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Electrically induced contraction of C2C12 myotubes cultured on a porous membrane-based substrate with muscle tissue-like stiffness

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ABSTRACT

A porous membrane-based cell culture device was developed to electrically stimulate a confluent monolayer of C2C12 myotubes. The device's cell culture substrate is a microporous alumina membrane-modified by attaching an atelocollagen membrane on the upperside and a hole-spotted poly(dimethylsiloxane) (PDMS) film on the underside. When electric current is generated between the device's Pt ring electrodes – one of which is placed above the cells and the other below the PDMS layer – the focused current at the PDMS hole can electrically stimulate the cells. C2C12 myoblasts were cultured on the substrate and differentiated into myotubes. When the electrical pulses were applied, myotubes started to contract slightly in and near the hole, and that the continuous stimulation increased both the number of stimuli-responding myotubes and the magnitude of the contraction considerably owing to the underlying atelocollagen membrane with muscle tissue-like stiffness. Also, the generation of contractile myotubes on a wider region of the membrane substrate was possible by applying the electrical pulses through the array of holes in the PDMS film. Using the present system, the glucose uptake by contractile myotubes was examined with fluorescence-labeled glucose, 2-NBDG, which displayed a positive correlation between the contractile activity of myotubes and the uptake of 2-NBDG.

1. Introduction

The use of *in vitro* cell culture models that exert physiologically relevant functions is a promising approach for investigations on biological processes as an alternative method of whole animal experiments [1,2]. Skeletal muscle is one of the major insulin-target tissues responsible for the maintenance of whole body glucose homeostasis [3,4], and defects in glucose uptake in skeletal muscle contribute to the insulin resistance characteristics of type 2 diabetes [5–7]. Also, adequate exercise is known to be highly helpful not only for preventing the development of type 2 diabetes but also for diabetic patients already suffering from insulin resistance though the precise mechanism has not been fully elucidated [8,9]. Analyses of the mechanistic details of insulin- and exercise-induced glucose uptake by skeletal muscle are often performed using whole animal experiments, because a conventional cell culture system lacks muscle contractile activity required for physiologically relevant energy expenditure and mechanical stress. Thus, the development of an *in vitro* culture system capable of inducing vigorous contraction of skeletal muscle cells has recently been attracting considerable attention. Besides, such a cell-based assay system will be beneficial to decrease costs while leading to more accurate prediction in the drug discovery process for screening candidate drugs against type 2 diabetes.

C2C12 myoblasts, a mouse skeletal muscle cell line, is one of the candidate cell types that can provide a skeletal muscle cell culture [10,11]. Myotubes differentiated from the myoblasts can be used to monitor skeletal muscle cell contraction and activity [12–19]. So far, a number of efforts have been made to generate *in vivo*-like structures and functions of skeletal muscle, including controlling alignment of myotubes [12,19–22], modulation of substrate stiffness [13,19,23,24], and creating 3D construct of myotubes [16–18,25,26]. Recent studies revealed that Ca^{2+} transients in C2C12 myotubes induced by electrical pulse stimulation can accelerate assembly of functional sarcomeres and stimulate contractile activity of the cells [14,15]. The fact that myotubes can

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contract in response to electrical stimulation signifies that contraction-inducible cellular responses such as glucose metabolism and protein secretion can be explored using such a contractile myotube model [15]. However, despite the strong demand for such culture system, an efficient way to reproducibly prepare contractile myotubes has not been well established. In most cases, the electrical stimulation of a myotube culture is conducted with a pair of stimulating electrodes placed at either side of the chamber. In such configuration, most of the current induced by the electrode polarization passes through the surrounding medium, which cannot cause cell depolarization.

To more efficiently stimulate C2C12 myotubes, we recently designed a porous alumina membrane-based cell culture device [27]. In this device, a porous alumina membrane is masked on its underside by an elastic thin film of poly(dimethylsiloxane) (PDMS) stenciled with a hole. Also, a pair of ring-type electrodes is placed at either side of the membrane on which a monolayer of myotubes is cultured. This configuration allows electric current generated between the electrodes to geometrically condense at the hole of the PDMS film and to perpendicularly pass through the cells, resulting in their effective depolarization, which has been demonstrated by visualizing intracellular Ca^{2+} transients with a Ca^{2+}-sensitive fluorophore. Moreover, in this configuration the voltage required for generating enough current to stimulate cells can be lower by ten or more times compared with conventional methods. However, in the previous study, very few cells contracted and the magnitude of contraction was smaller than expected, probably because the cell monolayer adhered strongly on the rigid membrane restricting the contractile degrees of freedom.

In this study, to facilitate the contraction of myotubes, the alumina membrane substrate was modified with an atelocollagen membrane. The muscle tissue-like stiffness of the atelocollagen membrane enabled the sarcomere assembly in myotubes during the differentiation from myoblasts, and resulted in the immediate contraction of myotubes with the electrical pulse. Then, the continuous stimulation developed the sarcomeric structures, allowing the vigorous contraction of the myotubes on the membrane with the proper stiffness. Additionally, the PDMS film with array of holes was employed to generate contractile myotubes on a wider region of the membrane substrate. Using the present system, the glucose uptake by myotubes was examined with fluorescence-labeled glucose, 2-NBDG.

2. Materials and methods

2.1. Materials

The following materials, reagents, and all other chemicals were purchased and used without further purification or modification: alumina membrane culture insert (pore size, 0.02 μm, thickness, 45 μm, NUNC cat. #161395, Thermo Fisher Scientific Inc., U.S.); poly(dimethylsiloxane) (PDMS) composed of prepolymer and curing agent (Silpot 184 W/C, Dow Corning Toray Co., Ltd., Japan); Atelocollagen Membrane (CM-6, KOKEN CO., LTD., Japan); Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemicals Industries, Ltd., Japan); fetal bovine serum (FBS, BioWest, U.S.); penicillin (Invitrogen Corp., U.S.); streptomycin (Invitrogen Corp., U.S.); 0.25% trypsin/0.01% EDTA solution (Invitrogen Corp., U.S.); calf serum (Biowest, U.S.); insulin (Sigma–Aldrich, U.S.); MEM amino acids solution (Invitrogen Corp., U.S.); MEM non-essential amino acid solution (Invitrogen Corp., U.S.); mouse monoclonal anti-sarcomeric α-actinin (Sigma–Aldrich, U.S.); Alexa Fluor 594 donkey anti-mouse IgG (Molecular Probes, U.S.); bovine serum albumin (Wako Pure Chemicals Industries, Ltd., Japan); DAPI (Dojindo, Tokyo, Japan); Vectashield (Vector Laboratories, Burlingame, CA, U.S.); 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG, Invitrogen Corp., U.S.).

2.2. Fabrication of the localized electrical stimulation device

The localized electrical stimulation device was fabricated as previously described [27], except for the modification with an atelocollagen membrane. Briefly, a commercially available, alumina membrane culture insert was modified by attaching an atelocollagen membrane (thickness, ca. 20 μm) on the upperside and a thin PDMS film (thickness, ca. 100 μm) on the underside (Fig. 1). The PDMS film has either one square hole of 1 mm^2 or nine holes of 0.3 mm in diameter arranged in a 3 × 3 array with an interpolar distance of 1.3 mm. The Pt ring electrode with the outer and inner diameters of 26 and 16 mm was fabricated on a glass plate; one electrode was placed above and another below the membrane substrate. The gap between the two electrodes is always 3 mm.

2.3. Measurement of the stiffness of atelocollagen membrane

Force spectroscopy measurements were conducted in DMEM using a NanoWizard atomic force microscope (AFM) system (JPK Instruments, Berlin, Germany) with a sharpened pyramidal cantilever (spring constant, 0.02 N m^{-1}, OMCL-TR400PSA-1, Olympus, Japan). Atelocollagen membrane was immersed in the medium for more than 3 h before the measurements. The resulting plot of the tip deflection and the cantilever position (Supplementary Fig. S1) yields an estimate of Young's elastic modulus of the membrane: $E = (\pi(1-\nu^2)k/2\tan\alpha)d/\delta^2$, where ν = Poisson's ratio of the sample (assumed here to be 0.5), α = the opening angle of the pyramid tip (18°), k = spring constant of the cantilever (0.02 N m^{-1}), d = tip deflection (m), and δ = the indentation of the sample (m) [28–30].

2.4. Cell culture

Murine C2C12 cells (less than six passages; American Type Culture Collection, U.S.) were maintained in a 37 °C, 5% CO_2 incubator and cultured in a growth medium: DMEM containing 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin [10,11]. When cell densities reached ~70% confluency, cells were detached by a 0.25% trypsin/0.01% EDTA solution and were either repropagated or used in experiments.

The alumina membrane-based substrate must be prepared before cell culture. To prevent bubble formation in the PDMS hole during the substrate pretreatment, the substrate was first treated with oxygen plasma (60 W, 30 s) to make the polymer surface hydrophilic. An atelocollagen membrane was then placed on the substrate in phosphate-buffered saline (PBS), followed by aspiration of the PBS. After the atelocollagen membrane was dried to allow attachment on the alumina surface, C2C12 myoblasts were seeded onto the membrane substrate at a density of 3×10^5 cells mL^{-1} and cultured until confluent. Once the cells achieved confluence, the differentiation to myotubes was induced by switching from the growth medium to differentiation medium: DMEM containing 2% calf serum, 1 nM insulin, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin [31]. The differentiation medium was changed daily. After 6 days, electrical stimulation studies were conducted.

2.5. Electrical pulse stimulation of myotubes

After assembling the device (Fig. 1), the camber was filled with DMEM containing 2% calf serum, 2% MEM amino acids solution, 1% MEM non-essential amino acid solution, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Current pulses

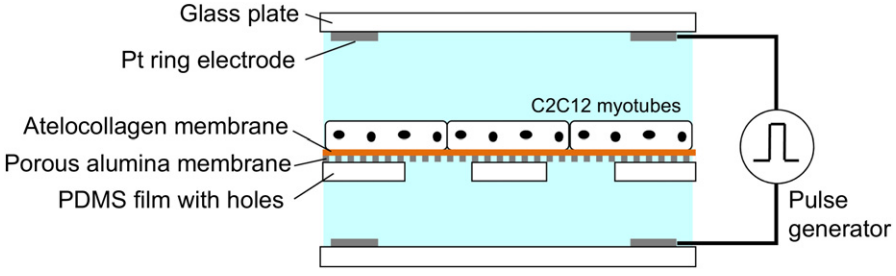

Fig. 1. Schematic illustration (cross section) of the porous membrane-based electrical stimulation device.