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Automatic fabrication of 3-dimensional tissues using cell sheet manipulator technique

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ABSTRACT

Automated manufacturing is a key for tissue-engineered therapeutic products to become common-place and economical. Here, we developed an automatic cell sheet stacking apparatus to fabricate 3-dimensional tissue-engineered constructs exploiting our cell sheet manipulator technique, where cell sheets harvested from temperature-responsive culture dishes are stacked into a multilayered cell sheet. By optimizing the stacking conditions and cell seeding conditions, the apparatus was eventually capable of reproducibly making five-layer human skeletal muscle myoblast (HSMM) sheets with a thickness of approximately 70–80 μm within 100 min. Histological sections and confocal topographies of the five-layer HSMM sheets revealed a stratified structure with no delamination. In cell counts using trypsinization, the live cell numbers in one-, three- and five-layer HSMM sheets were equivalent to the seeded cell numbers at 1 h after the stacking processes; however, after subsequent 5-day static cultures, the live cell numbers of the five-layered HSMM sheets decreased slightly, while one- and three-layer HSMM sheets maintained their live cell numbers. This suggests that there are thickness limitations in maintaining tissues in a static culture. We concluded that by combining our cell sheet manipulator technique and industrial robot technology we can create a secure, cost-effective manufacturing system able to produce tissue-engineered products from cell sheets.

1. Introduction

Tissue engineering is a technology that can fabricate 3-dimesional (3-D) functional tissues from cells *in vitro*, with the goal of regaining the lost functions of diseased organs. This technology is categorized by cell-based therapy in which its functionality and success is derived from the configurations or structures that can be produced. Until recently, the variety of tissue-engineered therapeutic products has been limited to only a few organs, such as skin or cartilage. In part, this was because the manufacturing of other tissues often requires complicated fabrication methods including very strict aseptic handling that the biological quality is not readily reproducible. Hence, we believe that automated manufacturing facilities will play a key role in tissue-engineered products to make them more common-place and economical.

When constructing 3-D tissues cell sheets are a very useful component. Temperature-responsive culture dishes have surfaces that are covalently grafted with poly (*N*-isopropylacrylamide) and can be used to make cell sheets without applying enzymes [1–5]. In these culture dishes confluently cultured cells detach from the surface as an intact cell sheet by simply reducing the temperature, which causes hydration of the polymer. Since these cell sheets retain their extracellular matrix and the adhesive proteins around them, their structural integrity and mutually adhesive properties allow us to fabricate 3-D constructs by simply stacking them layer upon layer [6–9]. There are many advantages to these multilayered cell sheets for use as therapeutic grafts, which include nonuse of excipients, a stratified structure mimicking physiological tissues, adhesiveness to organs, and efficient local administration of cells. In addition, we think this method is suitable for controlling the thicknesses of cell-dense constructs that might attenuate acute ischemic cell death after implantation caused by insufficient nutrient-waste exchange through solute diffusion. Despite such advantages, the challenges faced to stack cell sheets, either with supportive membranes or by liquid handling, has long hindered

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reproducible fabrication and any precise studies about multilayered cell sheets. However, a recently-developed cell sheet manipulator technique using cell-adherent hydro gels has now made it easier to fabricate 3-D constructs from cell sheets [10], and the method appeared to be enough simple to be done by industrial robots. Hence, we developed an automatic cell sheet stacking apparatus by exploiting the cell sheet manipulator technique, to produce an automatic manufacturing system for tissue-engineered products.

2. Materials and methods

2.1. Temperature-responsive culture dish

Temperature-responsive culture dishes were prepared by the method previously reported [1]. Briefly, a solution of *N*-isopropylacrylamide (NIPAAm) monomer was spread on commercially available tissue culture polystyrene dishes. Then, electron beam irradiation induced the monomer to be polymerized and covalently grafted to the culture surface. These dishes were washed vigorously by cold water to remove any ungrafted monomers, and finally they were sterilized.

2.2. Cell culture

Human skeletal muscle myoblasts (HSMM) were purchased from Lonza (Basel, Switzerland) and propagated through three passages to obtain the necessary cell number. HSMMs were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in SkGM-2 medium (Lonza).

2.3. Cell sheet manipulator

To retrieve the cell sheets from temperature-responsive dishes, plunger-like cell sheet manipulators coated with a cell-adhesive gelatin hydro gel were prepared similarly to our previous report [10], but with several modifications in order to improve reproducibility. To prepare the gelatin solutions, powdered gelatin from porcine skin (Sigma–Aldrich, St. Louis, Missouri, USA) was added to Hanks' balanced salt solution (Sigma–Aldrich) to a final concentration of 6 wt%, neutralized with a small volume of 1 N sodium hydroxide solution (Wako Pure Chemical Industries, Osaka, Japan) and dissolved completely in a 50 °C water bath. Then, the gelatin solution was sterilized by filtration using a bottle top filter with a 0.45 μm pore diameter, and its aliquots were stored at 4 °C until use. On the day before the stacking operation, the aliquot was warmed and melted again in a 37 °C water bath for 30 min, and the gelatin solution was dispensed into 2.5 mL cylindrical polytetrafluorethylene (PTFE) molds. Then, metallic plunger-like manipulators were immediately dipped in the solution and held 3 mm above the bottom. The manipulators were stored at 4 °C overnight to solidify (Fig. 2b). The next day, several minutes before the stacking operation, the manipulators were removed from the molds with the coating of gelatin gel and equilibrated to room temperature.

2.4. Optimization of cell sheet stacking conditions

To shorten the process time, the cell sheet manipulator weights and temperature profiles were manually optimized by measuring the area of cell detachment in each condition, prior to designing the apparatus. HSMMs were seeded in temperature-responsive culture dishes at 1×10^5 cells/cm^2 and cultured overnight. Based on the method previously reported [10], the cells were then stacked in three layers and transferred onto tissue culture polystyrene (TCPS) dishes with manipulators of the various weights (4, 12, 40, and 120 g) and with two types of temperature profiles: with or without a pre-cooling process. In the pre-cooling (−) group, the manipulators were held on the cells throughout the low-temperature treatment at 20 °C and the low-temperature treatment times were varied from 3, 8, 15, 30 and 60 min. In the pre-cooling (+) group, the low-temperature treatment time was fixed at 30 min and the manipulators were placed on the cells at 3, 8 and 15 min before the end of the low-temperature treatment; and the dishes were pre-cooled for 27, 22 and 15 min before applying the manipulators, respectively. After the cell sheets were stacked and transferred to the TCPS dishes, the cells remaining on the temperature-responsive culture dishes were fixed by a 4% formaldehyde aqueous solution for 15 min and stained with 0.08% Crystal Violet Staining Solution (Kanto Chemical, Tokyo, Japan) diluted by 20% ethanol for 15 min at room temperature. After the dishes were washed by distilled water twice, the stained dishes were placed on a white illuminator and pictures were taken by a digital camera. Then, the detachment areas were measured from these pictures using Image J software (public domain, distributed by the National Institute of Health of USA at http://rsbweb.nih.gov/ij/). In addition, the cell sheets transferred on the TCPS dishes were also stained by Crystal Violet to confirm that the transfer was successful.

2.5. Automatic cell sheet stacking apparatus

In pursuit of a method for the stable manufacturing of multilayered cell sheets, an automatic cell sheet stacking apparatus was designed and assembled. The stacking and transfer processes were automated, while the process of washing the melted gelatin was not incorporated because it was not an essential step for this method and required additional machinery for handling liquids. It was designed to accommodate both 3.5 and 6 cm dishes. To preparing for future extensions of this automated system, it was comprised of two modules: an incubation module and a stacking module (Fig. 2a). The incubation module is capable of incubating ten dishes in a humidified 37 °C/5% CO_2 environment. The stacking module handles the dishes and cell sheet manipulators with precise temperature control using Peltier elements. Both modules were connected to each other and installed in a commercially available clean bench (Fig. 2c). The machinery was operated by a laptop PC through a USB interface. All robotic sequences and temperatures were controlled by specially developed software.

2.6. Automatic cell sheet stacking process

The stacking process used by the apparatus was conducted largely as shown in Fig. 1. One modification from the original method previously reported [10] includes the use of seeding rings. Seeding rings were used to prevent cells from adhering at the peripheral areas of the dishes that did not make contact with the manipulator; seeding rings were placed in the dishes and then cells were only seeded within inner area of the ring (4 cm^2). The rings were removed just before stacking which prevents tearing of the cell sheets at the edges of the manipulator. Although the Fig. 1 shows only a four-layer cell sheet being transferred to the last dish, we conducted a number of experiments that varied the destination of the transferred cell sheets as well as the number of layers transferred.

2.7. Optimization of seeding density

To identify the maximum seeding density to reproducibly stack HSMMs, five-layer HSMM sheets were fabricated at various seeding densities by the apparatus and the resulting structures were then examined by a confocal microscopy. HSMMs were seeded in 3.5 cm temperature-responsive culture dishes with seeding rings at 1×10^5, 2.5×10^5, 5×10^5, 1×10^6 and 2×10^6 cells/cm^2 and cultured overnight. On the following day, the cells were marked with live cell fluorescent staining reagents of different colors (CellTracker™) (Life Technologies Corp., Carlsbad, California, USA) according to the instructions provided by the manufacturer. Then, the stained cells were stacked in five layers using the apparatus. The first four HSMM sheets were stacked on the manipulator, and then the four-layer HSMM sheets were transferred onto the fifth dish by melting the gelatin gel. The resultant five-layer HSMM sheets were adhered onto the last dishes and fixed with 4% formaldehyde aqueous solution for 30 min and transparentized by Sca*l*e U2 reagents [11] for 1 week. Cross-sectional views of the multilayered HSMM sheets were subsequently obtained by a confocal laser scanning microscope (LSM 510 META) (Carl Zeiss Microscopy, Cambridge, UK) using the Z-stack method.

2.8. Histological analysis of multilayered HSMM sheets

To examine more precisely the structure of the multilayered HSMM sheets, histological studies were performed for both the detached and adhered samples. To make the detached samples, HSMMs were seeded into 3.5 cm temperature-responsive culture dishes with seeding rings at 1×10^6 cells/cm^2 and cultured overnight. The cells were stacked in five layers and cultured for 1 h on the fifth temperature-responsive dish to allow adhesion. Then, the five-layer construct was again treated with low temperature to detach it from the surface. The detached cell sheets were fixed with a 4% formaldehyde aqueous solution. To make the adhered samples, HSMMs were seeded into 3.5 cm temperature-responsive culture dishes with seeding rings at 1×10^6 cells/cm^2, cultured overnight, stacked in five layers and transferred onto 12 mg/mL fibrin gels (Bolheal®) (Teijin Pharma Ltd., Tokyo, Japan) that were 2 mm thick. The cell sheets transferred onto fibrin gels were fixed with a 4% formaldehyde aqueous solution after 1-h, 2-day and 5-day culture. All samples were embedded in paraffin and subjected to histological examinations, including hematoxylin-eosin (H&E) staining, TUNEL staining and Ki67 immunohistochemistry by established standard procedures.

2.9. Static culture of multilayered HSMM sheets

Lastly, the functionality and survivability of the multilayered HSMM sheets were evaluated by *in vitro* static culture. HSMMs were seeded into temperature-responsive culture dishes with seeding rings at 1×10^6 cells/cm^2 and cultured overnight. The cells were stacked in one, three, and five layers using the apparatus, and then transferred manually onto 10 cm tissue culture polystyrene dishes. The multilayered HSMM sheets were then cultured in 10 mL of SkGM-2 medium. After 1-h culture (referred as day 0), half of the samples were digested by TrypLE Select reagent (Life Technologies Corp.) and then the live cell numbers were counted using Trypan Blue dye and hemocytometers. The other half of the samples was cultured for 5 days. During the culture, the media were changed every 24 h and supernatants were sampled at each medium change. At the end of the culture, the cell sheets were digested and live cell numbers were counted in the same manner as previously described. The collected supernatants were analyzed for glucose, lactate, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor 2 (FGF-2) and stromal cell-derived factor 1α (SDF-1α). The concentrations of glucose and lactate were measured by a BF-6iM medium analyzer (Oji Scientific Instruments, Hyougo, Japan). The concentrations of VEGF, HGF, FGF-2 and SDF-1α