

Research Article

Alignment of Skeletal Muscle Cells Cultured in Collagen Gel by Mechanical and Electrical Stimulation

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For in vitro tissue engineering of skeletal muscle, alignment and fusion of the cultured skeletal muscle cells are required. Although the successful alignment of skeletal muscle cells cultured in collagen gel has been reported using a mechanical force, other means of aligning cultured skeletal muscle cells have not been described. However, skeletal muscle cells cultured in a two-dimensional dish have been reported to align in a uniform direction when electrically stimulated. The purpose of this study is to determine if skeletal muscle cells cultured three-dimensionally in collagen gels can be aligned by an electrical load. By adding direct current to cells of the C2C12 skeletal muscle cell line cultured in collagen gel, it was possible to align C2C12 cells in a similar direction. However, the ratio of alignment was better when mechanical force was used as the means of alignment. Thus for tissue engineering of skeletal muscle cells, electrical stimulation may be useful as a supplementary method.

1. Introduction

In order to construct functional skeletal muscle in vitro for use in tissue engineering, it is important to align skeletal muscle cells in a uniform direction. In a three-dimensional (3D) collagen gel culture without any applied stress, skeletal muscle cells do not orient uniformly. Although some research groups have shown the alignment of skeletal muscle cells in collagen gel using mechanical force [1, 2], other means of aligning skeletal muscle cells in 3D culture have not been reported.

In monolayer culture, the application of a direct current (DC) electrical field to muscle cells causes myoblast axes to orient perpendicular to the electrical current [3–8]. However, it has not been determined if skeletal muscle cells will align in a 3D collagen gel culture using a similar electrical load. Thus we cultured skeletal muscle cells three-dimensionally under either a mechanical load or an electrical load and compared the rate of alignment.

2. Materials and Methods

2.1. Skeletal Muscle Cells. Mature murine myogenic cell line C2C12 cells were purchased from Riken Cell Bank (Tsukuba, Japan). The culture medium was Dulbecco's modified essential medium (DMEM, Sigma, St. Louis) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S).

2.2. Mechanical and Electrical Load. We made ring-shaped collagen gels containing C2C12 cells. Cells were suspended in 0.25% Trypsin/EDTA, centrifuged, and embedded in type I collagen gel solution (Cell-Matrix 3D cell culture kit, Nitta Gelatin Inc., Japan) at 3×10^5 cells/mL. The collagen gels were incubated at 37°C, 5% CO₂ for 30 min until gel became solid.

The collagen gel rings were made following Okano et al. [9] and were stretched every minute for 24 hours by a hook connected to a motor (Figure 1). Cells in the collagen gels were cultured in DMEM with 10% FBS and 1% P/S.

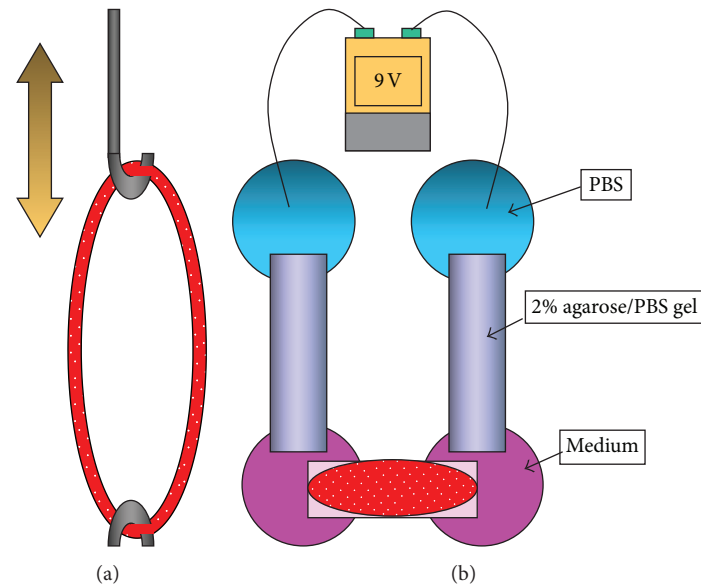


FIGURE 1: Schematic diagram of the methods used to apply mechanical force (a) and electrical load (b). For application of mechanical force, the hook moves up and down twice per minute in the direction of the arrow (a). For electrical stimulation, the electrical circuit consists of a pair of electrode baths containing DMEM with 10% FBS and 1% P/S, a pair of agar bridges (8 cm long) made of phosphate buffer saline (PBS) gelled by 2% agar, and a pair of electrode baths containing PBS; electrical current was supplied by a 9 V dry battery (b).

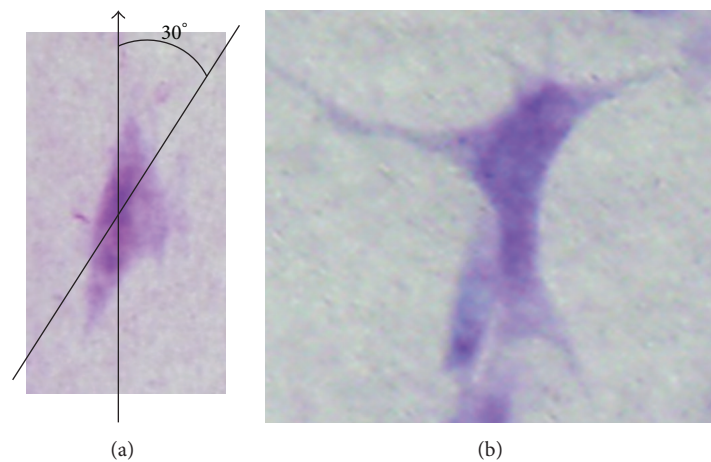


FIGURE 2: (a) Spindle-shaped cells that were inclined over 30° toward the applied electrical field or mechanical force were counted. (b) Spherical and multi-axis cells were not included in the cell count.

The equipment used to apply the electrical load is shown in Figure 2. The electrical circuit consisted of a pair of electrode baths containing DMEM with 10% FBS and 1% P/S, a pair of agar bridges (8 cm long) made of phosphate buffered saline (PBS) gelled using 2% agar, and a pair of electrode baths containing PBS. Electrical current was supplied by a 9 V dry battery. The voltage loaded between both sides of the collagen gels implanted with skeletal muscle cells was measured using an electrometer and was adjusted to 10 V/cm. The strength of the voltage loaded was the same as that used in two-dimensional cultures [10]. As for control, C2C12 cells in collagen gel were cultured without mechanical and electrical load.

After applying the mechanical or electrical load for 24 hours at 37°C and 5% CO_2 , gels were collected and each gel containing C2C12 cells was bisected. Half of each gel was fixed with 4% paraformaldehyde and then observed under a phase-contrast microscope. Gels were then air-dried on a cover slip and stained with Giemsa solution and used for scoring cell alignment. The remainder of the gels was used for analyzing the expression of proteins important for muscle differentiation.

2.3. Cell Scoring. All spindle-shaped cells were categorized as aligned cells. Spindle-shaped cells that were inclined more than 30° toward the applied electrical field or mechanical

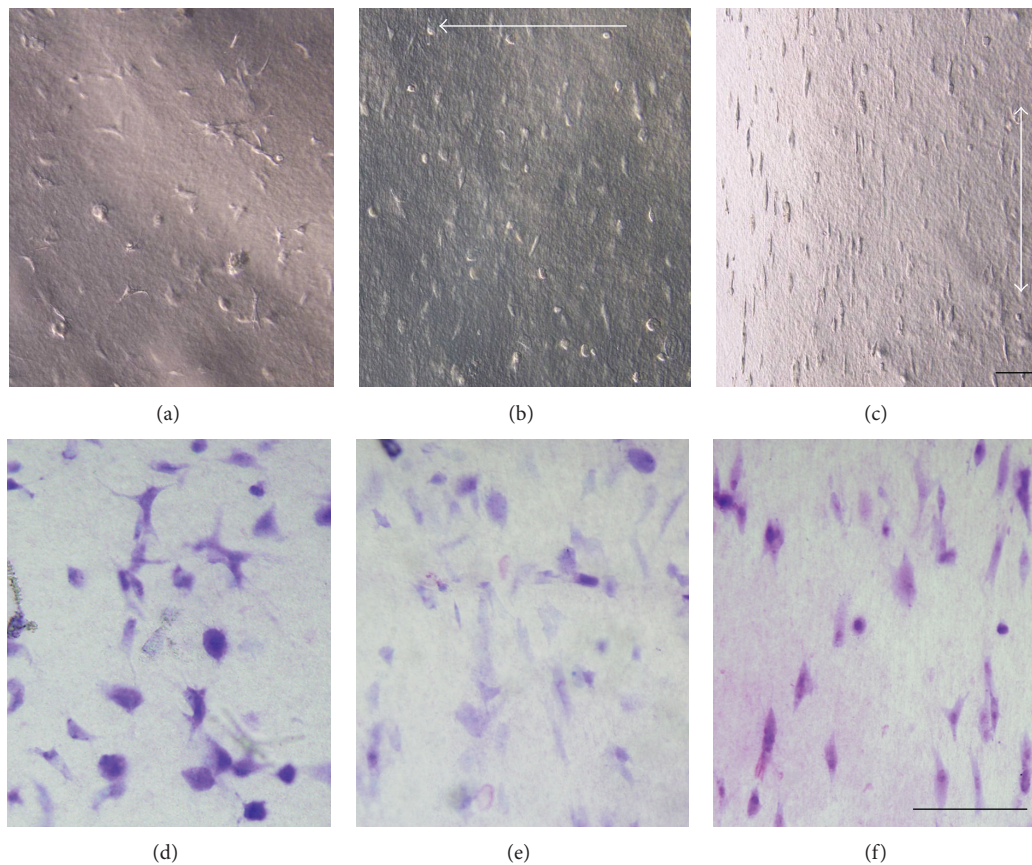


FIGURE 3: Phase-contrast microscopic views ((a), (b), and (c)) and Giemsa staining ((d), (e), and (f)) of C2C12 cells cultured under an electrical field ((b), (e)), with mechanical force ((c), (f)) and without electrical/mechanical load ((a), (d)). The arrows indicate the direction of either electrical current (b) or mechanical force (c). Scale bar = 50 μm .

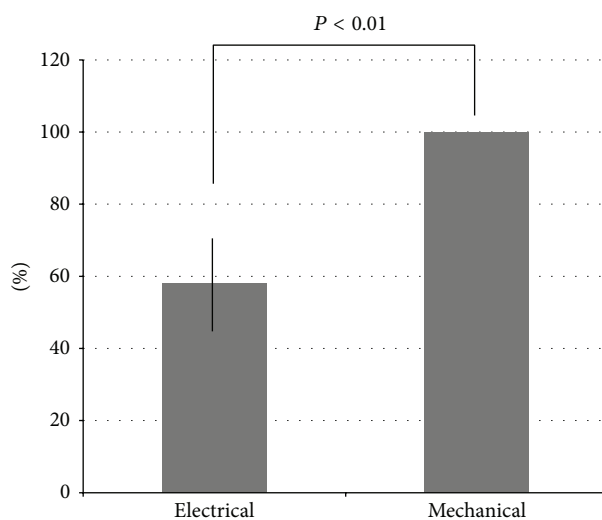


FIGURE 4: The rate of C2C12 myoblast cell alignment. When an electrical load was applied under the present conditions, cells tended to align perpendicular to the electrical field. The rate was $58 \pm 14\%$ (Electrical). In contrast, the rate of aligned cells along the direction of the mechanical force was 100% (Mechanical).

force were positively counted. Spindle-shaped cells that were inclined below 30° toward the applied electrical field or added mechanical force were not counted. Cells with spherical shapes or multiple axes were not included in the cell count. We counted 10 randomly chosen areas viewed at low magnification and compared the two groups. Statistical analysis was performed using Student's *t* test.

2.4. Immunocytochemistry. The rest of the gels were also air-dried on a cover slip and used for immunocytochemical study. The cover slips were immunostained using rabbit antibodies against MyoD and myogenin to observe the status of muscle differentiation.

3. Results

When mechanical stress was applied to the collagen gel rings, all cells were aligned in the same direction as the mechanical force (Figures 3 and 4). When viewed microscopically, C2C12 cells were observed to be stretched parallel to the mechanical force.

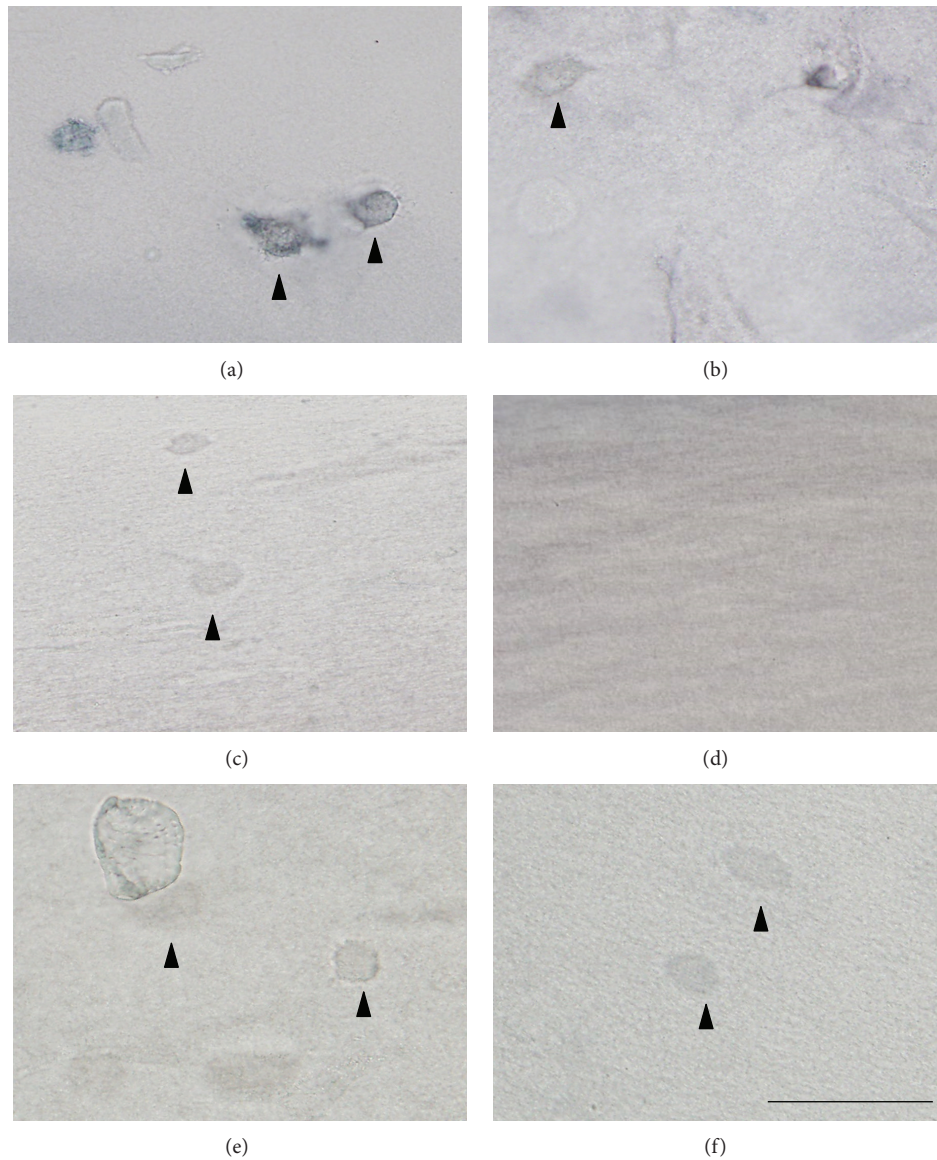


FIGURE 5: The expression of MyoD and myogenin. Almost all cells in three different conditions were positively stained with MyoD in the nucleus ((a), (c), and (e), arrow heads). The number of myogenin positive cells was rare ((b), (d), and (f), arrow heads). Bar=20 μ m.

In contrast, when a 10 V/cm DC electrical load was applied for 24 hours, many cells formed spindle shapes and their bipolar axes tended to be perpendicular to the applied electrical field (Figures 3 and 4). The ratio of alignment was $58 \pm 14\%$.

The expression of MyoD, a myoblast cell marker, was observed in the nucleus in almost all cells. Some cells were positively stained with myogenin, a differentiated skeletal muscle cell marker, but the positive cells were rare and there was no difference between three groups (Figure 5).

4. Discussion

In order to build skeletal muscle structure for the purpose of tissue engineering, it is important to culture cells

three-dimensionally. During in vivo skeletal muscle development many factors are reported to direct the alignment of skeletal muscle cells [11–20]. Among them, mechanical stress-like tension or movement of the fetus and electrical load have been reported to exert an influence on cell alignment in vivo. Consistent with this, some studies have successfully aligned skeletal muscle cells in collagen gel using mechanical stress [1, 2, 10]. In our study, the addition of mechanical stress resulted in almost all cells aligning in a uniform direction with minimum loss of cell viability.

In monolayer culture, various studies on the relationship between alignment and electrical load have been reported [3–8, 10]. Hinkle and colleagues showed that initially spherical myoblasts formed bipolar axes at right angles to an applied electrical field [3]. Moreover, concanavalin A receptors in

the membrane of cultured embryonic muscle cells were shown to incline in an electrical field [10]. It is hypothesized that the difference in electrophoretic redistribution of ion channels within cell membranes influences the orientation of myoblasts. However, skeletal muscle cell alignment in collagen gel had yet to be reported.

In the present study, when an electrical load was applied, skeletal muscle cells tended to align perpendicular to the electrical current. However, some cells appeared to lose viability and long term culturing with the aim of constructing muscle fibers oriented in the same direction proved to be difficult.

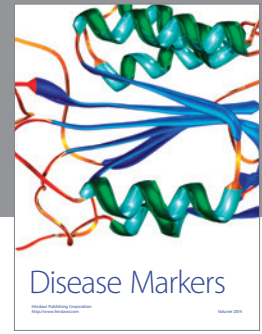
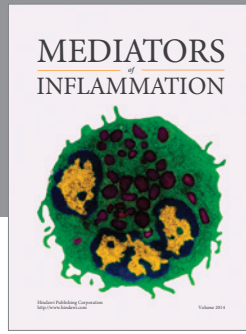
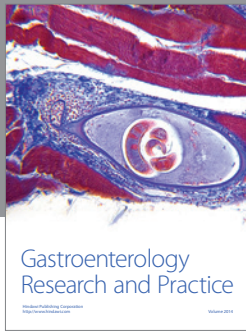
In conclusion, in order to achieve in vitro skeletal muscle cell tissue engineering, mechanical force is an effective means of cell alignment. While electrical load can also align skeletal muscle cells to some degree, the power of alignment is not sufficient. However, for tissue engineering of skeletal muscle cells, electrical stimulation may be used as a supplementary approach.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] H. H. Vandenburg, S. Hatfaludy, P. Karlisch, and J. Shansky, "Skeletal muscle growth is stimulated by intermittent stretch-relaxation in tissue culture," *American Journal of Physiology*, vol. 256, no. 3, part 1, pp. C674–C682, 1989.
- [2] M. S. Sakar, D. Neal, T. Boudou et al., "Formation and optogenetic control of engineered 3D skeletal muscle bioactuators," *Lab on a Chip*, vol. 12, no. 23, pp. 4976–4985, 2012.
- [3] L. Hinkle, C. D. McCaig, and K. R. Robinson, "The direction of growth of differentiating neurones and myoblasts from frog embryos in an applied electric field," *Journal of Physiology*, vol. 314, pp. 121–135, 1981.
- [4] K. R. Robinson, "The responses of cells to electrical fields: a review," *Journal of Cell Biology*, vol. 101, no. 6, pp. 2023–2027, 1985.
- [5] S. Ahadian, S. Ostrovidov, V. Hosseini et al., "Electrical stimulation as a biomimicry tool for regulating muscle cell behavior," *Organogenesis*, vol. 9, no. 2, pp. 87–92, 2013.
- [6] D. W. van der Schaft, A. C. van Spreuwel, K. J. Boonen, M. L. Langelaan, C. V. Bouten, and F. P. Baaijens, "Engineering skeletal muscle tissues from murine myoblast progenitor cells and application of electrical stimulation," *Journal of Visualized Experiments*, no. 73, article e4267, 2013.
- [7] S. Ahadian, J. Ramón-Azcón, S. Ostrovidov et al., "Interdigitated array of Pt electrodes for electrical stimulation and engineering of aligned muscle tissue," *Lab on a Chip*, vol. 12, no. 18, pp. 3491–3503, 2012.
- [8] M. Flaibani, L. Boldrin, E. Cimetta, M. Piccoli, P. De Coppi, and N. Elvassore, "Muscle differentiation and myotubes alignment is influenced by micropatterned surfaces and exogenous electrical stimulation," *Tissue Engineering A*, vol. 15, no. 9, pp. 2447–2457, 2009.
- [9] T. Okano, S. Satoh, T. Oka, and T. Matsuda, "Tissue engineering of skeletal muscle: highly dense, highly oriented hybrid muscular tissues biomimicking native tissues," *ASAIO Journal*, vol. 43, no. 5, pp. M749–M753, 1997.
- [10] M. Poo and K. R. Robinson, "Electrophoresis of concanavalin A receptors along embryonic muscle cell membrane," *Nature*, vol. 265, no. 5595, pp. 602–605, 1977.
- [11] R. C. Strohman, E. Bayne, D. Spector, T. Obinata, J. Micou-Eastwood, and A. Maniotis, "Myogenesis and histogenesis of skeletal muscle on flexible membranes in vitro," *In Vitro Cellular and Developmental Biology*, vol. 26, no. 2, pp. 201–208, 1990.
- [12] R. Shah, J. C. Knowles, N. P. Hunt, and M. P. Lewis, "Development of a novel smart scaffold for human skeletal muscle regeneration," *Journal of Tissue Engineering and Regenerative Medicine*, 2013.
- [13] M. E. Kolewe, H. Park, C. Gray, X. Ye, R. Langer, and L. E. Freed, "3D structural patterns in scalable, elastomeric scaffolds guide engineered tissue architecture," *Advanced Materials*, vol. 25, no. 32, pp. 4459–4465, 2013.
- [14] P. Y. Wang, T. H. Wu, W. B. Tsai, W. H. Kuo, and M. J. Wang, "Grooved PLGA films incorporated with RGD/YIGSR peptides for potential application on skeletal muscle tissue engineering," *Colloids and Surfaces B*, vol. 110, pp. 88–95, 2013.
- [15] R. Shah, D. Ready, J. C. Knowles, N. P. Hunt, and M. P. Lewis, "Sequential identification of a degradable phosphate glass scaffold for skeletal muscle regeneration," *Journal of Tissue Engineering and Regenerative Medicine*, 2012.
- [16] I. E. Palamà, A. M. Coluccia, G. Gigli, and M. Riehle, "Modulation of alignment and differentiation of skeletal myoblasts by biomimetic materials," *Integrative Biology*, vol. 4, no. 10, pp. 1299–1309, 2012.
- [17] S. L. Hume, S. M. Hoyt, J. S. Walker et al., "Alignment of multi-layered muscle cells within three-dimensional hydrogel macrochannels," *Acta Biomaterialia*, vol. 8, no. 6, pp. 2193–2202, 2012.
- [18] N. F. Huang, R. J. Lee, and S. Li, "Engineering of aligned skeletal muscle by micropatterning," *American Journal of Translational Research*, vol. 2, no. 1, pp. 43–55, 2010.
- [19] P.-Y. Wang, H.-T. Yu, and W.-B. Tsai, "Modulation of alignment and differentiation of skeletal myoblasts by submicron ridges/grooves surface structure," *Biotechnology and Bioengineering*, vol. 106, no. 2, pp. 285–294, 2010.
- [20] I. C. Liao, J. B. Liu, N. Bursac, and K. W. Leong, "Effect of electromechanical stimulation on the maturation of myotubes on aligned electrospun fibers," *Cellular and Molecular Bioengineering*, vol. 1, no. 2-3, pp. 133–145, 2008.



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