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Gene Electrotransfer in 3D Reconstructed Human Dermal Tissue

Moinecha Madi^{a,b}, Marie-Pierre Rols^{a,b*}, Laure Gibot^{a,b*}

^a IPBS-CNRS, 205 route de Narbonne, 31077 Toulouse, France; ^b Université de Toulouse, UPS, 31077 Toulouse, France

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corresponding author(s)
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Abstract: Gene electrotransfer into the skin is of particular interest for the development of medical applications including DNA vaccination, cancer treatment, wound healing or treatment of local skin disorders. However, such clinical applications are currently limited due to poor understanding of the mechanisms governing DNA electrotransfer within human tissue. Nowadays, most studies are carried out in rodent models but rodent skin varies from human skin in terms of cell composition and architecture. We used a tissue-engineering approach to study gene electrotransfer mechanisms in a human tissue context. Primary human dermal fibroblasts were cultured according to the self-assembly method to produce 3D reconstructed human dermal tissue. In this study, we showed that cells of the reconstructed cutaneous tissue were efficiently electroporated by applying millisecond electric pulses, without affecting their viability. A reporter gene was successfully electrotransferred into this human tissue and gene expression was detected for up to 48h. Interestingly, the transfected cells were solely located on the upper surface of the tissue, where they were in close contact with plasmid DNA solution. Furthermore, we report evidences that electrotransfection success depends on plasmid mobility within tissue-rich in collagens, but not on cell proliferation status. In conclusion, in addition to proposing a reliable alternative to animal experiments, tissue engineering produces valid biological tool for the *in vitro* study of gene electrotransfer mechanisms in human tissue.

Keywords: Delivery, plasmid DNA, electroporation, extracellular matrix, gene therapy, skin, tissue engineering

INTRODUCTION

Since few decades, a physical method named electroporation has been developed to locally deliver therapeutic drugs (cytotoxic molecules and nucleic acids) to a large range of targeted tissues [1-5]. This promising method is currently applied in human clinics. The major fields of application of DNA electrotransfer are vaccination and gene therapy in oncology [6-8]. Clinical applications have already began in veterinary and human oncology [9-11]. Hopeful results have been obtained with the first published phase-I trial on interleukin-12 electroporation, conducted in a cohort of patients with metastatic melanomas [12].

However, while gene electrotransfer is highly efficient in a large panel of cell lines *in vitro*, gene expression is usually less efficient *in vivo* in some tissues such as tumors and the skin [7,13]. Although the first study on DNA electrotransfer was initiated more than 30 years ago [14], underlying mechanisms are not yet fully understood, in particular in *in vivo* situations [15]. It is therefore still mandatory, for further developing the method in clinics, to increase our knowledge of the process.

The skin is an appealing tissue target for gene electrotherapy because of its large surface area, its accessibility and its immunological properties with antigen presenting cells present both in the epidermis and in the

dermis [16]. Gene electrotransfer to muscle tissue is highly effective and leads to long-term transgene expression [17,18]. With some plasmids and under optimized delivery conditions, it could achieve higher expression than in skin [19]. Nevertheless, high and/or long-term transfection are not always required [20]. For example, in wound healing, there is no need for therapeutic growth factor transgene expression when the wound has closed [21,22]. Similarly, DNA vaccination does not require a continuous production of antigens once the immune system has responded to the vaccine [4,23]. Donat and collaborators gave evidence that vaccine to the skin is as, if not more, efficient than delivery to muscle [24]. Gene electrotransfer to the skin can be as efficient as muscle as reported by Heller et al showing over 30% efficiency delivering to the skin [25].

Skin consists of distinct cell types, mainly keratinocytes, melanocytes, Langerhans cells, fibroblasts and endothelial cells. This diversity of cell types can differently respond to gene transfection depending on the plasmid construction (i.e., tissue-specific promoters), on the electrical parameters and/or on the type of electrodes used.

*Address correspondence to this author at IPBS-CNRS, 205 route de Narbonne, 31077 Toulouse, France; Tel/Fax: +33-561-175-827, +33-561-175-994; E-mails: gibot@ipbs.fr; rols@ipbs.fr

In order to understand the role of each parameter of the experimental setup on the distinct cell types present within the skin during the gene electrotransfer process, relevant biological models of human skin tissue have to be developed.

Using the tissue engineering self-assembly approach [26], we produced reconstructed human dermal tissue, devoid of any exogenous material, and rich in native extracellular matrix [27,28]. In this context, primary human dermal fibroblasts reproduce a native environment, by secreting and organizing a 3D extracellular matrix dense in collagens. These abundant collagen fibrils were clearly detected by second harmonic generation (SHG) [29]. In a previous study, we showed that this tissue-engineered model is efficiently electroporated with millisecond electric pulses [29]. Thus, we propose here to further examine gene electrotransfer mechanisms *in vitro* in human tissue by using this relevant and useful biological model.

MATERIALS AND METHODS

Cell culture. Primary human dermal fibroblasts were extracted from human child foreskin after posthectomy as previously described [27]. Primary cells were grown at 37°C in Dulbecco's Modified Eagles Medium (Gibco-Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Reconstruction of human 3D dermal tissue. Human dermal substitutes, devoid of any exogenous material, were reconstructed *in vitro* according to the self-assembly approach. In a few words, primary human dermal fibroblasts were grown for three weeks in cell culture medium supplemented with 50 µg/mL of ascorbic acid (Sigma-Aldrich) as already published [28,29]. After three weeks, enough extracellular matrix was secreted to produce a cell sheet that is easy to manipulate. Since the cells are primary dermal fibroblasts, extracellular matrix is comparable to human dermis.

Collagen detection by second harmonic generation (SHG). Stacks of 425x425x60 µm were acquired using a 7MP multiphoton microscope (Carl Zeiss, Jena, Germany) coupled to a Ti-Sapphire laser, Chameleon Ultra 2 (Coherent Inc) settled to 800 nm. Organized fibrillar collagens were detected by second harmonic generation at a wavelength of 400 nm. Imaris software (Bitplane AG) was used to analyze the acquired 3D images.

Electroporation and gene electrotransfer in 3D human dermal tissue. Two flat parallel stainless steel electrodes were placed on the top of a cell sheet and immersed in pulsing buffer (10 mM K₂HPO₄/ KH₂PO₄ buffer, 1 mM MgCl₂, 250 mM sucrose, pH 7.4) containing or not 100 µM propidium iodide (PI) (Sigma-Aldrich) or 2 µg of 4.7 kb pEGFP-C1 DNA plasmid (Clontech, #6084-1). Electroporation was performed with an ELECTRO cell S20 generator (βTech, Toulouse, France) which delivered square-wave electric pulses. The electrical conditions were the

following: 8 pulses lasting 5 ms at a frequency of 1 Hz applied from 0 to 800V/cm at room temperature.

Electroporation detection and quantification. Cell membrane permeabilization was analyzed on living tissues by multiphoton microscopy. Electroporation efficiency was investigated by adding PI to the pulsation buffer during electric field application. IMARIS software was used to draw an isosurface and to isolate the electroporated cells (i.e. cells in which PI was present).

Cell viability. Tissue viability was determined by adding PI 24h after application of the electric field. Viability was also quantified using PrestoBlue assay (Invitrogen) according to the manufacturer's protocol. Briefly, 24h after electric field application, the cell sheet was incubated for 30 min at 37°C in a PrestoBlue solution. Absorbance (excitation 570 nm, emission 600 nm) was then read using a plate reader (Clariostar, BMG). Controls, named "Dead" were cell sheets subjected to 3 freeze/thaw cycles).

Reporter gene electrotransfer efficiency. 3D stacks showing both SHG and efficiently electrotransfected GFP-positive cells were acquired using a multiphoton laser scanning microscope. Pictures were analysed with IMARIS software to show both top and side views of the cell sheet. Image J software (NIH, Bethesda, USA) was used to quantify the GFP-positive areas within total pictures (425x425 µm).

Cell proliferation within dermal tissue. Cell proliferation was assessed with Click-it® EdU Alexa Fluor® imaging kit (Molecular Probes). In a few words, cell sheet was incubated for 24h with 10 µM EdU before being processed according to the manufacturer's recommendations. Reconstructed dermal tissues were observed with a 7MP multiphoton laser scanning microscope.

DNA staining and gene electrotransfer. pEGFP-C1 plasmid was stoichiometrically labelled with TOTO-1 (Molecular Probes), a DNA intercalating dye as previously described [30]. The plasmid was stained with 2.3x10⁻⁴ M dye at a DNA concentration of 1 µg/µl for 60 min on ice. Cell sheet was incubated in labeled plasmid-containing pulsation buffer and exposed to 8 pulses each lasting 5 ms at a frequency of 1 Hz at 800V/cm at room temperature. Cell sheets were observed 1 minute after the pulses with the 7MP multiphoton laser scanning microscope.

Statistical analyses. Statistical significance in cell viability after different electrical treatments was determined using 1-way ANOVA with a Dunnett's post-test. Statistical analyses used GraphPad Prism software, version 4.02 (GraphPad Software, San Diego, USA). All plotted data were expressed as means ± SEM.

RESULTS AND DISCUSSION

The aim of our study was to examine the relevance of reconstructed human dermal tissue as a model to address

gene electrotransfer processes. To do so, we used standard electrical parameters for gene electrotransfer (i.e., 8 pulses lasting 5 ms at 1 Hz frequency with field intensities from 0V/cm to 800V/cm). Multiphoton imaging allowed visualization of membrane permeabilization, gene transfer and expression at the single cell level within tissue, on fresh human models. Cell uptake of propidium iodide was used to detect membrane permeabilization. A plasmid carrying the Green Fluorescent Protein gene allowed detection of gene transfer and expression. This methodology addresses the following issues: (i) Can the cells within reconstructed dermal tissue be efficiently electroporated and transfected? (ii) Does transfection affect cell viability? (iii) Does the location of the cells within the tissue and their proliferative status affect the process?

Cells within reconstructed human dermal tissue are efficiently electroporated by millisecond electric pulses. Electroporation was assessed by adding propidium iodide during electric field application. Images obtained by microscopy were analyzed by IMARIS software which allows cell sheets to be visualized both from top and side views (Fig. 1). In the control condition (0V/cm), a few permeable cells that correspond to dead cells were observed in the cell sheet. Indeed, cell renewal takes place in the reconstructed human dermal tissue model, and dead cells are entrapped within the extracellular matrix. By applying the electric pulses and increasing the electric field intensity, cells located both on the surface and in depth were efficiently and homogeneously electroporated. Thus, the whole cells embedded within the tissue model can be electroporated. We then checked whether membrane permeabilization was transient or not. Irreversible electroporation (IRE) offers great promise in interventional oncology with thermal or non-thermal tumor ablation [4,31]. However, IRE has to be avoided in gene therapy since it is not compatible with gene expression.

Cell viability within tissue is preserved after application of millisecond electric pulses. Membrane resealing occurs in about 20 minutes after application of millisecond pulses [32,33]. Thus, when added 24h after the electric pulses, PI only enters into irreversibly permeabilized cells i.e. dead cells. Control tissues revealed only a few positive cells while “dead” tissue was highly fluorescent. Images showed no significant increase in the number of dead cells after applying the electric field up to 600 V/cm (Fig. 2A). A resazurin-based functional test involving viable cell metabolic activity (Fig. 2B), was used to quantify tissue viability and showed no statistical difference between control cells (0 V/cm) and cells pulsed up to 600 V/cm. A statistically non-significant decrease was observed at 800V/cm, as confirmed by PI-positive cells increase on fluorescent pictures. At 800V/cm, side views indicated that dead cells were situated on the top of the cell sheet (data not shown). Previous histological analyses showed that several thousand cells are embedded in multiple layers within the 50 μ m thick sheet [29]. Thus, even though some cells appeared positive to PI at 800V/cm, their number was not significant.

Accordingly, millisecond electric pulses up to 800 V/cm did not significantly affect cell viability within the dermal tissue.

Gene electrotransfer is spatially and temporally limited within the tissue. GFP reporter gene plasmid was electrotransferred within 3D reconstructed dermal tissue (Fig. 3). No transfected cells were detected when no electric field was applied (0V/cm) or when no plasmid DNA was added (data not shown). GFP expression was observed 6h after gene electrotransfer and lasted up to 72h (data not shown). Electrotransfection was observed for electric field intensities above 200 V/cm, i.e. under electric field conditions leading to cell electroporation. GFP-expressing cells increased with the electric field intensity. Cross-sectional views showed that only cells located at the top of the cell sheet were efficiently transfected (Fig. 3A), whatever the electric field intensity. This could be due to the fact that only the top of the sheet was in contact with the plasmids during application of the electric field. Quantification of GFP-positive area 24h and 48h after gene electrotransfer revealed that the most efficient electric field intensity was 800 V/cm, leading to a 8% rate of transfection (Fig. 3B). This percentage is quite low compared to in vivo studies achieving 30% efficiency delivery to the skin [25]. Further optimization of both electrodes geometry and placement could improve electric field distribution and increase plasmid mobility within our tissue model, leading to a better gene electrotransfer efficiency.

Reporter gene expression depends on plasmid mobility within the tissue but not on cell proliferation status. A hypothesis to explain why transfected cells were solely located at the tissue surface is that only these cells are proliferative. Proliferative cells are usually more efficiently electrotransfected because, as previously reported [34], the disintegration of the nuclear membrane during mitosis helps the direct access of DNA plasmid to the nucleus, enhancing gene expression. In order to characterize cell proliferation within our 3D model, we aimed to visualize cells engaged in S-phase replication [35]. A nucleoside analog of thymidine was added to the culture medium. Proliferative cells were homogeneously distributed through the 3 dimensions within the reconstructed dermal tissue (Fig. 4A). This constant cell renewal cannot explain the localization of the transfected cells but could explain why transgene expression lasted for less than 72h.

We then aimed to determine if the accessibility of plasmid DNA to cells could explain why only cells in the upper part of the dermal tissue were transfected. To do so, cells were pulsed in the presence of a fluorescently labeled- plasmid DNA that allowed its 3D distribution within dermal tissue to be visualized (Fig. 4B). Under electrical parameters that induce efficient cell permeabilization and transfection (i.e. 800 V/cm), plasmid DNA was showed to interact with the cells. Interestingly, this interaction was limited to the cell side facing the cathode as previously described on cells grown in monolayers and in suspension [30]. Thus, for the first time we were able to observe DNA-membrane

interactions in a healthy tissue. Interestingly, by DNA spot patterns we were able to recognize fibroblast typical morphology. Indeed, these stromal cells are elongated cells growing oriented in parallel. Analysis of tissue cross-sectional views revealed that fluorescent plasmid DNA was confined to the tissue surface. It has been reported that DNA plasmid electrophoretic transport is inversely correlated with the amount of collagen in tumor tissue [36]. Thus, tumors containing only some proteoglycans and collagens were found to be more efficiently electrotransfected *in vivo* than solid tumors with a rich extracellular matrix [37,38]. Collagen content modulates tumor tissue resistance to macromolecule diffusion by linking and stabilizing glycosaminoglycans [39]. Since the reconstructed human dermal tissue is highly rich in collagens [29], it seems that this observation is also correct for solid healthy tissue.

We are now optimizing the electrical parameters to improve gene electrotransfer efficiency in a healthy tissue context. Indeed, some authors previously showed that an association of high voltage with low voltage pulses enhanced gene expression in skin [7,40-42]. Additionally, skin is a complex tissue composed of distinct cell types. Theoretically cell types with different morphological characteristics can be targeted and transfected by specific electrical parameters [32]. Thus, we plan to refine our skin tissue-engineered model by adding an epidermis compartment as previously described [27,28] in order to study cell targeting. Finally, we would like to investigate the role of plasmid construction on gene electrotransfer efficiency in skin. Indeed, tissue-specific promoters lead to smaller transfection rates but specific cells can be better targeted [43,44].

CONCLUSION

Reconstructed human dermal tissue appears to be a relevant tool to mimic and assess gene electrotransfer in human skin. Using this model, we will gain a better comprehension of the mechanisms involved in gene electrotransfer and its limits in tissues and propose strategies to improve its efficiency, while maintaining its safety, or to specifically target a particular cutaneous cell type.

CONFLICT OF INTEREST

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