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Electrical stimulation by enzymatic biofuel cell to promote proliferation, migration and differentiation of muscle precursor cells

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ABSTRACT

Electrical stimulation is a very important biophysical cue for skeletal muscle maintenance and myotube formation. The absence of electrical signals from motor neurons causes denervated muscles to atrophy. Herein, we investigate for the first time the utility of an enzymatic biofuel cell (EBFC) as a promising means for mimicking native electrical stimulation. EBFC was set up using two different enzymes: one was glucose oxidase (GOX) used for the generation of anodic current followed by the oxidation of glucose; the other was Bilirubin oxidase (BOD) for the generation of cathodic current followed by the reduction of oxygen. We studied the behaviors of muscle precursor cells (MPCs) in terms of proliferation, migration and differentiation under different electrical conditions. The EBFC electrical stimulations significantly increased cell proliferation and migration. Furthermore, the electrical stimulations promoted the differentiation of cells into myotube formation based on expressions at the gene and protein levels. The EBFC set up, with its free forms adjustable to any implant design, was subsequently applied to the nanofiber scaffolding system. The MPCs were demonstrated to be stimulated in a similar manner as the 2D culture conditions, suggesting potential applications of the EBFC system for muscle repair and regeneration.

1. Introduction

The physiology of skeletal muscle is mediated by two major factors: one is Electrical stimulation from nerve cells and the other is mechanical stimulation [1,2]. Electrical stimulation from motor neurons is known to be critical in regulating the development and maintenance of myoblasts, such as proliferation, migration and differentiation for myotube formation [3–6]. The skeletal muscle atrophy characteristic of the common motor neuron disease, amyotrophic lateral sclerosis (ALS), is caused by the lack of stimulation from motor neurons [7]. Most ALS patients die within 5–6 years after diagnosis [7,8]. Nevertheless, proper clinical therapy does not yet exist to relieve the symptoms of ALS [7].

To this end, several strategies to mimic electrical stimulation by neurons have been explored, including the use of electrical biophysical systems and electrical implantable biomaterials [9–12]. Histological electrical therapies such as pain management, improving neuromuscular functioning, and allowing joint motility and tissue repair are now clinically available [13–17]. Direct current is also used clinically for electrical stimulation [18,19]. However, the effective mechanisms governing those therapies have yet to be clarified [20–22]. More extensive studies *in vitro* have been piled up to investigate the electrical stimulatory effects and to elucidate the underlying mechanisms at the cellular level [1,6,14,23,24]. Cumulative findings have revealed that the exogenous electrical stimuli provided by electrotherapeutic devices significantly influence cell structure, movement, metabolism, replication, proliferation, and differentiation [14,16], which is reasoned to be primarily due to the stimulation of cell membrane receptors and alterations in membrane ion channel characteristics [25].

Systems for providing electrical stimuli have been possible through electrical circuits using electrical power sources mostly

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made of inorganic materials [17,26]. However, these have limited life time, low biocompatibility and often require a sizable device, restricting extensive use and ease of clinical applications. This is particularly limiting when designing electrical stimulation systems for implantable biomaterials. Implantable biomaterials in diseased and dysfunctional muscle tissues should be biocompatible (and possibly degradable) with sizes and shapes that allow for implantation as well as ease of repair and regeneration of associated tissues. Biomaterials (i.e., degradable biopolymers) generally available for implantable purposes meet those basic criteria. However, intrinsic formation of electrical circuits in the currently available biomaterials is impossible.

Here we present enzymatic biofuel cell (EBFC) as an electrical power source that can intrinsically generate electrical stimuli for muscle repair and regeneration. EBFC has recently been recognized as a promising power source for implantable biomedical devices in the living body [27]. In 1911, Potter introduced the first biofuel cell using cultured yeast and E. Coli cells on platinum electrodes [26]. During the 1960s in the United States, practical interest has been developed in the use of EBFC as an efficient energy production system for vehicle power suppliers and as an implantable power source for cardiac pacemakers [28]. However, the electrochemical performance in all of the early EBFC studies was very poor. Efforts have recently been devoted to improving EBFC and overcoming limitations such as the short active lifetime, low power density, and low efficiency for electrical devices [29–32]. With improvement, the applicability of EBFC to other kinds of designable formulations has great merit for implantable uses in tissue repair and regenerative therapy. Another important advantage of EBFC is good biocompatibility because the glucose/oxygen that exists at high concentrations in human body plasma can serve as fuel for EBFC. Despite these fascinating features of EBFC, no studies have yet been conducted using EBFC as an electrical stimulation system for the repair and regeneration of tissues including muscle.

Here we report the utility of EBFC for applications in tissue repair and regeneration targeting muscle tissue. Depending on the type of enzymes used, EBFC can be categorized into anodic, cathodic and full-set conditions, consequently providing electron-rich and electron-poor conditions to the cell and tissue microenvironment. In the cathodic compartment, Bilirubin oxidase (BOD) consumes electrons by reducing dioxygen (O_2) to water (H_2O), resulting in an electron-poor condition in the vicinity of the cathode [33,34]. In the anodic compartment, glucose oxidase (GOX) releases electrons by oxidizing glucose to gluconolactone, creating an electron-rich condition [33,34]. EBFC can therefore easily create two completely different electrical environments for the cell and tissue. The gradient electrical microenvironment is essential for action potentials in muscle cells.

Using EBFC-derived electron-rich or -poor conditions, we investigated the effects on the behaviors of the muscle precursor cell C2C12, such as cell proliferation, migration and differentiation. The results provided here will be highly useful for providing evidence on utilizing EBFC for the repair and regeneration of muscle as well as for possible extended utility in other tissues.

2. Materials and methods

2.1. C2C12 cells

Cells were purchased from American tissue culture collection (ATCC). Cells were thawed and plated at a density of 2×10^3 cells/cm^2 in culture dishes using Dulbecco's modified eagle medium (DMEM; LM 001-05, WelGENE, Daegu Korea) supplemented with 10% fetal bovine serum (FBS; Hyclone, Thermo), and 1% penicillin/streptomycin (Giboco) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Fabrication of EBFC

Screen-printed carbon electrodes (SPCEs) were prepared with Electrodag® 423SS (Acheson, Port Hurton, USA) on OHP film using a semi-automatic screen printing machine. The anodic enzyme was glucose oxidase (GOX) from *Aspergillus niger* (219 U/mg) purchased from Amano Enzyme Inc. (Japan). Cathodic catalyst was Bilirubin oxidase (BOD) from *Myrothecium verrucaria* (10.5 U/mg, Sigma enzyme). Poly (ethylene glycol) (400) diglycidyl ether (PEGDGE) supplied by Polysciences, Inc was used as a cross-linker. The loading solutions for anodic and cathodic electrodes were composed of enzyme, redox mediator, and cross-linker. The anodic catalyst consisted of the cross-linked adduct of 40 μg/ml of GOX, 2.0 mg/mL of PVI-[Os(4,4′-dimethoxy-2,2′-bipyridine)$_2$Cl]$^{+/2+}$, and 40.0 mg/mL of PEGDGE with the volume ratio (4: 4: 1). The cathodic catalyst consisted of the cross-linked adduct of 40 μg/ml of BOD, 2.0 mg/mL of PVI-[Os(4,4′-dimethoxy-2,2′-bipyridine)$_2$Cl]$^{+/2+}$, and 40.0 mg/mL of PEGDGE with the volume ratio (4: 4: 1). 10 μl of the mixture was placed on the SPCE, and dried for 24 h in the desiccator at room temperature (25 ± 1 °C).

2.3. Electrochemical characterization of EBFC

For electrochemical measurements, two SPCEs were glued onto the edge of the dish (35 pie dish). The anodic or cathodic electrodes used in this study were 15-mm-long and 2-mm-width, with active areas of 30 mm^2. A two-electrode electrochemical cell coupled to a CHI 660B potentiostat (*Austin, TX, USA*) was used for the open circuit potential (OCP) and *i-t* technique. The electrochemical characteristics of modified PVI-[Os(dmo-bpy)$_2$Cl]$^{+/2+}$ were studied with 3.5 mm-diameter working electrodes (SPCEs) on a flexible polyester film. A 0.5-mm-diameter platinum wire counter electrode and an Ag/AgCl micro-reference electrode (3.0 M KCl saturated with AgCl, Cypress, Lawrence, KS, USA) were used. The electrodes were placed on the culture dish with medium and cells similar to the cell culture conditions (detailed in the following sections), and the current density was monitored with respect to exposure time. The power density and polarization curves were obtained by a cyclic voltammetry technique [35]. The applied potential ranged from −0.4− to 0.6 V and the scan rate was 1 mV/s. The experiment was carried out under ambient air conditions at room temperature.

2.4. EBFC set-up for 2D cell cultures

C2C12 cells were seeded at 1×10^5/ml on the culture dish (35 pie dish) set up with three different EBFC designs or without EBFC. A 3 mm gap was left between the EBFC electrode and bottom of the culture dish. 2 ml of culture medium was added initially to level the medium below the electrodes, which is required for the cells to initially settle down and anchor to the culture dish. After 16 h, 3 ml of culture medium was added to completely cover the electrodes for enzymatic reaction. From this time point (t = 0), the cells were cultured for predetermined time points for required assays.

2.5. Live imaging of cells

A live image of cells was monitored to visualize the cell motility and adhesive protein arrangement. For this, cells were co-transfected with red fluorescent protein (RFP)-paxillin and green fluorescent protein (GFP)-actin. RFP-paxillin plasmid was kindly provided by Dr. Xiong from Georgia Health Science University. GFP-actin plasmid was purchased from BD pharmingen™ (Green FP vector-actin: 558721). Neon transfection system (Invitrogen life science) was used for the cell transfection under optimized electroporation condition (1100 V, 30 ms, and 1 pulse). After 3 days of culture, paxillin-RFP/actin-GFP co-transfected cells were trypsinized and prepared into a cell suspension (1×10^4/ml). The suspended cells were seeded on the confocal dish (100350, SPL Korea) for live imaging. Red and green positive cells were selected and the live image processed every 2.5 min for 20 cycles, by confocal laser scanning microscopy (META M700 ZEISS, Germany) for a total 50 min.

2.6. Cell count

After culturing the cells for different time points, cells were harvested by trypsinization and the cell number was determined by trypan blue exclusion and hemocytometer counting.

2.7. Cell migration study; wound closure model

For the *in vitro* cell migration study, the cell suspension was prepared at 5×10^5/ml in culture medium. Cell suspension of 75 μl was applied to each culture well. After culture for 24 h to attain a near cell-confluence, a cell-free gap was made using the culture-insert tool (80206, ibiTreat, ibidi, Germany), which creates a cell-free gap with a width of ~500 ± 50 μm. The medium was refreshed gently to eliminate any non-adherent cells. After 16 and 24 h of culture, the number of cells found within the gap was counted.

2.8. Adhesion protein rearrangement and cell polarization behaviors

To visualize the morphology of the cells, cultured cells were fixed with 4% paraformaldehyde. Next, the samples were subjected to regular immunocytochemistry procedures; treated with rabbit anti-focal adhesion kinase (FAK) antibody (A-17; 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C then with the anti-rabbit secondary FITC-conjugated antibody, phalloidin (A34055, Alexa Fluor 555 phalloidin, Invitrogen) for F-actin staining at room temperature for 1 h. The nuclei were counter-stained with DAPI. After washing with PBS, the sample was mounted by anti-faded medium (H-1000, Vectashield mounting media, Vector