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SHORT COMMUNICATION

Electroporation-mediated delivery of a naked DNA plasmid expressing VEGF to the porcine heart enhances protein expression

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Gene therapy is an attractive method for the treatment of cardiovascular disease. However, using current strategies, induction of gene expression at therapeutic levels is often inefficient. In this study, we show a novel electroporation (EP) method to enhance the delivery of a plasmid expressing an angiogenic growth factor (vascular endothelial growth factor, VEGF), which is a molecule previously documented to stimulate revascularization in coronary artery disease. DNA expression plasmids were delivered in vivo *to the porcine heart with or without coadministered EP to determine the potential effect of electrically mediated delivery. The results showed that plasmid delivery through EP significantly increased cardiac expression of VEGF compared with injection of plasmid alone. This is the first report showing successful intracardiac delivery, through* in vivo *EP, of a protein expressing plasmid in a large animal.*

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Advances in the treatment of coronary artery disease (CAD) have been attained through interventions, such as angioplasty and coronary artery bypass surgery.[1] However, some CADs are not amenable to these interventions, indicating that development of other therapies is required. One such therapeutic strategy is the induction of revascularization through targeted gene therapy.[2–6] Vascular endothelial growth factor (VEGF) is an angiogenic protein used to stimulate angiogenesis in models of CAD and, as such, has been evaluated as a therapeutic target. Genes encoding VEGF isoforms can potentially circumvent many of the obstacles presented by restenosis and other cardiovascular pathologies through the stimulation of potentially therapeutic collateral vessel formation.

Direct naked/nonviral DNA plasmid injection and adenoviral-mediated gene transfer[6,7] have shown promise for treating CAD. In addition to providing symptomatic relief during ischemia, studies indicated that VEGF stimulates collateral vessel formation even in nonischemic hearts.[8] However, some drawbacks exist for adenoviral-mediated and nonviral naked DNA direct injection gene therapy methods. For example, nonviral DNA delivery has been shown to often mediate low and short-term gene expression, whereas adenoviral vectors have some toxicity concerns. Toxicity issues for the adenoviral-based vectors include the generation of 'memory' immune responses, after delivery of the vector, against the adenoviral vector backbone. This is likely due to the previous exposure to a number of 'natural' adenoviruses during life.

The nonviral naked DNA approach, although somewhat inefficient, deserves further scrutiny because of its, to date, more favorable patient safety profile. In fact, numerous subjects have been 'vaccinated' with antigens expressed from naked DNA plasmids without the development of significant adverse events. Several nonviral methods have been developed in an attempt to enhance delivery of DNA and have been the focus of several reviews.[9–12] Both chemical and physical techniques including liposomes,[13–15] particle bombardment[16–18] and hydrodynamic delivery[19,20] have been described and used to increase the efficiency of tissue DNA uptake.

Another physical delivery method to circumvent problems of low protein expression associated with naked viral-based DNA plasmids is the delivery of genes through *in vivo* electroporation (EP). The demonstration that electric fields can be safely and effectively applied *in vivo* to deliver small molecules[21] and the widespread use of EP to deliver plasmid DNA to cells *in vitro* provided a foundation for the use of EP to deliver

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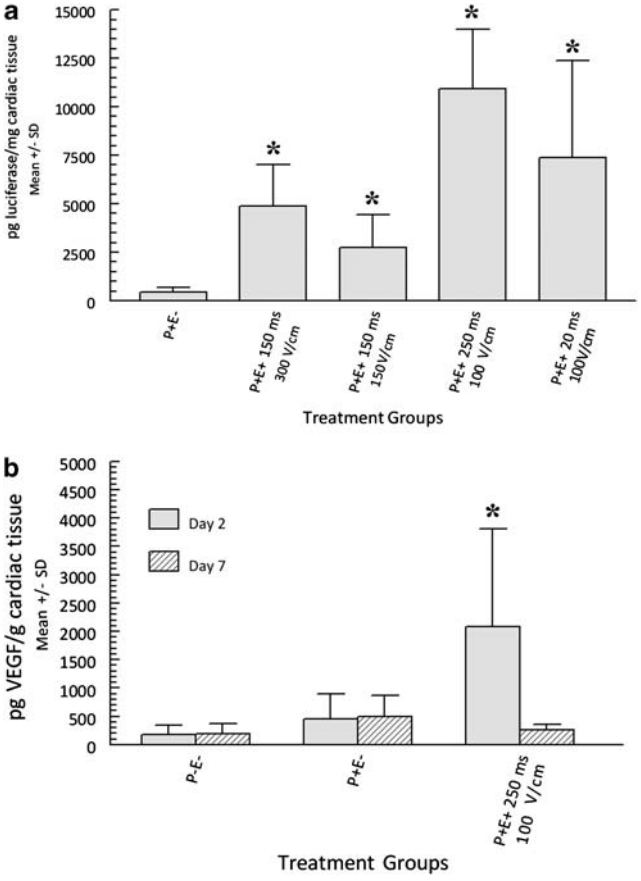
plasmid DNA *in vivo*. EP is a simple and direct, *in vivo* method used to deliver genes for therapy. The use of electric pulses for the *in vivo* delivery of plasmid DNA has been steadily increasing. Preliminary results indicate that therapeutic plasmid DNA delivery could potentially achieve similar success to conventional drug delivery. Multiple studies have shown the feasibility of this approach, primarily in skin, skeletal muscle and tumors.[22–25]

Importantly, the EP delivery method has recently been successfully evaluated in a clinical trial in which an interleukin-12-expressing plasmid was delivered to patients with metastatic melanoma.[26] A second clinical trial delivered a cancer vaccine to muscle.[27] In terms of the application of EP-mediated delivery of therapies against CAD, two studies have reported using electric pulses to transfer genes to the heart. Both of these studies used explanted/*ex vivo* heart samples collected either from mice or from embryonic chicks.[18,28]

Therefore, it was hypothesized that EP could deliver plasmid DNA to the heart for the potential treatment of CAD. The goal of the study presented here was to establish a safe and potentially therapeutic EP-based plasmid DNA delivery to the heart in a large animal model, which could potentially be translated to human medicine. The pig is a useful model for these studies because of similarities between pigs and humans in terms of cardiac and coronary artery anatomy, as well as physiology. This proof-of-concept brief communication describes the successful novel delivery of expression plasmids to the porcine heart.

The initial experiment involved injection of a plasmid-encoding luciferase, pLuc (gWizLuc, Gene Therapy Systems, San Diego, CA, USA), followed by the administration of EP. This in four pigs with injection of the plasmid made in two sites marked with polypropylene sutures in the anterior left ventricular wall in a line 1.5 cm lateral to the left anterior descending coronary artery ~2 cm apart. A second set of injection sites was made 2 cm lateral to the initial line with this line of sites now in the lateral ventricular wall supplied by the left circumflex artery. The sites were again 2 cm apart. A total of four injection sites were made in each heart. Three sites received EP delivered to individual sites after each injection. One site received plasmid injection without EP and a biopsy site was taken in the far lateral ventricular wall without plasmid injection or EP. This same delivery technique was used in each group of pigs described below.

These pulses were administered at a rate of 8 per second and were not synchronized with the heart rhythm (ASYNC). The pLuc delivery experiments were carried out to determine which of the EP parameters resulted in the highest levels of gene expression. The rationale for using ASYNC pulses was to assess whether such a delivery could be applied safely and whether it was effective in enhancing gene expression. Forty-eight hours after pLuc and EP administration, the myocardial expression of luciferase was quantified in cardiac biopsy samples (collected with a 6-mm biopsy punch) using commercial firefly luciferase (Sigma-Aldrich Chemical, St Louis, MO, USA). These data are shown in Figure 1a. There was a significant increase in expression when pLUC was delivered with EP compared with injection alone. The highest expression was obtained using an applied field strength of 100 V cm^{-1} and a pulse width of 250 ms. These optimal EP parameters (that is, 250 ms of pulse width and 100V cm^{-1} of applied field strength) were then subsequently used to deliver pVEGF. Two or seven days after the delivery of pVEGF to pig hearts (two injection and EP sites, one injection only site and one no-treatment site), a myocardial tissue punch (6 mm) of the treated sites was excised and the VEGF expression in the samples was measured (expressed as picogram of VEGF per gram tissue) using a commercial Quantikine human VEGF quantitative sandwich ELISA (enzyme-linked immunosorbent assay) kit (R&D Systems, Minneapolis, MN, USA). In these experiments, the VEGF expression was significantly higher at 2 but not at 7 days after treatment compared with the pVEGF treatment without EP (Figure 1b). In summary, for these initial EP delivery experiments, EP increased expression by 25- and 5-fold for luciferase and VEGF, respectively. Although both proteins were expressed from vectors containing the CMV promoter, differences in protein-fold increase levels and duration of expression were observed. Several possible variables may have produced this difference. Different CMV promoter/enhancers do not necessarily produce identical expression. Other plasmid structural elements, for example, the polyadenylation signal, may affect expression. Luciferase was detected using a functional assay, whereas VEGF was detected using an antibody assay. Finally, the proteins expressed may differ in half-life. Although the luciferase protein half-life in mammalian cell culture is 3 h,[29] the half-life of VEGF *in vivo* has been measured at 6 and 50 min.[30,31]



Although the primary objective of increasing protein expression was obtained, it was observed that ASYNC pulses resulted in ventricular fibrillation in all the pigs Although most of the pigs were successfully defibrillated, it was reasoned that the development of a synchronous (SYNC) EP pulse delivery method, in which the pulses could be delivered in synchrony with the normal heart rhythm would circumvent this problem. Therefore, in a subsequent set of plasmid injection experiments, electrical pulses were delivered to the heart synchronously with the QRS complex determined using a surface electrocardiogram. Importantly, the initial experiments indicated that delivering SYNC pulses eliminated ventricular fibrillation.

Synchronous electrical pulses, delivered during the absolute refractory period of the cardiac electrical cycle, have long been known to have minimal risk of causing electrical fibrillation; for example, as used to treat atrial arrhythmias. Hojman *et al*.,[32] have shown that EP induces changes in Na^+ and K^+ fluxes, and in Ca^{2+} content in the skeletal muscle with larger changes when DNA is present with EP. These effects may also be present in the cardiac muscle and in treatment of the heart, but have not been definitely shown. The larger and more determinant effect is the timing of the pulse delivery within the absolute refractory period of the cardiac cycle.

To determine the SYNC EP conditions that result in maximal expression, experiments were conducted again in six pigs with pLuc injection plus EP using six injection and EP sites, two injection-only sites and two no-treatment sites in each heart (half were evaluated for expression and the other half underwent histological evaluation). In these experiments, it was determined that the pulse length was required to be shortened to be synchronized with the QRS wave. In the experiment summarized in Figure 2a, expression of luciferase was measured in the porcine hearts either 2 or 7 days after treatment. In this experiment, the highest level of expression was 10 000- and 15-fold higher at day 2 or 7, respectively, compared with pLuc treatment without EP. Interestingly, using different pulsing conditions resulted in not only different peak levels of luciferase expression but also in different expression kinetics. Peak short-term expression (2 days) that was significantly higher than injection alone was obtained using an applied field strength of 80 V cm^{-1} and a pulse width of 20 ms. The expression was no longer significantly higher than injection alone by 7 days. In contrast, when pLUC was delivered with an applied field strength of 120 V cm^{-1} and pulse width of 20 ms, peak expression was delayed to 7 days and was significantly higher. This suggests the versatility of delivery with EP, as certain expression patterns can be obtained using different EP delivery parameters. In experiments in which pVEGF was delivered (in six pigs using five injection sites and one no-treatment site in each heart) with SYNC pulses, the maximal VEGF expression was fivefold higher than pVEGF injection alone, when measured at a 2-day post-treatment time point (Figure 2b). No episodes of ventricular fibrillation were experienced using the SYNC EP delivery technique. VEGF expression was evaluated at peak expression (day 2) on the basis of the results presented in Figure 1b, which showed a decrease in VEGF expression to background by day 7.

Figure 1 Cardiac expression of luciferase or VEGF following ASYNC EP-mediated delivery of pLuc or pVEGF pLuc (**a**) or pVEGF (**b**) were administered by intracardiac injection followed by ASYNC pulses. Expression for luciferase and VEGF are given as mean pg mg^{-1} and pg g^{-1} cardiac tissue sample ± s.d. respectively. In the figures P+ and P− designates with or without plasmid injection whereas E+ and E− designates with or without delivery via EP. Results for luciferase expression represent a mean and s.d. of three sites (four sites for injection only) and for VEGF the results are a mean and s.d. of six sites. A total of 10 pigs (30–35 kg) were used for these sets of experiments (four for luciferase and six for VEGF). Multiple sites were utilized on the heart of each animal. Statistical analysis: statistical comparisons for protein expression were determined between the groups receiving electroporation and P+E− (plasmid injection alone) by Student's unpaired *t*-test. For statistical comparisons which included greater than two groups (comparison between experimental groups) the analysis was done by nonparametric ANOVA. The asterisk symbol (*) indicates significantly elevated expression ($P<0.05$) compared with control. Plasmids: To construct pVax1-hVEGF165, a fragment containing hVEGF165 was subcloned from pBLAST49-hVEGFv2.0 (HYPERLINK 'https://webmail.odu.edu/owa/www.invivogen.com' InvivoGen, San Diego, CA, USA) into pVax1 (Invitrogen Corp., Carlsbad, CA, USA), which contains the CMV promoter and the BGH polyadenylation signal. The plasmid was commercially prepared (Aldevron, Fargo, ND, USA) and suspended in sterile injectable saline. Endotoxin levels were <0.1 EU/μg plasmid. The pLuc used in the studies was a gWizLuc plasmid (Aldevron). Anesthesia: animals were anesthetized during DNA injection and electric pulse administration. Pre-anesthetic agents: induction: Ketamine 10–20 mg kg^{-1} i.m. for immobilization, Atropine 0.02 mg kg^{-1} i.v., Sufentanil 0.015 mg kg^{-1} h^{-1} i.v. followed by 0.007 mg kg^{-1} Sufentanil bolus after 5 min. Maintenance: Sufentanil 0.015–0.030 mg kg^{-1} h^{-1} i.v. infusion supplemented by 0.5–1.0% isoflurane in oxygen via inhalation (that is, intubation). Monitoring: animals were monitored using EKG monitoring, pulse oximetry (O_2 sat), capnography (end-tidal CO_2), and rectal temperature. Medications: antibiotics: Cephalexin 10 mg kg^{-1} i.v. once for antibiotic prophylaxis, Anti-arrhythmic: Amiodarone 5–10 mg kg^{-1} was injected intravenously just before cardiac manipulation and every 30 min as required with use of Lidocaine intravenously p.r.n. Surgical procedure: Animals were placed in supine position and on a warming blanket. Steri-drape was used to cover from neck to mid-abdomen. Incision was made in skin from sterno-manubrial junction to xyphoid using a no. 10 Blade. The subcutaneous tissue was divided to sternum using electrocautery and then the sternum divided with a stryker oscillating saw. Arrhythmias were treated as indicated above and the sternum closed with 0 wire and soft tissues closed in layers with absorbable suture. Plasmid injection and electroporation: plasmid DNA (pLuc or pVEGF) was injected (200 μg in 100 μl of sterile saline) in multiple sites in the left ventricle followed by EP at specific delivery parameters that varied by field strength and pulse width. EP was administered via an epicardial probe at the designated parameters. The electric fields were applied using an applicator containing an array of 16 electrodes and a firing sequence of a series of 2×2 mm squares. Four pulses were fired in each direction (total of 72 pulses for entire array). Internal defibrillation was administered as needed (10–50 J) in all animals receiving asynchronous EP and the heart allowed to recover for 10–15 min following restoration of normal sinus rhythm. Location of treatment site(s) were marked with 4–0 prolene suture. Two controls were performed; plasmid delivery without EP and an untreated control, in which there was neither plasmid injection nor administration of EP. Evaluation: At 2 or 7 days post-operatively, the sternotomy was re-opened. The injection sites were excised with a 6 mm punch and sent for luciferase or VEGF assays. Luciferase expression was quantified using commercial firefly luciferase (Sigma, St Louis, MO, USA). A sandwich ELISA kit was used for detection of VEGF. The animals were euthanized with intracardiac-administered potassium chloride. This experimental animal surgery and treatment protocol was approved by the University of South Florida Institutional Animal Care and Use Committee and all experiments were performed in accordance with all relevant guidelines and regulations.

An ability to tailor EP parameters for differential expression may be useful for optimal results and to minimize complications in a clinical situation. It is unclear at this time when the most optimal delivery periods would be and how long they would persist. Further study is necessary. The VEGF levels in this study are higher than in a clinical study by Kastrup *et al*., but the levels in this study were myocardial tissue levels, whereas the clinical study measured serum levels.[33,34] No significant increase in serum levels of VEGF were observed in this study. The regional localization and lack of systemic increases in VEGF may have significant advantages in mitigation of complications due to increased VEGF levels with neovascularization in the retina in diabetes and neovascularity to support tumor formation/growth. More study is necessary to define the optimal target levels of VEGF both in the myocardium and in the serum, both in experimental and in clinical situations.

In addition to the assessment of expression, biopsy samples of the injection/EP sites were also examined histologically. There was evidence of pericarditis in all samples. This was an expected sequela of the cardiac manipulations. Some mild-to-moderate inflammatory responses were also noted in several samples, which could not be directly associated with treatment conditions.

To our knowledge, this is the first report of the successful delivery of a plasmid expressing a vascularization-modifying protein such as VEGF to the porcine heart through EP. The expression of VEGF was increased significantly in tissue surrounding the cardiac injection site that had received pVEGF plus EP. These studies indicate a proof-of-concept validation of this method and provide the impetus for further investigation. These studies will include safety analysis as an assessment of potential treatment-associated biological and clinical effects. Ultimately, these studies will indicate the potential for this technique and strategy for the treatment of CAD in humans.

Conflict of interest

Richard Heller has been funded by the National Institutes of Health. Drs Richard Heller, William G Marshall and Mark J Jaroszeski are coinventors on a patent application, which covers the technology that was used in the work reported in this paper. Drs Richard Heller and Mark J Jaroszeski are also inventors on other patents that were used in this work. Drs Richard Heller and Mark J Jaroszeski have ownership interest in RMR Technologies and own stock and stock options of Inovio Biomedical Corporation. Brian A Boone, Jose D Burgos, Sylvia I Gografe, Margaret K Baldwin, Michele L Danielson, Mary J Larson, Denise R Caretto, Yolmari Cruz, Bernadette Ferraro and Kenneth E Ugen declare no potential conflict of interest.

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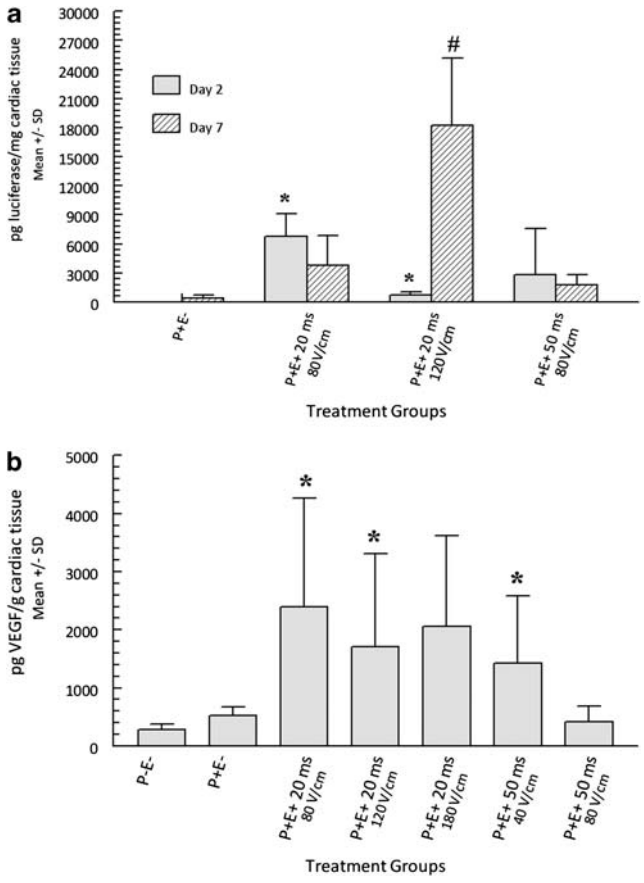

Figure 2 Cardiac expression of luciferase or VEGF following SYNC EP-mediated delivery of pLuc or pVEGF. pLuc (**a**) or pVEGF (**b**) was administered by intracardiac injection followed by SYNC pulses. The electric fields were applied using an applicator containing four needle electrodes that were 6 mm long and arranged to form a 5×5 mm square. Four pulses were fired in two perpendicular directions (total of eight pulses for the entire array). Expression for luciferase and VEGF are given as mean pg mg^{-1} and pg g^{-1} cardiac tissue sample ± s.d. respectively. In the figures P+ and P− designates with or without plasmid injection whereas E+ and E− designates with or without delivery via EP. Methods are as described in the legend for Figure 1 except that EP pulses were synchronized with the heart rhythm. Electroporation pulses were administered individually just before the peak of the r wave (within the qrs complex) and completed before the initiation of the t wave. Results for luciferase expression represent a mean and s.d. of three sites and for VEGF the results are a mean and s.d. of four sites except for injection only (five sites) and injection and electroporation with 20 ms and 120 V cm^{-1} (five sites). A total of 12 pigs (30–35 kg) were used for these experiments (six for luciferase and six for VEGF). Multiple sites were used on the heart of each animal. For histological evaluation, four sections were prepared from each biopsy and stained with hematoxylin and eosin and then assessed by pathological examination to determine any evidence of damage as well as immune or inflammatory reactions. Electroporation sites were treated before the non electroporated sites. *Statistical analysis:* Statistical comparisons were performed as described in Figure 1 legend. The asterisk symbol (*) on a column indicates that the expression for that treatment on day 2 was significantly elevated ($P < 0.05$) compared with day 2 control. The hash (#) symbol on a column indicates that the expression for that treatment on day 7 was significantly elevated ($P < 0.05$) compared with day 7 control.

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