Use of a Tendon/Ligament Cell Sheet with Mesenchymal Stem Cells and Cyclic Stretch Stimulus for Tendon/Ligament Tissue Restoration

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ABSTRACT

It can be relatively difficult for damaged tendon or ligament tissue to heal perfectly due to the inability to self-cure. Therefore, tendon and ligament tissue engineering has been used as a restorative method. Perfect tissue restoration, however, has yet to be achieved. In other research, cell sheets have been applied to various different types of tissues and organs for regeneration. In this study, we attempted to fabricate a tendon/ligament cell sheet for use in tissue engineering. Firstly, human bone marrow mesenchymal stem cells (hBMSCs), which have excellent proliferative abilities, were harvested and formed into a cell sheet. Then, a cyclic stretching stimulus was applied to the hBMSC sheet to enhance cell differentiation into tendon/ligament cells. The results showed that the work was successful in promoting differentiation, resulting in a tendon/ligament cell sheet. These results suggest that this method is effective for tendon/ligament tissue engineering.

Keywords: *Cell Sheet, Human Bone Marrow Mesenchymal Stem Cell (hBMSC), Differentiation, Tendon/ligament Cell, Mechanical Stimulus*

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Introduction

Tendons and ligaments are connective tissues that directly attach muscles to skeletal structures, and bones to bones, respectively, permitting locomotion and enhancing joint stability [1–3]. Tendon/ligament injuries, which can range from repetitive strain injury to complete rupture, are often seen in athletes and other highly active people, where reduced functionality of the injured tendon/ligament tissue can have a devastating effect on quality of life. Tendon/ligament injuries are difficult to manage; although spontaneous healing can occur, it often results in the formation of scar tissue that differs morphologically, biochemically, and biomechanically from healthy tendon/ligament tissue [4]. Repaired tendon/ligament tissue is also impaired in terms of its dynamic properties and strength [5–9]; hence, current treatments for tendon/ligament injury, both conservative and surgical, can be said to have limited effectiveness [9].

Thus, there is a pressing need for tendon/ligament tissue engineering. The main difficulty with such engineering is the small numbers of cells that can be obtained from explanted tissue, because tendons/ligaments are relatively acellular, containing few tendon/ligament cells [10], and are terminally differentiated with a very limited proliferative capacity [11]. Bone marrow mesenchymal stem cells (BMSCs) are more commonly used in this field due to their proliferation capability and pluripotency [12–16].

Various approaches, such as the application of a mechanical stimulus [17–20] or chemical stimulus [21–23], as well as coculture [19, 20, and 24], have been used to enhance ‘stem cell to tendon/ligament cell’ differentiation. In particular, mechanical stimulation is being investigated as a simpler and safer differentiation-inducing technique compared to chemical stimulation and coculture. Therefore, in this work we studied differentiation induction using a cyclic stretching stimulus [25–28]. However, tendon/ligament tissue restoration, which is one of the ultimate goals in the field of the regenerative medicine, has not yet been realised.

One major area of research in tissue engineering has concerned the use of cell sheets [29, 30]. For example, myocardial cell sheets [29, 31] and corneal cell sheets [32] are now under clinical investigation. Therefore, given their success in other areas, cell sheets could potentially be a technique with utility for tendon/ligament tissue restoration.

In this study, we attempted to fabricate a tendon/ligament cell sheet. Firstly, a cell sheet was made using human bone marrow mesenchymal stem cells (hBMSCs). Then, cyclic stretching stimulus was induced in the hBMSC sheet to promote the differentiation of hBMSCs into tendon/ligament cells. Given its simplicity, this method for creating cell sheets has the potential to become a powerful technique in tendon/ligament tissue restoration.

Materials and Methods

Cell preparation

hBMSCs (UE6E7T-3; Riken Cell Bank, Tsukuba, Japan) were cultured in accordance with the protocol of the supplier, and were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10 % new bone calf serum, 0.5 % GlutaMax, and 0.05 % gentamicin (Invitrogen, Carlsbad, CA, USA) at 37 °C under a 5 % CO_2 atmosphere in a humidified incubator. All hBMSCs used in this study were at or prior to the 30th passage to insure a high proliferative capacity. The hBMSCs were cultured in 25 cm^2 culture flasks (BD Biosciences, Franklin Lakes, NJ, USA) at an initial density of 1.0 × 10^4 cells/cm^2 for expansion without differentiation. Medium replacement was done every 3 days. Figure 1 shows images of the cells over time under a phase-contrast microscope. At near-confluence, occurring every 5–7 days, cells were detached from the culture flasks with 0.25 % w/v trypsin (Wako Pure Chemical Industries, Ltd.) –1 mM EDTA (ethylenediaminetetraacetic acid; Takara Bio, Shiga, Japan) and seeded into the new culture flasks. The trypsinised hBMSCs from the cultured flasks were plated onto the bottom of the UpCell® device (CellSeed Inc., Tokyo Japan), used for fabricating cell sheets, as described in the following section.

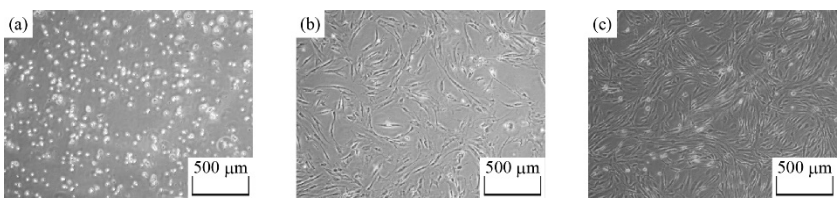

Figure 1: Human bone marrow mesenchymal stem cells (hBMSCs) seeded onto a flask. (a) 0 day. (b) 2 days. (c) 6 days.

Fabrication of cell sheet

The commercially available UpCell® system (CellSeed Inc.) was used to fabricate the hBMSC sheets. Temperature-responsive polymers, PIPAAm, were coated onto the bottom surface of the UpCell® culture dish, and cell-adhesive proteins were then used to coat the polymers. Firstly, the trypsinised hBMSCs, described in the previous section, were seeded onto the UpCell® culture dish (Figure 2(a)) and cultured as normal up to a confluent state at 37 °C under a 5 % CO_2 atmosphere in a humidified incubator. Secondly, the culture medium was removed and the hBMSCs were covered with a CellShifter™ (CellSeed Inc.; left figure in Figure 2(b)). Thirdly, when the temperature was reduced to 20–25 °C, the chemical properties of the

PIPAAm coating changed from hydrophobic to hydrophilic, allowing it to be separated from the population of hBMSCs and resulting in an hBMSC sheet on the bottom of the UpCell® culture dish (right figure in Figure 2(b)). Fourthly, the hBMSC sheet was transferred onto the fibronectin-coated bottom surface of a PDMS elastic chamber and left unaltered for 1 day (left figure in Figure 2(c)). Then, the CellShifter™ was removed from the hBMSC sheet, and the sheet was cultured at 37 °C under a 5 % CO_2 atmosphere in a humidified incubator for 2 days. Finally, cyclic stretching stimulation was induced, as described in the following section.

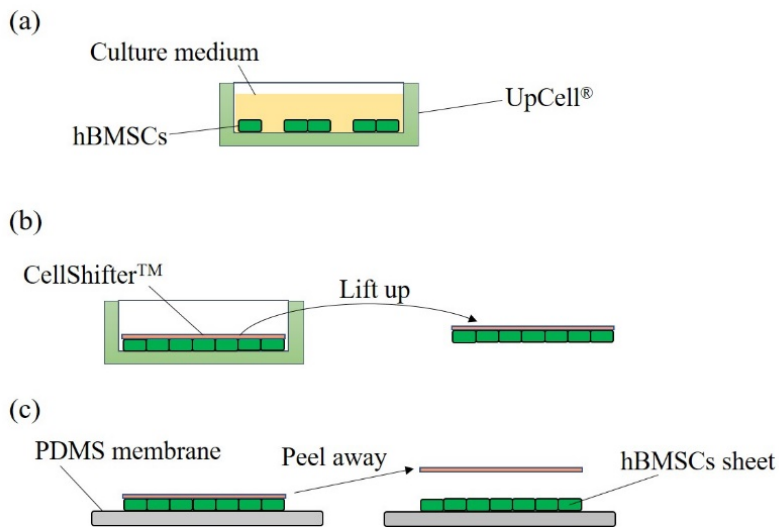

Figure 2: Fabrication procedure for cell sheet. (a) hBMSCs culturing using the UpCell®. (b) Cell-sheet collection after confluent growth. (c) Transferal of the cell sheet onto the PDMS elastic chamber for cyclic stretching stimulus.

Cyclic stretch condition

A simple stretching device (ShellPa; Menicon Co., Ltd., Aichi, Japan), fitted with polydimethylsiloxane (PDMS) elastic chambers, was used to apply uniaxial cyclic deformation. The PDMS elastic chambers have a transparent bottom (20 × 20 mm^2, 400 μm thick) and 9.6-mm high walls (Figure 3). The chamber was deformable by up to 20 % in a single axis by use of a stainless steel grappling-hook device. Uniaxial deformation of the bottom membrane of the PDMS chamber was accompanied by a small degree of subsidiary deformation in the orthogonal direction, as the sides of the membrane were allowed to deform. Although the orthogonal deformation was suppressed by

the thick walls of the PDMS elastic chamber, elongation of 10 % induced up to approximately 4.3 % perpendicular retraction.

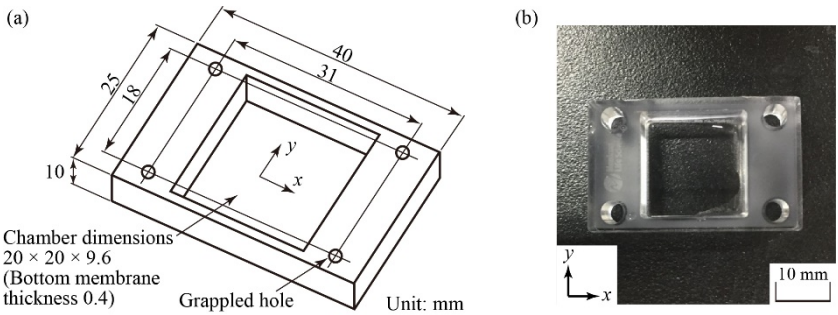

Figure 3: PDMS elastic chamber for cyclic stretch stimulus. (a) The geometry. (b) The top-side image.

The PDMS elastic chambers were rinsed in distilled water in an ultrasonic bath for 15 min, placed in sterile phosphate-buffered saline (PBS; Cosmo Bio, Tokyo, Japan), and sterilised by exposure to ultraviolet light in a sterile hood for 30 min. They were then coated with human fibronectin (R&D Systems, Minneapolis, MN, USA) at a concentration of 1 μg/cm^2 and incubated for 3 h. The hBMSC sheets were plated onto the bottom membrane of the PDMS elastic chamber and cultured for 2 days without cyclic stretching, and 1 Hz, 10 % and 48 h were used as the cyclic frequency, stretch ratio and duration, respectively. The cyclic frequency of 1 Hz was based on the physiological motions of tendon (e.g. walking can be approximated to 1 Hz), which is also optimal for the proliferation of hBMSCs [33]. The stretch ratio and duration (10 % and 48 h, respectively) were based on preliminary experiments for effective tendon/ligament cell differentiation [25]. A control sample was prepared in the same manner but was not subjected to cyclic stretching stimulus. Table 1 lists the experimental conditions of this study.

Table 1: Experimental conditions of the cyclic stretching stimulus

Stretch ratio [%]	**Cyclic frequency [Hz]**	**Stretch duration [h]**
0 (Control) 10	1	48

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

When the cyclic stretching period was completed, the cells were lysed and their total RNA isolated using an RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). The purity and concentration of the RNA were assessed by determining the absorbance ratio at 260/280 nm. Reverse transcription was performed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh; internal control), scleraxis (Scx), tenascin-C (Tnc), type III collagen (Col III), type I collagen (Col I), and catenin β1 (Ctnnβ1) gene expression levels were analysed using pre-designed minor groove binder probes (Applied Biosystems), TaqMan PCR Master Mix (Applied Biosystems), and Light Cycler apparatus (ABI 7300; Applied Biosystems). Gene expression levels were calculated using the standard curve method and normalised relative to the levels of Gapdh gene expression. The qRT-PCR was performed using three independent samples to confirm reproducibility, with the data expressed as means and standard deviation (SD). A paired student's *t*-test was used for the analysis and p-values < 0.05 were considered to indicate statistical significance.

Immunofluorescence staining

At the end of the cyclic stretching period, the hBMSC sheet was fixed in 4 % paraformaldehyde (Wako Pure Chemical Industries, Ltd.) for 15 min and permeabilised in 0.25 % Triton X-100 (Wako Pure Chemical Industries, Ltd.) in PBS (Cosmo Bio) for 15 min. A cytoskeleton was stained with FITC (fluorescein isothiocyanate; Enzo Life Sciences, New York, NY, USA) in PBS with 5 % mouse serum at room temperature for 1 h. The hBMSC sheet was washed three times with PBS for 5 min after each step. The PDMS chamber with the stained cell sheet was cut like a glass slide and encapsulated with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). The periphery of the sample was sealed with nail polish to prevent it from drying out before observation. Finally, the sample was observed with a confocal microscope (A1Rsi-N; Nikon Instech. Co, Ltd., Tokyo, Japan).

Results and Discussion

The aim of this work was to fabricate an hBMSC sheet through the protocol described above. Figure 4(a) shows the hBMSC sheet on the CellShifter™. The hBMSCs were under confluence and formed a cell sheet with a monolayer (Figure 4(b)).

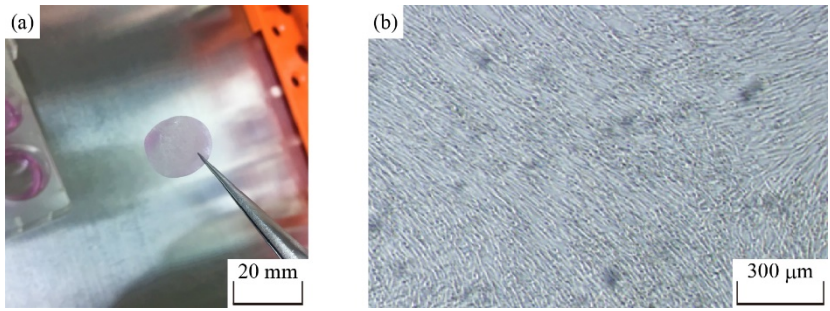

Figure 4: hBMSC sheet. (a) hBMSC sheet transferal onto the CellShifter™. (b) Phase-contrast photomicrograph of the hBMSC sheet.

The hBMSC sheet was transferred onto the bottom surface of a PDMS elastic chamber and applied the cyclic stretching, as described in Table 1. The results of the mRNA expression levels are shown in Figure 5. The longitudinal axis represents the relative mRNA expression levels normalised by the mRNA expression of the hBMSC sheet onto a PDMS elastic chamber under no-mechanical stimulation. All markers of tendon/ligament cell differentiation, namely Scx, Tnc, Col III and Col I, were promoted by the cyclic stretching. Particularly, the results suggest that hBMSC-to-tendon/ligament cell differentiation is encouraged by the mechanical stimulus, since the expression level of Scx, one of the crucial markers of differentiation [34], was significantly increased. Meanwhile, despite cyclic stretching, the results suggest that the hBMSCs maintained a sheet-like state, since Ctnnβ1, one of the important intracellular proteins for cell-cell adhesion, did not significantly decrease.

Additionally, one result of particular interest was that the cellular orientation could be observed in the cell sheet by stimulating the cyclic stretching, as the cells in the cell sheet not subjected to cyclic stretching were randomly aligned, as shown in Figure 6. This kind of orientation behaviour can typically be seen in cells at confluency under moderate cyclic stretching [25, 26]. This orientation behaviour in a cell sheet is a novel observation in hBMSC-to-tendon/ligament cell differentiation. In-vivo tendon/ligament cells are regularly aligned along the collagen fibres of actual tendon/ligament tissue. Therefore, artificially oriented cell sheets with tendon/ligament cell differentiation hold great potential for rebuilding injured tendon/ligament tissue, effectively owing to the implantation.

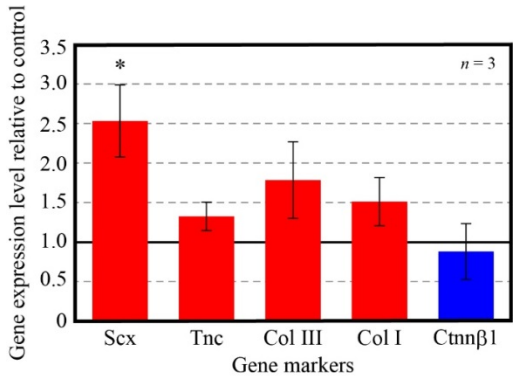

Figure 5: mRNA expression levels in a stretched cell sheet. Scx, Tnc, Col III and Col I are tendon/ligament cell differentiation markers. Ctnnβ1 is the cellular adherence marker. Data are normalised to the corresponding gene expression levels in non-stretched cell sheet (defined as 1). *p<0.05 relative to control.

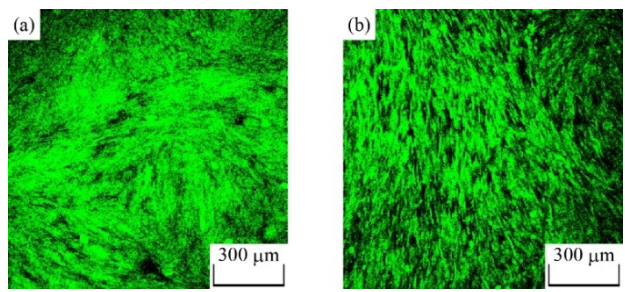

Figure 6: Confocal fluorescent micrographs of the cell sheets. (a) Non-cyclic stretched. (b) Cyclic stretched.

Conclusion

The hBMSC sheets were successfully fabricated and differentiated into tendon/ligament cells with application of a cyclic stretching stimulus. Additionally, the cells of the cell sheets were regularly aligned in one direction. These results hold great promise for restoration of injured tendon/ligament tissue through implantation, since such properties are quite similar to the in-vivo tendon/ligament tissue environment. Further investigation will need to be carried out into implantation experiments using animals.

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