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Development of automated 3-dimensional tissue fabrication system

~ Tissue Factory - Automated cell isolation from tissue for regenerative medicine~

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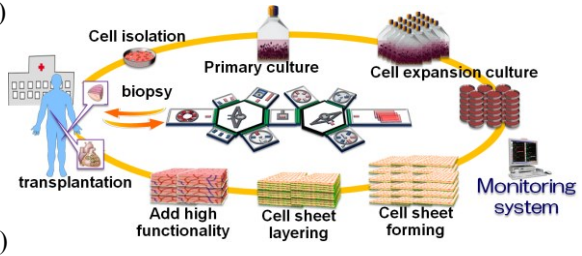
***Abstract*— We have developed a new automated cell isolation system as one of the modules of automated cell sheet production system named Tissue-Factory (T-Factory). This system enables isolation of the target cells from tissue. Using this new system, we successfully isolated skeletal myoblast from skeletal muscle tissue. The cell isolation system makes us stably prepare cell suspension from each tissue automatically and safely. Isolation of skeletal myoblasts will contribute to labor-saving cell cultivation and operational stability, and lead further process in tissue engineering and regenerative medicine.**

I. Introduction

Cell therapy is currently one of the most effective treatments for damaged tissue. Since the cell sheet engineering has been established [1,2,3],This technology has been applying for various cells such as cornea and periodontal ligament cells [4,5]. Some of these have reached clinical trial stage [6, 7]. This technology established application of regenerative medicine to a new stage. In present study, the cell sheet of human cells for clinical trial fabricated by educated and well-trained technicians in facilities called Cell Processing Center (CPC) whose cleanliness is highly managed for clinical use. In the future, the cell sheet engineering technology will become popular and manual cell sheet fabrication will not keep up with increased demand and stability of product quality. Therefore, our automated cell sheet fabricating system is considered necessary for industrial use and is acquired for mass production and quality control. In this study, we introduced automated cell sheet fabrication system named "Tissue–Factory" consisting of modules for isolation, cell expansion, cell sheet fabrication and cell sheet layering. This system enables to fabricate cell sheet using specimen from a patient automatically and safely. This report describes cell isolation module in which the first process of cell sheet fabrication is conducted. Cell isolation from tissue is the first and the most important process in cell sheet fabrication. However, most part of the process depends on manual operations that require highly sophisticated techniques to stably acquire high quality cells. In this study, we introduce a newly developed cell isolation system which enables isolation of target cells from tissue specimen. Skeletal myoblasts were isolated from skeletal muscle tissue in the series of procedures of mechanical mincing, enzyme digestion, etc to prepare cell suspension under aseptic conditions. We compared the numbers of isolated cells per weight of tissue specimen between the operations of our new system and the conventional method. Skeletal myoblasts were isolated with this system, which contributes to labor-saving and operational stability and leads to development of tissue engineering and regenerative medicine.

(a)



(b)

Figure 1. Process of the cell sheet fabrication system[8] (a). Automated cell sheet production system (T-Factory)(b).

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II. Materials and Method

All animal experiments were performed according to "Guidelines of Tokyo Women's Medical University on Animal Use," "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

A. Preparation of skeletal muscle tissue from Sprague-Dawley rats

Skeletal muscle of about 4 g is prepared from the femoral region of Sprague-Dawley rats (Nisseizai, Tokyo, Japan) by biopsy and immersed in Dulbecco's modified Eagle's medium-high glucose (DMEM; Sigma-Aldrich, St. Louis, MO, USA) with 1% penicillin–streptomycin solution as the transport solution .

B. Cell isolation and Primary culture of skeletal myoblast from skeletal muscle using manual method

Primary skeletal myoblasts were prepared according to previously published procedures.[9] Skeletal myocyte specimens from Sprague-Dawley rats (Nisseizai, Tokyo, Japan) were briefly digested at 37°C in collagenase (class II, Worthington Biochemical, Lakewood, NJ, USA) and TripLE Select™(Gibco:12563-029, Life Technologies Corporation, CA, USA). Isolated cells were suspended in culture medium composed of 5% fetal bovine serum (FBS, Lonza, Walkersville, MD, USA), SkBM Basal Medium (Lonza, Walkersville, MD, USA), SmGM-2 SingleQuot Kit Suppl. & Growth Factors (contains hEGF,0.5 ml; Fetuin, 5.0 ml; BSA, 5.0ml; Dexamethasone, 0.5 ml;Insulin, 5.0 ml; GA, 0.5 ml Lonza, Walkersville, MD, USA), and 0.2% penicillin–streptomycin solution. Isolated cells were filtered(Millex-GV PVDF0.22um Millipore: SLGVM33RS), and washed with Hanks' Balanced Salt solution (Sigma-Aldrich St. Louis, MO, USA :H9394) after harvesting onto culture dish (Cell Seed Inc. Tokyo Japan) with a seeding density of 0.25×10^4 cells/cm^2 , then incubated at 37°C in a humidified atmosphere with 5% CO_2 cultured until becoming sub-confluent.

C. Automated cell isolation system

After biopsy from skeletal muscle, we put the specimens in the automated cell isolation system (Figure 2(a)). This system has five functional units; a cell isolation unit, a pipetting unit for cell suspension, a liquid pump unit for supplying medium, a centrifugation unit for cell washing and cell isolation, and a capper unit for capping centrifugation tubes. Supplementary items such as culture dish and centrifugation tubes were linked with a 6-axis robot handling unit (Figure 2(b)). The cell isolation unit has a stirrer with four knives, a chamber (Figure 2(c)), and a rotation controller. The stirrer was attached to the rotation controller before use. Specimens were put into a vessel to which pre-warmed enzyme solution was added (Figure 2(c)). Then the motor of the isolation unit rotated the stirrer intermittently (0.5 s rotation with 1.0 s rest interval) at 37°C. After this process was completed, skeletal myoblasts were acquired after membrane filtration, washed, and suspended in a culture medium.

D. Evaluation of automated cell isolation system

The number of isolated cells per weight of tissue, the relative ratio of target cells per a specific number of isolated cells, and the viabilities of target cells were examined by comparing our new method and the conventional manual method. The number of acquired cells was counted using a hemocytometer (C-Chip, NanoEnTek Inc., Guro-gu,Seoul, Korea) and the relative ratio of viable cells were determined using Trypan-blue stain solution (Sigma-Aldrich St. Louis, MO, USA). These were observed with phase-contrast microscope (CKX31N, Olympus Corporation, Shinjuku-ku, Tokyo, Japan).

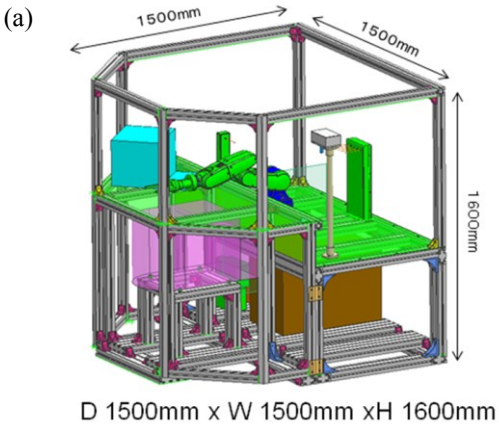

Figure 2. 1st prototype of automated cell isolation and primary culture system. (a) Overview of entire automated cell isolation system. The system has five functional units; a cell isolation unit, a pipetting unit for cell suspension, a liquid pump unit for medium supplying, a centrifugation unit for cell washing and cell isolation, and a capper unit for capping centrifugation tubes. Supplementary items such as culture dish and centrifugation tubes were linked with 6-axis robot handling unit (b).

(c)

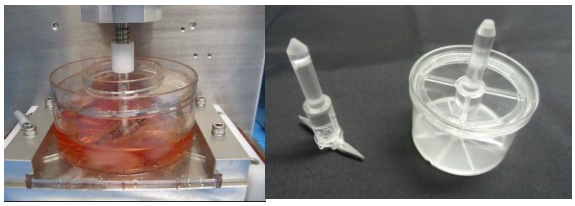

Figure 3(c). Cell isolation vessel with stirrer with four knives. Specimens were put into a vessel, minced by knife rotation, and treated by enzyme digestion(c).

E. Histological analysis

For histological analysis, the cultured cells were fixed with 4% paraformaldehyde and permeabilized with 0.15% TritonX-100 (Sigma-Aldrich, St. Louis, Missouri USA) in phosphate-buffered saline. Then, indirect immunofluorescent assay was performed. For myoblast staining, anti- desmin antibody ([Y266] ab32362, abcam Inc., Cambridge, UK) specific for myoblast was used as the primary antibody. Alexa-Fluor 488-labeled anti-mouse IgG antibody (Life Technologies Corporation, CA, USA) was used as the secondary antibody. Prepared specimens were observed with a fluorescence microscope (ELIPSE TE2000-U, Nikon, Tokyo, Japan) with a CCD camera (Axio Cam HRc, Carl Zeiss, Hallbergmoos, Germany)

III. RESULT

A. Comparison of number of isolated cells between automated cell isolation system and manual method.

The number of isolated cells per weight was 4.58±1.45 $\times 10^4$ cells/g with our new automated cell isolation system and 5.57±1.08 $\times 10^4$ cells/g with the conventional manual method (mean±SD) (Figure 3). The number of cells isolated by the automated method was almost equal to the number obtained by the conventional manual method.

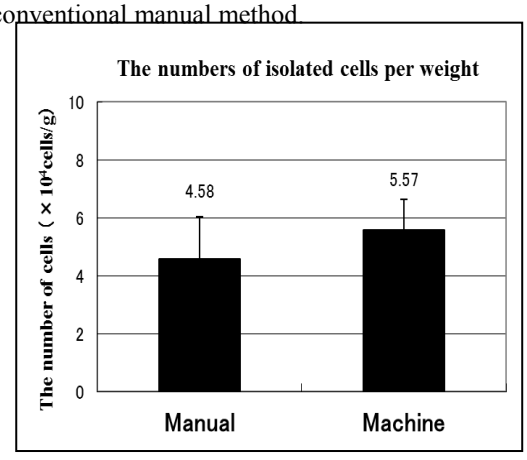

Figure 3. Number of isolated cells in control and machine specimens

B. Optimization of cell isolation conditions

To increase the number of isolated cells, we have optimized the speed of the rotating knives of the cell isolated unit in the range of 100-500 rpm. Figure 4 shows that the numbers of isolated cells and dead cell ratio using trypan-blue staining (Wako Pure Chemical Industries, Ltd. Osaka, Japan). Isolated cell count increases as the blade rotation speed increased from 100 to 300 rpm. The maximum count, 4.73±1.32 $\times 10^4$ cells/g was acquired at 300 rpm. The dead cell ratio increases over 300 rpm. Therefore, the optimal condition of cell isolation unit setting was set at 300 rpm in this system for the SD rat muscle specimens.

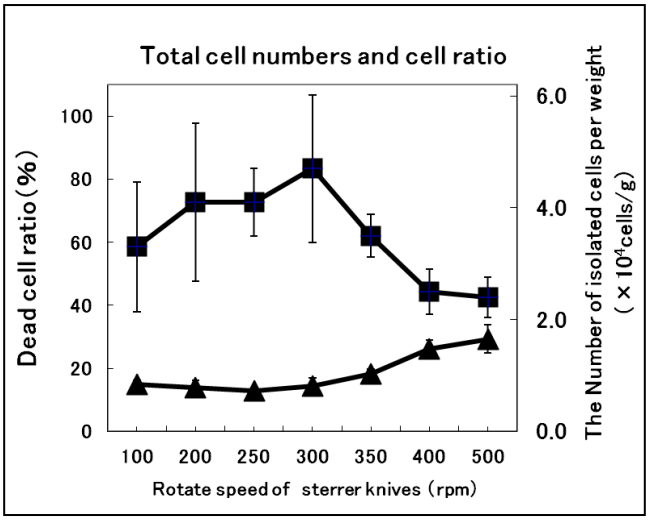

Figure 4. Optimization of cell isolating performance. we have optimized speed of rotate knives of cell isolated unit (from 100rpm to 500rpm) and observed the number of isolated cells and dead cell ratio using trypan-blue staining

C. Immunofluorescent staining of isolated cells

We immnofluorescently stained isolated cells with anti-desmin antibody and identified well differentiated myoblasts (red in Figure 5). The blue in Figure 5 are Nuclei stained with Hoechst 33342.

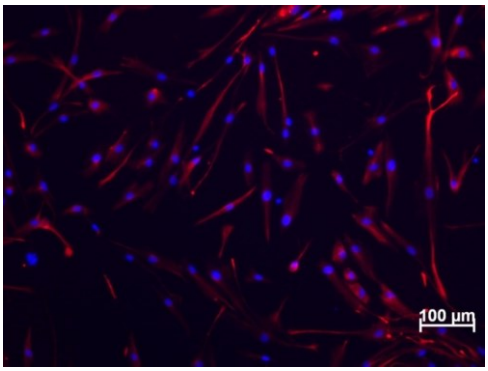

Figure 5. Immnofluorescent staining for anti-desmin antibody (red) and Nuclei were stained with Hoechst 33342 (blue), which demonstrated well differentiated myoblasts.

D. Factory model of automated cell isolation module in T-Factory.

After evaluation of 1st prototype of cell isolation and primary culture module, we have developed 2nd prototype module as one of the modules of automated cell sheet production system (Figure 6).

Figure 6. 2nd prototype of cell isolation module in T-Factory.

IV. DISCUSSION

We have successfully developed an automated cell isolation system and a primary culture system, which allows us to isolate skeletal myoblasts from skeletal muscle specimens.

Mechanization and automation have been recently required for labor-saving cell culture to improve operational stability, as well as to avoid contamination risk. Automated culture systems, such as specimen homogenation devices and automated expansion culture systems have been developed by some manufacturers. However, there have been few reports on automating cell isolation process due to cell isolation difficulty. Since isolation cell process differs for each cell source and cell quality heavily depends on quality of host tissue, for instance freshness and volume of the host tissue. Many sensitive controls have been required for cell isolation. Shioyama and his colleagues created a cell isolating machine and accomplished Sprague-Dawley neonatal rat cardiomyocyte suspension from ventricle muscle [10]. Our study has accomplished the whole process of cell isolation and primary culture process including cell isolation, centrifugation, liquid handling and acquired cell suspension without human intervention. Our system allows us to isolate skeletal myoblasts from tissue specimens without specific technicians, enables to reduce contamination risk due to no human intervention in the whole process, and reduce workload of technicians. These greatly contribute to labor-saving and cost reduction, and play an essential role in spreading regenerative medicine.

V. CONCLUSION

In this study, we introduced a newly developed cell isolation system and succeeded in automatically isolating skeletal myoblasts from Sprague-Dawley rat muscle tissue. The system is labor- and cost-saving, and will be applied to obtain isolated cells well-qualified for clinical use.

In conclusion, the new system may significantly contribute to the development of tissue engineering and regenerative medicine.

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