GEL SHEET BASED SKELETAL MUSCLE CELL CULTURE SYSTEM INTEGRATED WITH THE MICROELECTRODE ARRAY DEVICE

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ABSTRACT

We developed the fibrin gel-based C_2C_{12} myotube culture system integrated with the microelectrode array device to afford a physiologically relevant and stable bioassay system. Myotube/fibrin gel culture system was prepared by transferring a myotube monolayer from a glass substrate to a fibrin gel while retaining the original line patterns of myotubes. By attaching the myotube/fibrin gel onto the microelectrode array, we demonstrated site-specific electrical pulse stimulation of each myotube line pattern. This device would enable highly-accurate bioassay of contraction-mediated glucose metabolic alterations in myotubes by patterning test and control cells stimulated with different contraction/exercise stress next to each other.

KEYWORDS: skeletal muscle cell, type 2 diabetes, cell transfer, fibrin gel, microelectrode arrays

INTRODUCTION

In vitro bioassay system incorporating skeletal muscle cells could be required to reveal the complex mechanisms involved in the development and maintenance of type2 diabetes because type2 diabetes is closely associated with defection of insulin- or contraction-induced glucose uptake via glucose transporter (GLUT4) in skeletal muscle cells. An assay system, currently available, that is used to monitor skeletal muscle cell contraction and activity consists of myotube monolayer cultured on substrates with a pair of electrodes for electric stimulation. By applying electric pulse stimulation, sarcomere assembly is accelerated and allows for cell contraction. However, the contracting myotubes were difficult to maintain their structure for a long period of time because they readily detached from the substrate within a few days.

In this study, we have developed the contractile C_2C_{12} myotube line patterns transferred onto a fibrin gel and succeeded in maintenance of stable contractile activity for a longer period of time (one week) than the myotubes on the conventional dish system (less than a few days) [1]. Using this gel-based culture, contraction-induced GLUT4 translocation from intracellular vesicles to the plasma membrane was detected by immunofluorescent analysis. Besides, the myotube/fibriin gel integrated microelectrode array device was fabricated for a physiologically relevant and stable bioassay system.

EXPERIMENTAL

Preparation of the contractile C_2C_{12} myotube / fibrin gel sheet

Figure 1(A) shows the process of preparing the C_2C_{12} myotube/fibrin gel sheet [1,2]. 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer-patterned substrate was prepared by using the micromold method, and then sterilized under UV light for 15 min. The myoblasts suspended in the growth medium ($1x10^5$ cells mL^{-1}) were poured over the substrate and left over night during which time cells adhered to the exposed glass. Nonadherent cells were removed by washing with the growth medium. Cells were cultured in the growth medium until fully confluent. Once confluent, the myoblasts were induced to differentiate into myotubes by replacing the growth medium to the differentiation medium. Then, the myotubes were transferred onto a fibrin gel. A fibrinogen mixture solution was poured over the cells, and the substrate was left undisturbed for 2 h at 37 °C under a 5% CO_2 atmosphere to facilitate the mixture gelation and to allow the cells to adhere to the gel. By gently detaching the gel from the substrate, the gel sheet with myotube line patterns (250 μm line width) were prepared (Fig. 1(B), (C)). The myotube/fibrin gel sheet was placed in the carbon electrode chamber (Fig. 3(A)), followed by applying the electrical pulse (amplitude, 0.7 V mm^{-1}; duration, 2.0 ms; frequency, 1.0 Hz) against the myotubes on the fibrin gel to endow the cells with contractile activity.

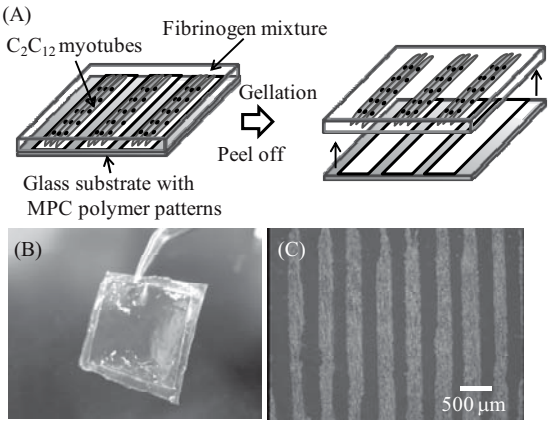

Figure 1: (A) preparation of line-patterned C_2C_{12} myotubes transferred onto a fibrin gel. (B) A photograph of a fibrin gel. (C) Myotube line patterns on a fibrin gel.

GLUT4 translocation assay with immunofluorescent analysis

Figure 2 shows the principle of GLUT4 translocation assay with immunofluorescent analysis. In the basal state, GLUT4 is stored in intracellular vesicles. Upon myotube contraction, these vesicles translocate to the plasma membrane where GLUT4 becomes inserted in the membrane and glucose uptake is facilitated. In this study, GLUT4 fully incorporated in the plasma membrane was detected by immunostaining with fluorescently-labeled antibody.

Transfected myotube line patterns stably expressing rat GLUT4, possessing the c-*myc* epitope tag in the first extracellular loop and enhanced cyan fluorescent protein (ECFP) at the carboxyl terminus[3], were transferred onto a fibrin gel. After 6 days of electrical stimulation in the carbon electriode chamber (amplitude, 0.7 V mm^{-1}; duration, 2.0 ms; frequency, 1.0 Hz), the cells were then fixed with 1% paraformaldehyde in PBS(-), followed by immunostaining with the mouse anti-c-*myc* antibody, and Alexa594-conjugated anti-mouse IgG antibody. Fluorescent images were monitored and analyzed using a confocal microscope.

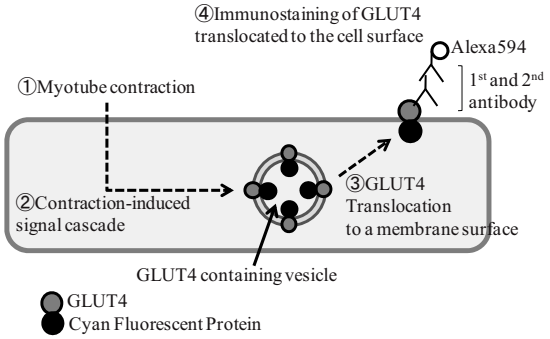

Figure 2: Principle of GLUT4 translocation assay with immunofluorescent analysis.

Site-specific electrical pulse stimulation of C_2C_{12} myotubes on a fibrin gel using the microelectrode array chip

Semicircular-formed microelectrode arrays (gap between the paired electrodes: 400 μm) was fabricated on a glass substrate by conventional photolithography and lift-off techniques. The myotube/fibrin gel sheet was attached on the microelectrode array chip, and then, each myotube line pattern was carefully aligned with the paired microelectrode patterns under microscope observation using a tweezers. Electrical pulses (amplitude: 2.0 V vs. ground electrode, frequency: 1.0 Hz, duration: 10.0 ms) were applied to an arbitral microelectrode pairs to induce contraction of each myotubes line pattern individually.

RESULTS AND DISCUSSION

Figure 3(B) shows the contractile properties of myotubes on a fibrin gel. The myotube line patterns on the gel exhibited vigorous twitch contraction depending on electrical pulse stimulation. The frequencies of contractions were completely synchronized with the electrical pulse frequencies (1Hz). The ability to control the myotube contraction would be beneficial for quantitative investigation of contraction-mediated metabolic alterations in myotubes. We found that the myotubes supported by an elastic fibrin gel maintained their contractile activity for a longer period of time (one week) than myotubes on a conventional culture dish, suggesting that the myotube/fibrin gel culture system would be better suited, than a conventional dish-based culture, to stable skeletal muscle cell based bioassay.

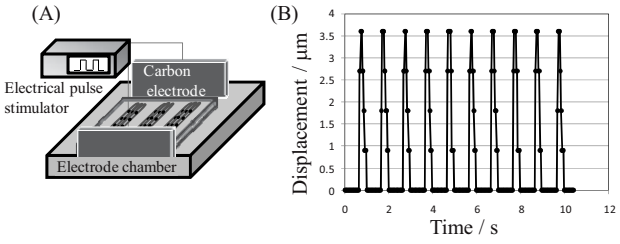

Figure 3: (A) Set up for entire electrical pulse stimulation of myotube line patterns on the gel. (B) The time course of contractile displacement of myotube line pattern on a fibrin gel when stimulated with continuous electrical pulse (amplitude, 0.7 V mm-1; duration, 2.0 ms; frequency, 1.0 Hz)

Figure 4 shows the fluorescent images of immunostained myotube line patterns on the fibrin gel stimulated with (A, B) or without (C, D) electrical pulse. Fig. 3(A), (C) represent the fluorescence of ECFP fused to GLUT4, suggesting expression of GLUT4 in the myotubes. Under basal condition, myotubes displayed no detectable surface expression of GLUT4 as assessed by immunostaining (Fig. 4(D)). After electrical pulse stimulation, myotubes displayed marked cell-surface expression of GLUT4 (Fig. 4(B)). These results suggested that myotube/fibrin gel culture system could be applicable to the contraction-induced GLUT4 translocation assay.

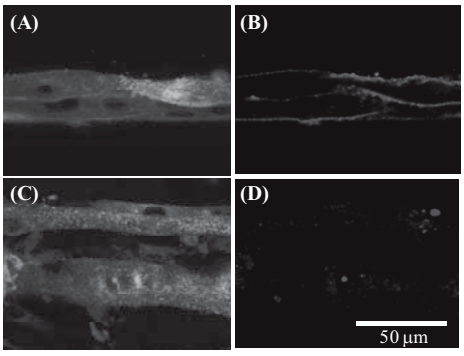

Figure 4: Fluorescent images of immunostained myotube line patterns on a fibrin stimulated with (A,B) or without (C,D) electrical pulse. (A),(C) represent the fluorescence of ECFP, and (B), (D) the Alexa594 fluorescence.

The C_2C_{12} myotube/fibrin gel sheet was easy to handle, allowing to be attached to various electric devices. Figure 5(A) shows the setup of myotube/fibrin gel combined with the microelectrode array device, and Fig 5(B) shows the micrograph of myotube line patterns aligned onto the microelectrode arrays. Fig. 5 (C) shows the time course of contractile

displacements of myotube line patterns. (1)-(3) in Fig. 5(C) represent the contractile behaviors of myotube line patterns shown in Fig. 5(B) (1)-(3). At first all the myotube line patterns were electrically stimulated, followed by site-specific electrical stimulation against myotube (2), exhibiting independent contractile behavior of each myotube line pattern on the microelectrode arrays. This device would enable highly-accurate bioassay of contraction-mediated metabolic alterations in myotubes by patterning test and control myotubes with different exercise stress next to each other. Now, we are investigating contraction-mediated translocation of GLUT4 in myotubes using this device.

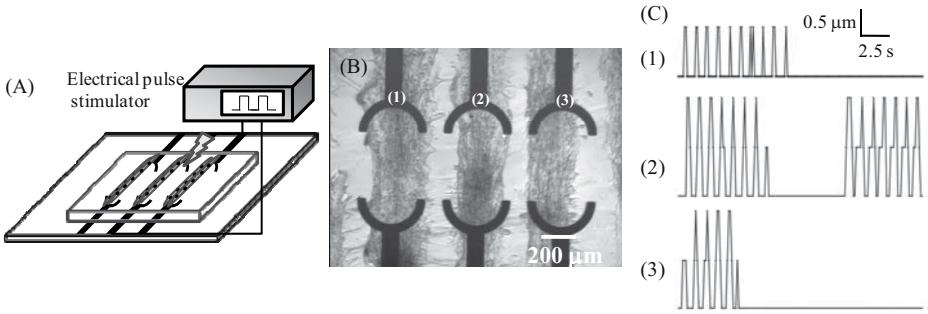

Figure 5: (A) the set up for site-specific electrical stimulation of myotube line using the microelectrode arrays. (B)the micrograph of myotube line patterns aligned with the microelectrode arrays. (C) the time course of myotube line patterns contraction stimulated with the electrical pulse (applied potential, 2.0 V vs. ground electrode; frequency, 1.0 Hz; duration, 10.0 ms).

CONCLUSION

In this study, we prepared micropatterns of contractile C_2C_{12} myotube on a fibrin gel by means of the cell transfer technique and the electrical pulse stimulation method. The myotube/fibrin gel system showed flexible contraction without detachment and longer-term stability sustaining contraction than the conventional dish-based system. Using this gel-based culture system, we succeeded in detection of contraction-induced GLUT4 translocation by immunostaining method. Finally, the myotube/gel culture system combined with the microelectrode arrays was fabricated and demonstrated site-specific electrical pulse stimulation of each myotube line pattern on the gel. Now, we are planning to investigate contraction-mediated translocation of the GLUT4 in myotubes using this device and immunocytiochemical GLUT4 translocation assay method.

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