in response to plasmids encoding IL-12 [26-29], IFN-α [30,31], and to DNA containing CpG motifs [32]. In general, when localised tumour expression is induced, the severe systemic side effects associated with cytokines are not observed. In spite of the minimal systemic expression, effects against additional untreated tumours have been reported after intratumour delivery of IL-12 [27] or IL-12 and -18 [33]. These encouraging results indicate that host immunity against metastatic disease can be stimulated after localised therapy.

Combined therapies may also induce complete tumour regression followed by long-term antitumour immunity. Two studies in mouse melanomas [34,35] monitored the effects of cytokine plasmid delivery combined with electrochemotherapy; that is, the delivery of the chemotherapeutic agent bleomycin with electric pulses [36,37]. In each study, the combined therapies were more effective than either therapeutic agent alone. In theory, the delivery of the chemotherapeutic agent results in complete regression of the primary tumour, whereas the addition of cytokine expression induces an antitumour immune response. A second set of studies combined delivery of a plasmid encoding IL-12 with recombinant Bacillus Calmette–Guerin protein [38]. The combination acted synergistically to induce tumour regression in a bladder cancer model.

In general, direct intratumour plasmid delivery is more effective in inducing a direct antitumour effect than the intramuscular delivery, which results in systemic transgene expression. However, serum expression of cytokines may be advantageous for the prevention of metastases when the expressed protein can be kept below toxic levels. In a model with established melanoma tumours, expression of IL-12 [26] or IFN-α [30] following intramuscular delivery does not effect tumour growth. However, in a squamous cell carcinoma model, these cytokines significantly reduced tumour growth [39,40]. In subcutaneous lymphoma, colon adenocarcinoma and melanoma with significantly smaller tumours, IL-12 induced tumour regression and increased survival time [41].

Transgenes other than cytokines have also been tested using intramuscular delivery of plasmid DNA. Delivery of plasmids encoding tyrosinase-related protein-2 and gp100 (the marker for melanoma cells) increases survival of mice bearing B16 lung metastases [42], whereas endostatin expression reduces the number of lung metastases [15]. In a human breast tumour model in nude mice, tumour growth is inhibited by intramuscular electrotransfer of plasmids encoding plasminogen domains, which inhibit endothelial cell proliferation [43]. These plasmids also acted as vaccines, preventing lung metastases after injection of B16 melanoma

Table 1. Antitumour effects following electrically mediated intratumour delivery of therapeutic plasmid DNA.

Experimental model*	Therapeutic gene/cDNA	Most significant antitumour observation	Ref.
Orthotopic C6 rat glioma	Monocyte chemoattractant protein-1	Macrophage and lymphocyte infiltration	[8]
B16 mouse melanoma	Dominant-negative Stat3	Significant tumour regression	[18]
CT26 mouse colon adenocarcinoma	Diphtheria toxin A fragment or HSV-TK	Significant tumour growth inhibition	[10]
C6 rat glioma	Diphtheria toxin A fragment	Massive death in tumour tissue	[9]
MH134 mouse hepatocellular carcinoma	IL-12	Significant tumour growth inhibition, significant inhibition of lung metastases	[23]
B16 mouse melanoma	IL-2 or -12	Significant tumour growth inhibition	[21]
CT26 mouse colon adenocarcinoma Renca mouse renal cell carcinoma	IL-12	Significant tumour growth inhibition	[24]
B16 mouse melanoma	IL-12 or IL-12/IL-18	Significant tumour growth inhibition, significant increase survival	[25]
T.Tn human oesophageal tumour cells	GM-CSF or IL-2	Significant tumour growth inhibition, significant increase survival	[20]
MH134 mouse hepatocellular carcinoma	TRAIL/Apo2 ligand	Significant tumour growth inhibition, significant inhibition of lung metastases	[12]
MH134 mouse hepatocellular carcinoma	Tie2 vascular endothelial receptor	Significant tumour growth inhibition	[17]
PC-3 human prostate cancer	Wild-type p53	Significant tumour growth inhibition	[13]
SCCVII mouse squamous cell carcinoma	IL-12	40% Long-term tumour regression, 50% long-term antitumour immunity	[28]
B16 mouse melanoma	IL-12	47% Long-term tumour regression, 70% long-term antitumour immunity	[26]
B16 mouse melanoma	IFN-α	70% Long-term tumour regression, 50% long-term antitumour immunity	[30]
B16 mouse melanoma	CpG motif DNA	83% Long-term tumour regression, 70% long-term antitumour immunity	[32]
BJMC3879 mouse mammary carcinoma	HSV-TK	Significant tumour growth inhibition	[11]
Renca mouse renal cell carcinoma	Endostatin	Significant tumour growth inhibition	[15]
SCCVII mouse squamous cell carcinoma	IFN-α	20% Long-term tumour regression, significant increase survival	[31]
B16 mouse melanoma	IL-12	80% Long-term tumour regression, 100% long-term antitumour immunity	[27]
1MEA.7R1 mouse hepatoma	IL-2/GM-CSF	Marked tumour growth inhibition	[22]
A549, MDA-MB-453, Detroit 562 carcinonas, primary stomach cancer	Antisense Plk1/Bcl-2	Significant tumour regression	[14]
CT26 mouse colon adenocarcinoma	IL-12/IL-18	Significant tumour growth inhibition, significant suppression contralateral tumours	[33]
CT26 mouse colon adenocarcinoma	Angiostatin/endostatin	Significant tumour growth inhibition	[16]
CT26 mouse colon adenocarcinoma	Il-12/HSV-TK	91% Long-term tumour regression, 75% long-term antitumour immunity	[29]
Lewis lung adenocarcinoma	GHRH	Significant tumour growth inhibition in males	[19]

*Tumours were generated by subcutaneous injection unless otherwise noted.

Apo: Apolipoprotein; GHRH: Growth hormone releasing hormone; GM-CSF: Granulocyte macrophage colony stimulating factor; HSV-TK: Herpes simplex virus thymidine kinase; TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand treatment.

cells. Interestingly, repeated intramuscular delivery of plasmids coding for Her-2/*neu* oncogene domains halts mammary tumour progression in mice transgenic for the rat Her-2/*neu* oncogene [44]. One study investigated electrically mediated gene transfer as a palliative for symptoms related to cancer and cancer therapy, rather than as a direct therapeutic agent. After intramuscular delivery of a plasmid encoding GHRH, companion dogs with naturally occurring tumours [45] were able to maintain their weights, thus ameliorating cancer-associated cachexia and weight loss.

In vitro data do not necessarily predict the therapeutic potential of a transgene. For example, transfection with tissue inhibitor of metalloproteinase 4 (TIMP-4) before injection of tumour cells results in inhibited tumour growth in a human breast cancer cell model in nude mice [46]. However, in the same model, the systemic expression resulting from intramuscular electroporation of a plasmid encoding TIMP-4 surprisingly stimulates tumourigenesis [47]. Conversely, in a Wilms' tumour model, also in nude mice, intramuscular delivery of TIMP-4 significantly slows tumour growth [48]. These results also indicate that therapeutic proteins do not act equally in all experimental models. This emphasises the importance of preclinical research.

Thus far, IL-12 is the transgene most successful in inducing a medley of antitumour effects. Although the effect of the IL-12 plasmid has been demonstrated in several models, the absolute response has varied from simple slowing of tumour growth to high percentages of complete long-term regression accompanied by long-term antitumour immunity. Several variables may play a role in these observed differences. For example, each tumour model employed may differ in its response to the antiangiogenic factors of IL-12, as well as to the immune response induced. The size of the tumour at the initiation of treatment can be a large contributing factor. The plasmid dosage, purification method and the specifics of plasmid construction may influence transgene expression. The electroporation parameters and electrode used are also important variables, although as long as adequate expression is achieved, therapeutic results have been observed after delivery with both short, high voltage pulses and long, low voltage pulses. Finally, the number and timing of treatments has been optimised for particular models in several studies.

The clinical applicability of these remarkable experimental results has not been demonstrated so far. In particular, IL-12 has demonstrated a recurring and dramatic antitumour effect in several cancer types including melanoma, squamous cell carcinoma and colon adenocarcinomas, without inducing the traumatic side effects observed in systemic recombinant protein therapy [49,50].

3. Protein replacement therapies

An important area of development for gene transfer applications is the treatment of protein deficiencies. Current therapies typically utilise injections of recombinant proteins. In addition to the need for frequent administrations, these therapies are typically expensive. A gene transfer approach has the potential to minimise the number of required administrations as well as reduce the cost. In order to reach this potential, it is critical to demonstrate that a gene-based approach can safely achieve expression of the delivered transgene to obtain therapeutic levels of the protein of interest and to maintain those levels with a reduced number of administrations. There have been several studies that have utilised *in vivo* electroporation to facilitate gene delivery to not only obtain the desired levels, but also to maintain those levels for significant periods of time. The vast majority of these studies have used intramuscular delivery.

A focus of many studies has been the delivery of a plasmid encoding for erythropoietin (EPO): a 30.4 kDa glycosolated protein. Recombinant EPO is currently used to treat many acquired and inherited disorders. However, due to the cost and frequency of injections, a gene transfer approach would be advantageous for most of these treatment protocols. To obtain necessary expression levels and to increase the length of expression, *in vivo* EP following intramuscular injection has been used. It was demonstrated in mice that this delivery approach could result in haematocrit levels being significantly elevated by day 7 and maintained for as long as 6 months [51,52]. This was accompanied by a 100-fold increase in serum levels of EPO following the delivery of plasmid with EP when compared with plasmid injection alone, and clinically effective quantities of circulating EPO were observed from day 7 to $\geq$ 6 months [51]. EPO levels in the muscle were 10-times higher than serum concentrations, thus demonstrating that the muscle acted as a reservoir [52]. When comparing a single intramuscular plasmid delivery with EP with daily subcutaneous injections of recombinant EPO (5 days) in rats, the daily injections resulted in highly elevated levels for the first couple of days and then quickly dropped to control levels, whereas the EP gene approach resulted in maintained levels of serum EPO [53]. Although the effect is not as long-lived, delivery to the skin using EP can also enhance expression and result in increased levels of serum EPO and a corresponding increase of haematocrit levels [54].

A greater assessment of EP-mediated delivery is to evaluate its potential in clinically relevant models. Two models for severe anaemia are a mouse model for β-thalassemia and nephrectomised rats as a model for chronic renal failure. In the β-thalassemia mice, haematocrit levels more than doubled within 2 weeks in mice that received intramuscular injection of plasmid encoding EPO followed by EP. This high level was maintained for 2 months followed by a gradual decrease, although at 4 months these levels were still above background levels [55]. Interestingly, EPO levels began to drop after 2 weeks, but this did not affect the increased haematocrit. A second treatment at 8 months restored the high haematocrit levels seen in the first 2 months. Similar results were noted in the nephrectomised rats. Haematocrit levels were significantly increased following the intramuscular injection of plasmid encoding EPO followed by EP [56]. Both of these studies demonstrated that the level of haematocrit increase was directly related to the plasmid dose. Another study demonstrated that the levels of EPO could be regulated by utilising an inducible plasmid [57]. A recent study demonstrated another method of regulating expression levels.

Haemophilia B is a disorder that results from a deficiency of blood clotting factor IX (FIX). As with EPO, therapy consists of injections of recombinant forms of FIX. Protein

replacement therapy could also be accomplished using a gene transfer approach. Two studies evaluated the delivery of a plasmid encoding for human FIX delivered intramuscularly with EP. Both studies showed increased serum levels of FIX [58,59]. Peak expression was seen at 7 days followed by a rapid drop. The drop may have been due to antibodies against the human FIX protein. When delivered to severe combined immunodeficient or nude mice, serum FIX levels were maintained for > 8 weeks [58]. Another interesting aspect was that serum levels were found to be higher when the delivery was performed in the mouse tibialis compared with the gastrocnemius muscle [59]. Interestingly, increased serum levels could also be obtained when the procedure was performed in dogs; however, there was not a muscle-dependent difference in muscle [59]. Another area for which this delivery approach may be useful is the treatment of Type I diabetes. The EP enhanced intramuscular delivery of a plasmid encoding for a furin-cleavable proinsulin resulted in prolonged survival of mice with severe diabetes. Delivery resulted in increased levels of insulin and maintenance of body weight [60]. In a non-obese diabetic mouse model, combined gene transfer of preproinsulin and mutant B7-1 altered the autoimmune anti-insulin response [61].

Localised protein replacement is another potential application. The enhanced intramuscular delivery of EP mediated delivery may make it suitable for possible therapies for Duchenne muscular dystrophy. Studies have evaluated the delivery of plasmids encoding for dystropin, utropin or microdystropin to mouse models of muscular dystrophy [62-66]. Long-term expression was achieved. There was an observed difference in longevity and levels of expression between immunocompetent and immunodeficient mice, thus demonstrating that control of the immune response will be a critical component of this approach. Molnar *et al.*, also demonstrated that levels of expression were directly related to the size of plasmid. Expression levels were reduced when full-length dystropin was delivered compared with microdystropin or a reporter gene. Utilising a different electrode and pulsing conditions, Murakami *et al.* delivered full-length dystropin to 56% of muscle fibres.

4. Vaccines

Delivery of DNA plasmids as vaccines has been demonstrated to elicit moderate to potent immunity in small animals such as rodents (i.e., rats and mice), but has proven to be less effective in primates. Several potential mechanisms for this inefficiency have been proposed, including inefficient presentation of antigen through typical T_H1 and T_H2 immune mechanisms. This problem has been somewhat obviated through the use of codelivered cytokines and costimulatory molecules that function as adjuvants and can be administered as either proteins or DNA expression plasmids [67-69]. Another potential mechanism for the diminished efficacy of DNA vaccines is the inefficient uptake of the DNA plasmids by the cells *in situ*. One delivery method utilised to address this problem has been *in vivo* EP. The goals of EP mediated vaccine delivery studies have been to significantly enhance either or both arms of the immune system (i.e., humoral and cellular) in order to more efficiently mediate protection against the particular infectious agent. Specifically, *in vivo* EP has been utilised to enhance immune responses including the development of antibody levels that correlate with protection.

In vivo EP has been performed using DNA plasmids expressing antigens from a number of viral pathogens including, importantly, the hepatitis B virus (**Table 2**). Specifically, the *in vivo* EP delivery of a weakly immunogenic hepatitis B surface Ag (HBsAg) DNA vaccine was significantly increased in mice as measured by a greater magnitude of antihepatitis B antibodies [70]. It was also shown that in mice and pigs immune responses against the HBsAg were enhanced after delivery of a DNA plasmid expressing this antigen plus EP [71-74]. Finally, enhancement of the effectiveness of EP augmented cutaneous DNA vaccination against hepatitis B was demonstrated when adjuvanted by a gold particle [75].

Enhancement of humoral- and cell-mediated immune responses against the hepatitis C virus E2 glycoprotein with *in vivo* EP in mice, rats and rabbits was demonstrated [76]. Other viral targets used in DNA vaccine efficacy studies with *in vivo* EP include bovine herpesvirus [71,74], influenza [77-79] and Japanese encephalitis [80]. The specific vaccine targets expressed a glycoprotein for bovine herpesvirus, neuraminidase or haemagglutinin for influenza and envelope protein for Japanese encephalitis virus.

The development of a successful vaccine against the retrovirus HIV is of paramount importance for the ultimate control of the devastating AIDS pandemic. A number of different vaccine approaches have been utilised against HIV, including recombinant proteins, live attenuated viral vectors and recently DNA vaccines that express relevant and immunogenic proteins from the virus. In fact, the world's first human clinical trials using DNA vaccines were conducted with plasmids expressing HIV-1 antigen [81,82]. Although the vaccines appeared to be safe, cellular immune responses were low with no significant humoral immunity being induced. Low immune responses were also noted experimentally in studies with these vaccines in non-human primates. Recently, *in vivo* EP has been utilised in various animal models. Widera *et al.* demonstrated in mice that the immunogenicity of a gag HIV-1 DNA vaccine was increased significantly in mice as measured by increases in antibody titres, a substantial reduction in the dose of the DNA vaccine to elicit an immune response and an increase in HIV-1 gag-specific $CD8^+$ T cells [70]. This was accompanied by the demonstration of significantly higher expression levels with the EP procedure. Similarly, specific antibody responses were significantly enhanced against HIV gag and env DNA vaccines in guinea-pigs and rabbits by *in vivo* EP [70]. Importantly, Otten *et al.* demonstrated

Table 2. DNA vaccine delivery for infectious diseases by *in vivo* electropration.

Experimental model	Plasmid encoded gene/cDNA	Most significant therapeutic observation	Ref.
Mice	Hepatitis B surface antigen*	Increased humoral immune response	[70]
Guinea-pigs, rabbits	HIV gag and envelope proteins*	Increased humoral and $CD8^+$ T-cell immune responses	
BALB/c mice	Influenza A haemagglutinin*	Protection against sublethal challenge	[77]
BALB/c mice	Influenza A subtype neuraminidases*	Protection against lethal challenge within subtype	[78]
Mice, rats, rabbits	Hepatitis C virus E2 glycoprotein*	Increased humoral and cell-mediated immune responses	[76]
BALB/c mice	Haemagglutinin gene of influenza virus A*	Increased humoral and cell-mediated immune responses	[79]
B6D2 or BALB/c mice	*Mycobacterium tuberculosis* 85b antigen*	Increased humoral and cell-mediated immune responses	[84]
Pigs	Hepatitis B surface antigen; glycoprotein D of bovine herpesvirus*	Increased humoral and cell-mediated immune responses	[71,72,74]
Pigs	Hepatitis B surface antigen‡ with protein boost	Protective antibody levels	[73]
Mice	Hepatitis B surface antigen‡ with gold particles	Increased percentage of responding animals; shortened time to maximum antibody response	[75]
C3H/HeN mice	Japanese encephalitis envelope protein*	Protection against lethal virus challenge	[80]
Sheep	Two *Haemonchus contortus* antigens*	Increased humoral responses; vaccine-specific immune memory	[85]
Rhesus macaques	HIV gag and envelope proteins*	Increased humoral and cell-mediated immune responses	[83]

*Muscle delivery. ‡Intradermal delivery.

that *in vivo* EP enhanced antibody, T_H cell responses and cytotoxic T-cell responses against plasmids, which express HIV gag and env [83].

In addition, *in vivo* EP has been shown to enhance immune responses against antigens from other infectious agents besides viruses when expressed from DNA vaccines. Specifically, Tollefsen *et al.* have demonstrated improved cellular and humoral immune responses against *Mycobacterium tuberculosis* antigens after intramuscular DNA immunisation combined with intramuscular EP [84]. Similarly, Scheerlinck and colleagues demonstrated an enhancement of immune responses to antigens of the ruminant parasite *Haemonchus contortus* when the specific DNA expression plasmids were delivered with *in vivo* EP [85].

In summary, *in vivo* EP has been effectively utilised as a method to enhance immune responses against antigens expressed by DNA plasmid based vaccines. Several potential mechanisms have been put forth for the immune enhancing effects of EP. The major ones are the enhancement of uptake of the DNA plasmid vaccines as well as increased expression of the antigens within the plasmid. In addition, it has been hypothesised that EP generates a local inflammatory response, which stimulates the recruitment of lymphocytes, with the resultant enhancement of immune responses.

5. Growth factors

As growth factors may be short-lived, multiple injections of recombinant protein may be necessary to maintain therapeutic levels. Gene transfer enhanced by muscle EP may result in long-lived systemic expression, thus avoiding the necessity of repeated injections. In cases where systemic expression is not desirable due to toxicity, EP may possibly be performed directly on the target tissue. Plasmids may be engineered for localised expression. In some instances, decreased growth factor expression may be desirable. In these cases, the delivered constructs may be designed to interfere with or cleave specific mRNAs, thus decreasing protein levels, or to bind the protein directly to block signal transduction.

Electrically mediated gene transfer of several growth factors has been tested as a therapeutic agent in a wide variety of clinical applications (**Table 3**). A variety of kidney disorders in experimental models have been ameliorated by interference with platelet derived growth factor [86] or transforming growth factor-β (TGF-β) expression [87,88], or by expression of hepatocyte growth factor (HGF) [89-92]. Wound healing in diabetic mice is enhanced by the delivery of plasmids encoding keratinocyte growth factor [93] or TGF-β [94]. After optic nerve injury, apoptosis in retinal ganglion cells is decreased by the delivery of brain-derived neurotrophic factor [95]. Muscle regeneration has

Table 3. Electrically mediated delivery of therapeutic plasmid DNA encoding hormones and growth factors.

Growth factor	Experimental model	Most significant observation	Ref.
PDGFR–IgG chimaera	Anti-Thy1 treated Sprague-Dawley rats*	Decreased experimental glomerulonephritis	[86]
GDNF	SOD1 mice*	Delayed deterioration of motor performance	[104]
Cardiotropin-1	pmn mice*	Delayed deterioration of motor performance, increased survival	[105]
BDNF	Wistar rats‡	Decreased apoptosis in retinal ganglion cells after optic nerve injury	[95]
NT-3	OF1 mice	Partially prevented cisplatin-induced neuropathy	[102]
SCF	Sl mutant mice§	Rescued defective spermatogenesis	[103]
HGF	C57Bl/6J mice*	Decreased apoptotic hepatocytes and ALT activity after CCl_4 intoxication	[100]
	5/6 nephroctomised Sprague-Dawley rats*	Increased body weight, reduced histological changes	[89]
	Cardiomyopathic TO-2 hamsters*	Increased myocardial function and capillary density	[99]
	Sprague-Dawley rats*,¶	Suppressed cyclosporin nephrotoxicity	[90,91]
	Diabetic rat*	Regression of advanced diabetic nephropathy	[92]
	C57Bl/6 mice*	Reduced bleomycin-induced pulmonary fibrosis; increased survival	[101]
bFGF	Japanese white rabbits*	Increased development collateral vessels in ischaemic limbs	[98]
IGF-1	BALB/c mice*	Increased number and diameter of regenerating muscle fibres and compound muscle action potential after injury	[96]
	Diabetic ICR mice*	Increased angiogenesis and arterial flow to limb	[97]
KGF	Diabetic mice#	Accelerated wound closure	[93]
TGF-βR–IgG chimaera	ICR mice*	Suppressed cyclosporin nephrotoxicity	[87]
anti-TGF-β DNAzyme	Anti-Thy1 treated Sprague-Dawley rats*	Decreased experimental glomerulonephritis	[88]
TGF-β1	Type II diabetic mice#	Accelerated wound healing	[94]

Delivery to: *muscle; ‡vitreous; §testes; ¶kidney; #excisional wound.

ALT: Alanine aminotransferase; BDNF: Brain-derived neurotrophic factor; bFGF: Basic fibroblast growth factor; CCl_4: Carbon tetrachloride; GDNF: Glial cell line-derived neurotrophic factor; HGF: Hepatocyte growth factor; IGF: Insulin-like growth factor; KGF: Keratinocyte growth factor; NT: Neurotrophin; PDGFR: Platelet-derived growth factor receptor; pnm: Progressive motor neuronopathy; SCF: Stem cell factor; TGF-β: Transforming growth factor-β; TGF-βR: Transforming growth factor-β receptor.

been observed after the delivery of a plasmid encoding insulin-like growth factor-1 (IGF-1) [96]. Localised expression of IGF-1 also increased angiogenesis in an ischaemic limb model [97], as did basic fibroblast growth factor [98]. HGF gene transfer also increases heart function and capillary density in a cardiomyopathy model [99].

Growth factors may also be used to reduce the adverse effects associated with therapeutic agents. Delivery of several plasmids encoding growth factors decrease toxicities associated with carbon tetrachloride (CCl_4) intoxication [100] or with the clinical use of cyclosporin A [90,91,87], bleomycin [101] or cisplatin [102].

Electrically mediated testicular delivery of a plasmid encoding stem cell factor has been used in direct tissue-specific gene replacement in a mutant mouse model to successfully restore Sertoli cell function [103]. An indirect approach has been tested in motor neuron disease models. Muscle gene transfer of glial cell line-derived neurotrophic factor (GDNF) hampered the decrease of GDNF observed during the progression of muscle pathology in SOD1 mutant mice [104]. Deterioration of motor neurons was delayed. However, no significant effect on survival was observed. In the progressive motor neuronopathy (pmn) mouse model, intramuscular delivery of a plasmid encoding cardiotropin-1 resulted in increased survival and muscle function [105].

6. Additional electroporation facilitated therapies

In vivo EP has been utilised to enhance the delivery of plasmid DNA in a variety of tissues including muscle, skin, liver and tumour [4,5]. The ability to use this delivery tool in multiple tissue systems makes it applicable to a variety of potential therapeutic applications. Dependent on the specific tissue

chosen for delivery, a transgene could be delivered for either a localised or systemic effect.

An interesting application of EP-assisted plasmid DNA delivery has been the enhancement of DNA vaccines. As summarised in Section 4 both cellular and humoral responses were obtained against antigens from a variety of infectious agents. The induction of monoclonal antibodies against a specific antigen can have significant therapeutic potential beyond the prevention of infections including potential efficacy against cancer and autoimmune diseases. In addition to inducing production of antibodies in response to an antigen, it may also be advantageous to deliver a plasmid encoding for the antibody and directly increase the serum levels of the desired antibody. Intramuscular delivery of plasmids encoding heavy chain, light chain or both resulted in serum levels of the desired monoclonal antibody. Antibody levels were significantly higher when the plasmids were delivered with EP [106,107]. Using an inducible Tet promoter allowed control over antibody production, but overall levels were low. Higher levels may be achieved by further optimising the delivery parameters [107]. Tjelle *et al.* achieved sustained serum levels of antibody following EP-mediated intramuscular delivery in both mice and sheep [106].

Rheumatoid arthritis (RA) is a chronic inflammatory disease. IL-1, TNF-α and matrix metalloproteinases are involved in the detrimental effects noted in RA. Damage is seen in cartilage and joints. Studies have evaluated using plasmids encoding for either IL-1 receptor antagonist (IL-1Ra) or TIMP-4 using intramuscular delivery with EP. TIMP-4 therapy abolished the development of RA [108] and IL-1Ra inhibited the onset of collagen-induced arthritis [109]. Delivery of pro-opiomelanocortin by intramuscular delivery and EP reduced the pain associated with arthritis [110]. A similar approach was used to suppress neuropathic pain in rats [111].

Cardiovascular disease is a major worldwide healthcare issue. In this area conditions such as viral myocarditis and atherosclerosis have been targeted for gene therapy regimens. Gene therapy approaches are being evaluated for their effectiveness in treating or preventing these diseases. *In vivo* EP has been used to deliver calcitonin gene-related peptide [112] or platelet-activating factor acetylhydrolase [113] to limit the progression of atherosclerosis. Both studies delivered the plasmids intramuscularly followed by EP. In both cases, this approach inhibited thickening of the aorta. To reduce the effect of viral myocarditis, either IL-1Ra or viral IL-10 was delivered intramuscularly with EP. There was a significant improvement in survival and overall effectiveness in treating viral myocarditis [114].

The utility of EP was also demonstrated for potentially delivering agents for the treatment of stroke. A plasmid encoding protein disulfide isomerase was delivered directly to the hippocampus. Subsequent expression of this protein reduced the number of ischaemia-induced apoptotic cells in the treated area [115]. *In vivo* EP has also been used to intramuscularly deliver a plasmid encoding a dominant negative inhibitor of monocyte/macrophage chemoattractant protein-1 and demonstrated that this approach could inhibit progression of pulmonary hypertension [116]. Direct delivery to the lungs was performed delivering a plasmid encoding the beta1 subunit of the Na^+,K^+-ATPase. When delivered with EP there was an increase in alveolar fluid clearance [117]. EP has also been used to deliver agents for treatment of autoimmune diseases such as lupus. Intramuscular delivery of a plasmid encoding IFN-R/Fc reduced disease manifestations when administered in both predisease and advanced disease stage in a mouse model of lupus [118].

Recently, a plasmid encoding for the muscarinic M_3 (M_3) receptor was delivered using EP directly to the bladder in a rat model. The investigators found that overexpression of M_3 resulted in enhanced smooth muscle contractility in the bladder and may be useful as a treatment for reduced bladder function [119]. Delivery of a plasmid encoding matrix metalloprotease-3 into rabbit conjunctiva with EP was examined in combination with trabeculectomy for glaucoma filtering surgery. This combination resulted in reduced levels of intraocular pressure and longer survival of filtering bleb [120].

7. Expert opinion and conclusions

EP is a physical method that has been widely used for the *in vitro* transfection of cells. Because of its physical nature, it was evident that EP could be applied to virtually any cell or accessible tissue. In the early 1990s the delivery of molecules to cells within tissues *in vivo* by EP was demonstrated. *In vivo* EP effectively delivered chemotherapeutic agents to a variety of different tumours in experimental models and in humans [1]. The large number of studies that demonstrated that EP could be safely and effectively applied *in vivo* to deliver small molecules in both animal and human studies provided the foundation for the use of EP to deliver plasmid DNA *in vivo*.

The use of EP for the *in vivo* delivery of plasmid DNA has seen tremendous growth in the past few years. The published studies demonstrate that therapeutic plasmid DNA delivery can potentially achieve the same success as drug delivery [4,5]. With electrically mediated gene therapy, both localised and systemic effects can be elicited. Gene therapy is applicable to many diseases including metabolic diseases and cancers. The translation of EP gene delivery to clinical applications may not necessarily utilise the same EP protocols as seen with drug delivery and will require additional pharmacological and toxicological studies. However, it is encouraging that as *in vivo* EP has been successfully translated into the clinic for drug delivery, electric fields can be applied safely.

The increased use of EP plasmid DNA delivery is related to its versatility. Plasmids have been delivered to a wide range of targets including internal and surface tissues. In addition to target versatility, EP delivery also allows control over expression profiles [121]. Careful selection of electrical parameters, electrode configuration and tissue site can result in high or low

expression levels of long or short duration. Choosing the appropriate EP parameters to achieve the right expression profile can mean the difference between success or failure of a particular therapeutic application. Short-term or low-level expression may be an advantage, particularly for plasmids encoding potentially toxic molecules such as immune modulators. In addition, it has recently been reported that by using a particular intramuscular EP delivery protocol, integration of the plasmid DNA into genomic DNA was observed [122]. The versatility of this delivery approach is a major advantage. However, when evaluating a new application for EP delivery, several variables should be considered to achieve the appropriate expression profile for a particular application.

Electrically mediated delivery of plasmid DNA is actually a two-step process. This concept has been evaluated in several studies, which have indicated that electroporative plasmid delivery does involve two distinct components [123,124]. The concept of using an EP pulse to permeabilise the membrane followed by an electrophorectic pulse to move the plasmid through the membrane was first tested *in vitro* and reported in 1992 [125]. This concept has also been demonstrated in plasmid DNA delivery to muscle *in vivo* [126]. When applied in combination similar expression levels can be achieved as with a single pulse type. The advantage is that each component can utilise milder EP pulsing parameters. This may lessen any real or perceived discomfort from the pulsing protocol and may facilitate translation of this delivery approach into the clinic.

As the use of EP delivery has grown, investigators often compare new delivery conditions with previously published results. In these comparisons, it is critical to evaluate all EP parameters. In addition to voltage (field strength), the discussions should include differences in additional electrical parameters such as pulse width, number and frequency (pulses/second), as well as electrode configuration/instrumentation and targeted tissue. Electrode configuration is very important: particularly comparisons of plate- and needle-type electrodes [127]. Currently, a number of types of equipment and electrodes for performing *in vivo* EP are available from several sources [128]. As the use of EP for gene transfer protocols continues to grow, so will the importance of utilising the appropriate electrode configuration. For example, one promising application for this delivery approach is the treatment of cancer. However, subcutaneous implants have primarily been used for *in vivo* EP delivery in the experimental models of cancer investigated so far. Orthotopic studies have been rare. For future advancement, it will be important to develop electrodes for effective delivery to internal tumours. Another advance that may facilitate the translation of this technology, particularly for the delivery of DNA vaccines, is needleless injections. Recent studies have shown the utility of combining jet injection with EP for delivery to both muscle and skin [73,129]. Several studies have evaluated the mechanisms and theories involved in the use of *in vivo* EP for the delivery of plasmid DNA [2,4,5].

It is evident from the growing number of studies utilising EP-mediated delivery of plasmid DNA that this approach is a useful tool for nonviral gene transfer. Although the concept of *in vivo* EP is relatively recent, a great deal of work on a large number of potential applications has been performed. The first *in vivo* EP studies describing the delivery of plasmids encoding for proteins with therapeutic potential were not reported until 1999. During the last few years, there has been a tremendous increase in the number of studies evaluating the therapeutic potential of this delivery approach and developing potential clinical protocols. Based on the accumulated positive results, it would appear that the first clinical application would be for the treatment of solid tumours. Another strong candidate for a clinical application of this approach is the delivery of DNA vaccines. Preclinical studies in both of these areas strongly suggest that *in vivo* EP will be useful in the implementation of successful gene therapy protocols. However, the true potential of this approach cannot be fully evaluated until clinical studies are actually initiated.

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