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The Method of Primary Cell Culture of Rat's Gastrocnemius Muscle and Comparison between Skeletal Muscle Cell and Tendon Cell in Protein Expressions of Myogenic Factor 5, Paired Box 7, and Desmin

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Original Article

The Method of Primary Cell Culture of Rat's Gastrocnemius Muscle and Comparison between Skeletal Muscle Cell and Tendon Cell in Protein Expressions of Myogenic Factor 5, Paired Box 7, and Desmin

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Skeletal muscle cells generate force and produce joint movement and are easily injured. A good understanding of the biological, physiological, and pathological mechanisms of skeletal muscle cells is essential for exploring the regulation of muscle repair, which occurs in the event of an injury. A method for reproducing skeletal muscle cells is essential for studying skeletal muscles. Most studies use cell lines as study models because these are capable of reproducing numerous times. However, the function of cell lines is markedly deviated from that of normal cells. Skeletal muscle cells from primary cell culture more closely represent muscle cells *in vivo*. They are better sources for the further study of the skeletal muscles, including the evaluation of the mechanisms underlying muscle damage and repair.

In the present study, we used the gastrocnemius muscle from Sprague Dawley rats to establish a protocol for primary skeletal muscle cell culture *in vitro*. Initially, the gastrocnemius muscle was excised from the rats and placed in a centrifuge tube. We used enzymes to separate the cells. After centrifugation, the supernatant was collected on a special medium. The first adherent cells on the culture plate were fibroblasts. The non-adherent cells were collected for further culture. After 24 hours of incubation, the adherent cells were washed with phosphate-buffered saline and kept cultured in the medium until confluence. These cells then underwent western blot analysis. The results revealed expressions of myogenic factor 5 (Myf5), paired box 7 (Pax7), and desmin proteins, which are only usually present in skeletal muscles. These findings provided evidence of the presence of skeletal muscle cells in the culture.
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Key Words: skeletal muscle cell, Myf5, Pax7, desmin, primary culture

INTRODUCTION

A skeletal muscle is enclosed in a sheath of connective tissue called epimysium. Bundles of muscle fibers and their individual contracting units are surrounded by partitions of the connective tissue constituting the perimysium and endomysium, respectively.[1] The muscle

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fiber is a multinucleated tubular structure formed by the fusion of multiple mononucleated muscle cells. Muscle precursor cells are generated by muscle stem cells and can differentiate into muscle cells.[2] A satellite cell is a mononucleate muscle precursor cell defined by its position beneath the basal laminae of myofibers. Satellite cells become activated, proliferate in response to muscle injury, and generate myoblasts by asymmetric division and differentiation. Myoblasts can generate new differentiated myofibers that fuse to repair or replace damaged myofibers.[3]

Skeletal muscles generate joint movement, provide joint stability, and assist in joint protection.[4] Muscle strains are a result of overstretching and can occur during participation in daily tasks; they are not limited to athletic activities. Muscle damage is characterized by the disruption of muscle fibers and its impact on contractile strength.[5] When a muscle fiber is damaged, satellite cells are activated; they replicate and then differentiate to form new fibers, thus permitting muscle repair.[6] Animal cell culture is commonly used to investigate this physiologic response and to test the effect of various chemical compounds or drugs on these muscle progenitor cells.

Animal cell culture has been a common laboratory technique since the 1940s. Cell culture is a complex process by which cells are grown under controlled conditions. Cultures normally contain only one type of cells. Most *in vitro* studies that investigated the repair process of skeletal muscle injury used cell lines as the material.[7] Cell lines can be subcultured numerous times. Cell lines also offer suitable characteristics, similar to muscle cells, and quality control. However, cell lines may undergo genetic changes, and therefore, their use is not recommended for a predictive screening purpose.[8,9] The primary culture cells have key characteristics similar to cells *in vivo*. They can be widely applied for basic studies that require precise cell characteristics.[10] However, cells are usually heterogeneous and have a limited lifespan. Care should be taken regarding the consistency and reproducibility of results using primary cell cultures obtained from different parents. Therefore, standardizing the procedure for primary cell culture is important.

In the present study, we aimed to establish a protocol for primary skeletal muscle cell culture *in vitro* that can be used for further study of the skeletal muscle, including the evaluation of mechanisms underlying muscle damage and repair.

MATERIALS AND METHODS

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Chang Gung Memorial Hospital.

Primary culture of rat gastrocnemius muscle cells

We used Sprague Dawley rats (age, 7–8 weeks; weight, 200–250g) in this study. The gastrocnemius muscle was excised, and the surrounding fascia and adipose tissue were removed. Each muscle was then cut into small pieces of approximately 1.5–2.0 mm^3. These pieces were individually placed in a 15 mL centrifuge tube. Thereafter, 0.2% collagenase type I in TESCA buffer (50 mM N-[Tris(hydroxymethyl)methyl]-2- aminoethanesulfonic acid, 0.36 mM $CaCl_2$) (Sigma, St. Louis, MO) was added to the centrifuge tube, and the cells were incubated for 45 minutes at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were then treated with 0.1% trypsin-ethylenediaminetetraacetic acid (trypsin-ETDA) (Gibco, Big Cabin, Oklahoma) and were incubated for another 45 minutes. The supernatant was collected and centrifuged at 1000 rpm for 5 minutes. The cell pellet was re-suspended in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Logan, Utah), with 10% fetal bovine serum (FBS) (Cansera, Rexdale, Ontario, Canada), 5% chick embryo extract (Gibco), 100 U/mL penicillin, and 100 g/mL streptomycin. After 1 hour of incubation, the fibroblast-shaped cells that adhered to the plate were discarded. The non-adherent cells were transferred to another plate for further culture. After incubation for 24 hours, the supernatant was removed, and the adherent cells were washed with 1× phosphate-buffered saline (PBS) and cultured in DMEM with 10% FBS (Cansera, Rexdale, Ontario, Canada) and 5% chick embryo extract (Gibco). These cells were used for the following experiment.

Primary culture of rat Achilles tendon cells

The Achilles tendons from Sprague-Dawley rats

were excised. The excised tendons were soaked in povidone-iodine for 3 minutes and washed twice with PBS. Each tendon was then cut into small pieces of approximately 1.5–2.0 mm^3 (6 pieces in total). Each piece was individually placed in a 6-well culture plate. After 5 minutes of air-drying for better adherence, 0.5 mL of Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) with 10% fetal bovine serum (Cansera, Rexdale, Ontario, Canada), 100 U/mL penicillin, and 100 g/mL streptomycin were added to each well. The explants were then incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. After migrating from the explants, the cells started to grow rapidly and the confluent culture was then subcultured at a 1:3 dilution after trypsin digestion. Tendon cells with appropriate growth rates and normal fibroblast-shapes were used in the following experiments.

Western blot analysis

Cell extracts were prepared in lysis buffer containing Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM phenylmethanesulfonyl fluoride (PMSF), and 1% Triton X-100 followed by sonication. The protein concentration of the cell extracts was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Samples with identical protein quantities were then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membrane was incubated at room temperature in blocking solution (1% BSA, 1% goat serum in PBS) for 1 hour, followed by 2-hour incubation in blocking solution containing an appropriate dilution of primary antibodies, anti-tubulin, Myf5, Pax7, and desmin (NeoMarks, Fremont, CA). After washing, the membrane was incubated in PBS containing secondary antibodies conjugated with horseradish peroxidase (Sigma, St. Louis, MO) for 1 hour. The membranes were washed and positive signals were detected using an enhanced chemiluminescence reagent (Amershan Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). The expression of each protein was normalized to the level of tubulin.

RESULTS

The growth of rat skeletal muscle cells isolated from the gastrocnemius muscle after a 1-day culture was not significant (Figure 1). After 6 days of growth, some square-shaped cells were noted in the culture medium (Figure 2). After 10 days of growth, a much greater number of cells was obtained in the culture medium (Figure 3). Almost 3 weeks were required to achieve confluency in the culture dish (figure was not shown).

Figure 4 reveals the result of the western blot analysis regarding the comparison of skeletal muscle cell and tendon cell expressions of Myf5, Pax7, and desmin proteins. Skeletal muscle cells demonstrated significant expressions of Myf5, Pax7, and desmin proteins.

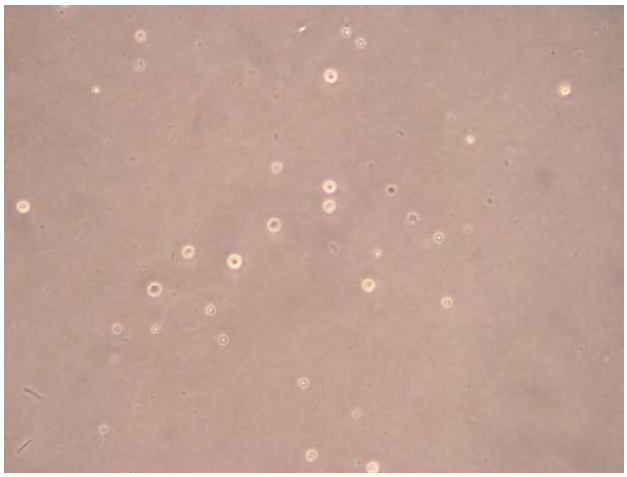

Figure 1. Skeletal muscle cells in the culture medium after 1-day culture. (100X)

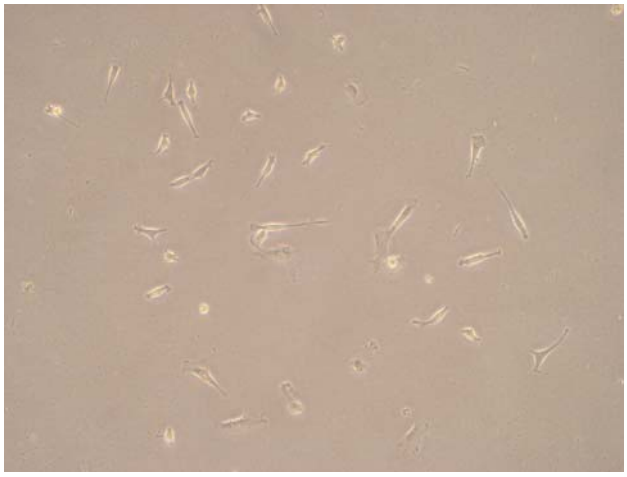

Figure 2. Skeletal muscle cells in the culture medium after 6-day culture. (100X)

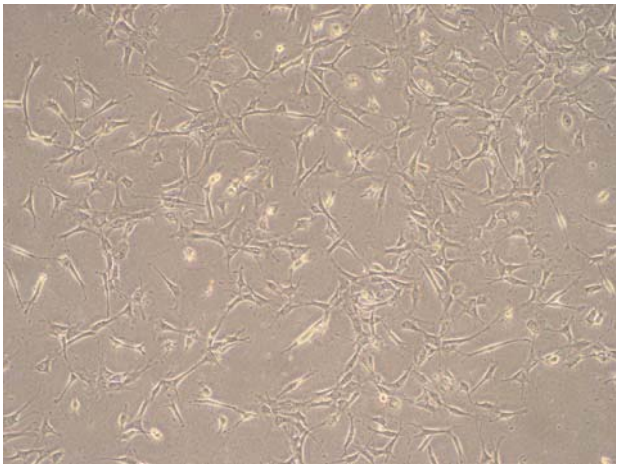

Figure 3. Skeletal muscle cells in the culture medium after 10-day culture. (100X)

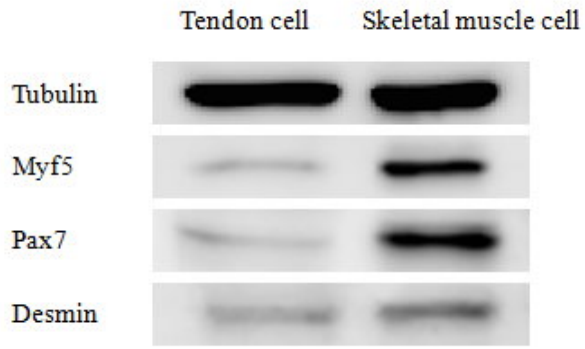

Figure 4. A comparison of skeletal muscle cells and tendon cells. Skeletal muscle cells demonstrated significant expressions of Myf5, Pax7, and desmin proteins in the western blot analysis.

DISCUSSION

Cells and tissues taken from animal or human donors can be widely used for a study of *in-vitro* systems to explore biological, pharmacological, and toxicological applications. It is important to have a sufficient number of replicates for these studies. In addition, the replicates should function in a manner that is similar to the normal function because cells undergo many changes when cultured. Primary culture and cell lines are the main types of cell culture in the *in-vitro* system.[11] Primary culture is the initial *in-vitro* culture of cells and tissues harvested directly from humans and animals. Primary cultured cells are usually heterogeneous but still closely represent the parent cell types and expression of tissue-specific properties. Most primary cultures have a limited lifespan and are known to change their differentiating characteristics over time. A cell line is a permanently established cell culture that will proliferate indefinitely given the appropriate fresh medium, space, and conditions.[10] Cell lines have a long lifespan and they reproduce easily. In addition, cell lines have good qualities control. However, cell lines are often genetically abnormal and are under artificial conditions in culture for a long time, which can cause a marked deviation of their function compared to that of normal cells.[8]

Most studies on skeletal muscle cells use cell lines because the reproduction of primary cultures is difficult owing to uncontrollable variations between preparations. In addition, cell lines offer characteristics of consistency, availability, and easy reproduction of massive cells. Yaffe developed mouse C2C12 cell lines.[12,13] The skeletal muscle C2C12 cell line had been widely used in the last few decades by laboratories worldwide to investigate key aspects of skeletal muscle physiology and plasticity.[14] Other cell lines have been used to explore the molecular mechanisms of muscle differentiation and function.[15]

In the past decade, primary skeletal muscle cells were not available for studies due to the difficulties encountered in reproducing these cells. Recently, an *in-vitro* system for primary skeletal muscle cell culture for several animals has been developed. Perruchot et al used the muscle tissue from pig for a culture of primary skeletal muscle cells.[16] Muscle tissue from other animals such as sheep[17], dog[18], and turkey[19] have also been used for primary skeletal muscle cell culture. Such cells may be applied for studying the mechanisms underlying muscle differentiation, and for identifying and characterizing novel therapeutic approaches for the treatment of age- and injury-induced muscle disorders.[7] In the present study, we have described the first feasible protocol of primary skeletal muscle cell culture from rat.

Cells in a culture usually proliferate following a standard growth pattern.[20] The first phase of growth after the culture is seeded is a lag phase, which is a period of slow growth. When the cells have adapted to the culture environment, their proliferation is greatly increased. In the present study, some cells were noted in the 6-day culture, which was still in the period of slow growth. In the 10-day culture, the cells proliferated exponentially

and a larger number of cells were seen. Finally, the cells reached confluence in the 21-day culture.

The cells from the primary cell culture showed expressions of Myf5, Pax7, and desmin proteins in the western blot analysis. Previous studies have shown that Myf5, Pax7, and desmin are the key factors of protein expression in skeletal muscles.[21,22,23] The Myf5 is a transcriptional factor in skeletal muscles and plays a key role in muscle cell determination in response to signaling cascades that lead to the specification of muscle progenitor cells.[21] Pax7 is a transcriptional factor in quiescent satellite cells, which are related to growth and regeneration of the skeletal muscles.[22] Desmin is a muscle-specific protein and a key subunit of the intermediate filament in cardiac, skeletal, and smooth muscles. Desmin filaments are mainly located at the periphery of the Z-disc of striated muscles and at the dense bodies of smooth muscle cells; they have been postulated to play a critical role in the maintenance of structural and mechanical integrity of the contractile apparatus in muscle tissues.[23] As Myf5, Pax7, and desmin are markers for skeletal muscle cells, the cells from our primary culture were proven to be skeletal muscle cells. We can therefore conclude that the protocol used in this study for primary culture of skeletal muscle cells is reliable.

CONCLUSION

This study revealed a protocol for primary rat skeletal muscle culture, which can be applied for further study of the skeletal muscle, including the evaluation of mechanisms underlying muscle damage and repair. Additionally, we also showed that in contrast to tendon cells, skeletal muscle cells exhibit the expression of Myf5, Pax7, and desmin proteins.

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大鼠腓腸肌細胞原代培養方法及生肌因數 5(Myogenic Factor 5)、配對盒基因 7(Paired Box 7)和肌間線蛋白(Desmin)在骨骼肌細胞與肌腱細胞上的蛋白質表現

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骨骼肌最主要的功能是讓肢體產生力量並使關節運動。當骨骼肌受損時，骨骼肌細胞的修復會開始進行。因此，了解骨骼肌細胞的生物、生理、及病理機轉有助於調控骨骼肌細胞修復。爲了要進行骨骼肌細胞的相關研究，我們需要培養大量的骨骼肌細胞。目前最常用的方式是利用細胞株培養來產骨骼肌細胞。藉由細胞株培養的優點是能產生大量的細胞。然而，這些培養出的細胞的部分特性跟原來的細胞已有些許不同。原代培養所產生細胞的主要特性與原來的細胞較類似。經由原代培養所產生的細胞適合用於骨骼肌損傷及修補等相關研究。

我們的研究目的是用大鼠腓腸肌細胞來建立一個骨骼肌細胞的原代培養的流程。一開始先取出大鼠腓腸肌細胞放在離心管內，然後利用酵素將細胞切割開。經過離心後，將上清液放置在特殊的培養基上。第一個附著在培養基上的是纖維母細胞。將未附著的細胞放到另一培養基培養。經過二十四小時培養後，將附著於培養基上的細胞用磷酸類溶液沖洗後再培養即得所要的細胞。藉由這個流程所培養出的細胞經過西方點墨法分析後，發現有生肌因數 5、配對盒基因 7 及肌間線蛋白所產生的蛋白質。這三個蛋白質只會出現在骨骼肌細胞。所以，我們可以藉由這三個蛋白質的產生，確認所培養出的細胞爲骨骼肌細胞。（台灣復健醫誌 2014；42(1)：23 - 29）

關鍵詞：骨骼肌細胞(skeletal muscle cell)，生肌因數 5(Myf5)，配對盒基因 7(Pax7)，肌間線蛋白(desmin)，原代培養(primary culture)

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