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Electro-gene transfer to skin using a noninvasive multielectrode array☆

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ABSTRACT

Because of its large surface area and easy access for both delivery and monitoring, the skin is an attractive target for gene therapy for cutaneous diseases, vaccinations and several metabolic disorders. The critical factors for DNA delivery to the skin by electroporation (EP) are effective expression levels and minimal or no tissue damage. Here, we evaluated the non-invasive multielectrode array (MEA) for gene electrotransfer. For these studies we utilized a guinea pig model, which has been shown to have a similar thickness and structure to human skin. Our results demonstrate significantly increased gene expression 2 to 3 logs above injection of plasmid DNA alone over 15 days. Furthermore, gene expression could be enhanced by increasing the size of the treatment area. Transgene-expressing cells were observed exclusively in the epidermal layer of the skin. In contrast to caliper or plate electrodes, skin EP with the MEA greatly reduced muscle twitching and resulted in minimal and completely recoverable skin damage. These results suggest that EP with MEA can be an efficient and non-invasive skin delivery method with less adverse side effects than other EP delivery systems and promising clinical applications.

1. Introduction

In the past two decades electroporation (EP) has received increased attention for its advantages compared to viral vectors for use in gene delivery. EP has been demonstrated to be an efficient non-viral *in vivo* gene delivery method by several independent research groups [1–5]. Diverse electrodes such as calipers, tweezers, needle arrays and microneedle arrays have been designed and tested in different species [6–10]. Various electrical parameters have been studied for their expression efficiency and adverse effects [6,11]. *In vivo* gene delivery by EP has been reported to achieve effective gene expression in various tissues and organs [12], such as liver [1], skin [13], muscle [14], brain [15], eye [16], lung [17], spleen [18], kidney [19], bladder [20], testis [21], artery [22], and tumors [2].

The skin contains large numbers of potent antigen-presenting cells, Langerhans cells and dermal dendritic cells, as well as an abundant blood supply in the dermal layer of the skin [23], which may help transgenic products distribute into distant organs through circulation [24]. These advantages make delivery of therapeutic genes to the skin very attractive, particularly, for i) the treatment of local diseases including skin cancer, chronic ulcer, burn, psoriasis; ii) vaccination against infectious diseases such as HIV, anthrax, malaria, as well as non-infectious diseases like cancer; iii) the correction of systemic or metabolic disorders like anemia in chronic kidney disease. Previous studies have shown that EP efficiently delivers plasmid DNA to the skin resulting in a 10–1000 fold increase of local and serum expression [24–27]. Skin EP delivery was successfully performed in rodent, porcine and non-human primate model systems [13,24,25]. Intradermal delivery of plasmid VEGF (165), FGF-2 or TGF-β by EP has been observed to promote wound healing in rat or mouse models [28–30]. Significant serum levels were achieved by EP delivery of both EPO and IL-12 plasmid DNA to the skin [24,31–33]. A number of studies demonstrated that significant tumor regression could be achieved by electrically mediated delivery of plasmids expressing IFN-α, IL-12, IL-2, IL-15, IL-18, GM-CSF and other transgenes to cutaneous tumors (melanoma, squamous cell carcinoma) [6]. In our mouse melanoma model [32,34], intratumoral EP of IL-12 plasmid resulted in complete tumor regression rates of 80%. Those mice were also resistant to subsequent tumor challenge. Moreover, our phase I human trial of IL-12 EP treatment of metastatic melanoma showed that distant untreated lesions could also regress, suggesting that not only had a local response been mounted against treated tumors but also a systemic memory response had been generated [35].

Current skin EP systems, utilize, for example, invasive needle electrodes as well as plate electrodes (calipers, forceps, etc.) and typically induce significant muscle twitching and discomfort and treatment can result in skin damage [25]. To overcome the pitfalls of these electrode designs, we developed a new non-invasive electrode known as multielectrode array (MEA). In previous studies [27], we reported that skin EP with the MEA could achieve comparable (in rat)

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or higher expression (in guinea pig) as compared to plate electrodes, while the applied voltage and muscle stimulation was greatly reduced. In the current study, we further modified the MEA to include flexible spring electrodes in the substrate to assure a full contact between all of the electrodes and the skin. We then characterized several critical aspects relevant to therapeutic applications. DNA delivery was tested in a guinea pig model, which has similar skin thickness and structure to human skin [36,37]. Localized transgene expression and kinetics were assessed by the measurement of luciferase activity with an *in vivo* bioluminescence scan. The evaluation of the MEA has also included the correlation between expression and the size of the treated area, potential tissue damage, DNA distribution and localization of gene-expressing cells.

2. Materials and methods

2.1. Animals

Female Hartley guinea pigs used in this study were 4 to 6 weeks old from Elm Hill Labs (Chelmsford, MA, USA). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Old Dominion University.

2.2. Plasmids

The reporter plasmids encoded luciferase (gWiz-Luc) and green fluorescent protein (gWiz-GFP) were both from Aldevron (Fargo, ND, USA). Fluorescein-labeled plasmid MIR 7907 and Cy^{TM}3-labeled plasmid MIR7905 (Mirus Bio LLC, Madison, WI, USA) were used to observe DNA distribution.

2.3. DNA injection and in vivo electroporation

Prior to delivery, animals were anesthetized in an induction chamber charged with 3% isoflurane in O_2 then fitted with a standard rodent mask and kept under general anesthesia during the procedure. Guinea pigs received intradermal (i.d.) injections of 50 μL or 200 μL plasmid DNA (2 μg/μL dissolved in saline) on the left and right flanks. Immediately after DNA administration, a MEA electrode with 4×4 2-mm-apart pins was placed over the injection site(s). Voltage was applied (each pair of electrodes was programmed to administer four pulses with total 72 pulses [27], electric field was 250 V/cm, pulse duration 150 ms and 150 ms delay). Electroporation was performed using the UltraVolt Model: Rack-2-500-00230 (UltraVolt, Inc. Ronkonkomo, NY, USA). The electroporation parameters we chose here were based on our recently published study [38] in which we evaluated the effect of different electrotransfer parameters on transgene expression and skin damage using a similar designed MEA electrode in the guinea pig model. The pulse parameters of 250 V/cm and 150 ms were found to give the highest expression with minimal damage to the skin. Increasing the field strength did not result in increased expression. For a single 200 μL injection or four 50 μL adjacent injections, four individual pulse applications were applied without change of pulse parameters.

2.4. Living imaging of luciferase expression

At different selected time points after delivery, animals were anesthetized then administrated intradermally with the same DNA volume of D-luciferin with 7.5 mg/mL in PBS buffer (Goldbio, St. Louis, MO, USA). Assessment of photonic emissions using the IVIS Spectrum system (Caliper Life Sciences, Hopkinton, MA, USA)) was performed 1.5 min after injection of D-luciferin. Background luminescence was determined by measuring luminescence from area without DNA injection.

2.5. GFP expression

Each excised sample was immediately frozen on dry ice. After visualization of GFP expression was observed and obtained by a fluorescence stereoscope (Leica Model MZFL III, Leica, Heerbrugg, Switzerland), the specimens were embedded in tissue freeze media OCT compound (Electron Microscopy Sciences, Hatfield, PA) and frozen at −80 °C freezer. Several frozen sections (8 μm thickness) were cut from each sample. Each section was fixed in 25% Acetone + 75% Ethanol 20 min and then washed twice in PBS. It was dried under dark and mounted into a coverslip with VECTASHIELD® mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Sections were examined by Olympus BX51 fluorescent microscopy (Olympus, Tokyo, Japan) for the presence of GFP.

2.6. Histological analysis

Each specimen was embedded, sectioned and fixed as mentioned above. Sections were dehydrated in 95% ethanol for 30 s, stained in hematoxylin solution for 5 min, rinsed with tap water for 3 min, classified in 1% acid alcohol for 10 s, washed with running tap water for 1 min, blued in 0.2% ammonia solution for 30 s, washed in running tap water for 3 min, rinsed in 95% alcohol, 10 dips, counterstained in eosin Y solution for 45 s, dehydrated through 95% alcohol, 2 changes of absolute alcohol, 10 dips each, cleared in 2 changes of xylene, 10 dips each, mounted with xylene based mounting medium. Sections were examined by Olympus BX51 microscopy.

2.7. Statistical analysis

All values are reported as the mean ± SD. Analysis of luciferase activity was completed using a 2-tailed Student's t-test when comparing two groups. Statistical significance was assumed at $p<0.05$. All statistical analysis was completed using the SigmaPlot 10.0.

3. Results

3.1. The level and duration of gene expression were significantly increased by intradermal DNA injection and non-invasive skin EP

The correlation between the level and duration of gene expression to the size of the treated area when delivering by EP with the MEA was evaluated by *in vivo* bioimaging. As shown in Fig. 1A, the maximum level of luciferase expression was achieved one day after delivery. While expression in the non-electroporated sites decreased dramatically by day 2 the expression of EP-treated sites was stable until day 15. The average levels of gene expression in the EP-treated groups were 2 to 3 logs higher than in the non-EP-treated groups from days 2 to 15. Among the different EP-treated groups, luciferase expression increased 3.7 to 6.3 fold in 200 μL DNA with one EP application compared to 50 μL DNA with one EP application from days 1 to 8 after delivery. However, the skin receiving 200 μL DNA and four EP applications expressed the highest level of protein with a 4.5 to 15.8 fold increase in expression compared to 50 μL DNA with one EP application from day 1 to day 12 ($P<0.05$ for the most time points). (Table S1). At day 22 after delivery, the luciferase expression of EP-treated skin decreased to the level of DNA injection only, both of which were still slightly increased as compared to background.

Given these findings, we wanted to address whether we could achieve long-term gene expression by repeated deliveries with MEA EP delivery. Based on the previously stated results, a one-time delivery would result in maximum gene expression within 24 h and would remain relatively constant through day 15. Therefore, we aimed to attempt three deliveries at the same site and to produce longer-term expression. The delivery time points were selected to be