Journal of Bioscience and Bioengineering
VOL. 108 No. 6, 538 – 543, 2009

www.elsevier.com/locate/jbiosc

Preparation of artificial skeletal muscle tissues by a magnetic force-based tissue engineering technique

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Received 3 April 2009; accepted 29 May 2009

Artificial muscle tissues composed of mouse myoblast C2C12 cells were prepared using a magnetic force-based tissue engineering technique. C2C12 cells labeled with magnetite nanoparticles were seeded into the wells of 24-well ultralow-attachment culture plates. When a magnet was positioned underneath each plate, the cells accumulated evenly on the culture surface and formed multilayered cell sheets. Since the shapes of artificial tissue constructs can be controlled by magnetic force, cellular string-like assemblies were formed by using a linear magnetic field concentrator with a magnet. However, the resulting cellular sheets and strings shrank considerably and did not retain their shapes during additional culture periods for myogenic differentiation. On the other hand, when a silicone plug was positioned at the center of the well during the fabrication of a cell sheet, the cell sheet shrank drastically and formed a ring-like assembly around the plug. A histological examination revealed that the cells in the cellular ring were highly oriented in the direction of the circumference by the tension generated within the structure. Individual cellular rings were hooked around two pins separated by 10 mm, and successfully cultured for 6 d without breakage. After a 6-d culture in differentiation medium, the C2C12 cells differentiated to form myogenin-positive multinucleated myotubes. Highly dense and oriented skeletal muscle tissues were obtained using this technique, suggesting that this procedure may represent a novel strategy for muscle tissue engineering.

[**Key words:** C2C12 cell; Skeletal muscle; Magnetite; Magnetic force; Tissue engineering]

There is a substantial need for replacement of muscle tissues following traumatic injury, tumor ablation or functional loss caused by muscle diseases such as muscular dystrophy (1). To treat the loss of skeletal muscle function, muscle tissue engineering approaches have been widely attempted. To construct artificial skeletal muscles that are physiologically equivalent to native muscles, mimicking of the natural skeletal muscle is believed to be crucial. For this purpose, tissue-engineered skeletal muscle should have the following two structural features: 1) a high cell density that may lead to cell fusion, resulting in multinucleated myotube formation; and 2) a highly unidirectional orientation that facilitates large muscular forces.

Skeletal muscle tissue-like constructs have been produced from mammalian cells using established skeletal muscle myoblast cell lines (2, 3), including C2C12 cells, or primary cells from neonatal rats (4). For the fabrication of these tissue-engineered constructs, artificial scaffolds such as biodegradable sponges (5) and hydrogels composed of collagen (6) and/or Matrigel (7) have been employed. Nevertheless, since the native skeletal muscle tissue is constructed with a high cell density, the use of synthetic scaffolds may interfere with cell–cell interactions, thereby resulting in the inhibition of multinucleated myotube formation. Consequently, achievement of successful muscle tissue engineering is associated with how a higher density of skeletal muscle cells can be achieved within artificial muscle tissues. On the other hand, to fabricate highly oriented artificial muscle constructs in which the myotubes are aligned, controlled uniaxial mechanical strains have sometimes been applied. Okano and Matsuda (8) reported that highly dense and oriented muscle tissues composed of a mixture of C2C12 cells and type I collagen were successfully prepared by sequential procedures of centrifugal cell packing and mechanical stress-loading.

Magnetite cationic liposomes (MCLs), which are cationic liposomes containing 10-nm magnetite nanoparticles, were previously developed to improve the accumulation of magnetite nanoparticles in target cells through electrostatic interactions between the MCLs and the cell membrane (9). We have developed a tissue engineering technique using magnetic force based on the fact that cells labeled with MCLs can be manipulated using a magnet (10). This methodology has been designated the magnetic force-based tissue engineering (Mag-TE) technique. With this technique, target cells such as human keratinocytes (11), rat neonatal cardiomyocytes (12) and human mesenchymal stem cells (13) labeled with MCLs were accumulated using a magnet. Subsequently, stratification was promoted by the

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doi:10.1016/j.jbiosc.2009.05.019

magnetic force, leading to the formation of multilayered sheet-like constructs without using any artificial scaffolds.

In the present study, we propose a novel procedure for the fabrication of highly dense and oriented muscle tissue constructs based on the Mag-TE technique.

MATERIALS AND METHODS

Preparation of MCLs The magnetite (Fe_3O_4; average particle size, 10 nm) used in the present study was donated by Toda Kogyo (Hiroshima). The magnetic characteristics of the magnetite at 796 kA/m (room temperature) were 2.0 kA/m, 63.9 Am^2/kg and 2.6 Am^2/kg for coercivity, saturation flux density and remanent flux density, respectively. MCLs were prepared from the colloidal magnetite and a lipid mixture consisting of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride, dilauroylphosphatidylcholine and dioleoylphosphatidyl-ethanolamine in a molar ratio of 1:2:2 as described previously (9).

C2C12 cell culture Mouse C2C12 myoblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml potassium penicillin G and 100 mg/ml streptomycin sulfate and cultured at 37 °C in a 5% CO_2 incubator.

MCL uptake by C2C12 cells C2C12 cells (7×10^5 cells) were seeded into 100-mm cell culture dishes (Greiner Bio-one, Frickenhausen, Germany) containing 10 ml of culture medium in the presence of MCLs (net magnetite concentration, 100 pg/cell) and incubated for 8 h to allow MCL uptake. To assay the amount of MCL uptake, the iron concentrations in the cells were measured using the potassium thiocyanate method (14).

Fabrication of C2C12 cell sheets For fabrication of C2C12 cell sheets, cells labeled with MCLs were harvested by trypsin treatment, and a predetermined number of cells (2 or 4×10^6 cells) were reseeded into the wells of 24-well ultralow-attachment culture plates (cat. no. 3473; Corning, New York, NY, USA; culture area, 200 mm^2/well), whose surfaces were composed of a covalently bound hydrogel layer that was hydrophilic and neutrally charged. Next, a cylindrical neodymium magnet (diameter, 30 mm; height, 15 mm; magnetic induction, 0.4 T) was placed on the reverse side of each plate to apply a vertical magnetic force to the plate, and the cells were cultured to allow the formation of C2C12 cell sheets.

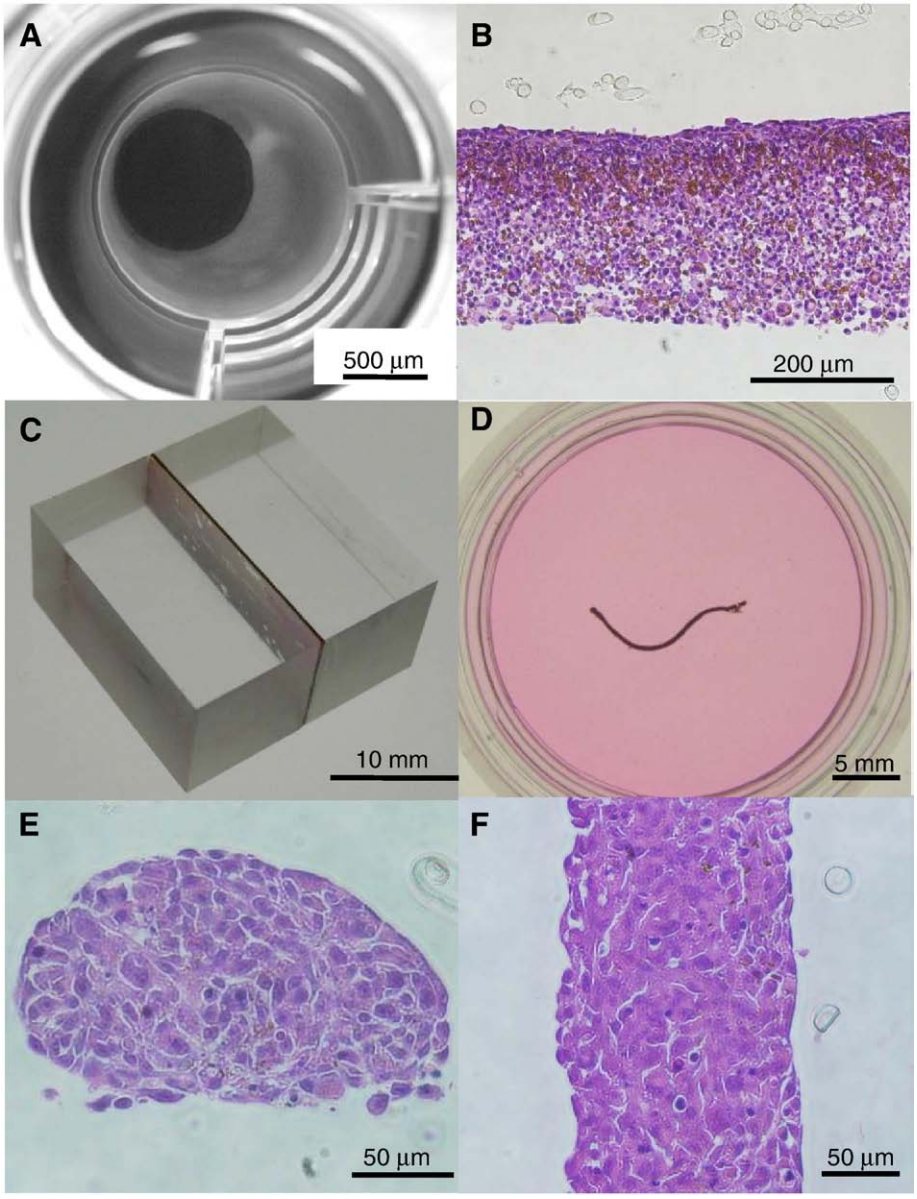

FIG. 1. C2C12 cell sheets and strings constructed using the Mag-TE technique. To construct cell sheets, C2C12 cells (4×10^6 cells) labeled with MCLs were seeded into the wells of ultralow-attachment culture plates and cultured on a magnet for 24 h. (A) A bright-field photograph of a cellular sheet. (B) A bright-field micrograph of a hematoxylin and eosin-stained cross-section of the cellular sheet. (C) To construct cellular strings, a magnetic field concentrator, in which a steel plate of 200-µm thickness was sandwiched between acryl resin plates, was used. C2C12 cells labeled with MCLs were seeded onto agarose-coated culture dishes and cultured in the presence of the magnetic field concentrator on a magnet. (D) A bright-field photograph of a cellular string. (E) A bright-field micrograph of a hematoxylin and eosin-stained axial section of the cellular string. (F) A bright-field micrograph of a hematoxylin and eosin-stained longitudinal section of the cellular string.