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Development and Characterization of Cell Sheet/PLCL sheet For Patch Tissue Engineering

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Abstract

Recently, cell sheet technology has been reported to repair many of partial tissue defects. However, it is difficult to construct organ-like 3D structures using cell sheet because high cell density of a thick layered cell sheet may trigger necrosis due to poor supply of oxygen and nutrient. To overcome those limitation, we constructed a hybrid structure by layering cell sheet on porous polymer sheet. SEM observation revealed that cell sheet was actively proliferating to the entire surface of polymer sheet. This is also confirmed by cell proliferation assay that shows the increasing number of live cells during 14 days of *in vitro* study. More interestingly, histological staining showed that cell sheet was able to penetrate into the porous structure and form a new layer of cell sheet on the bottom of polymer sheet, thus resulting a unique structure-like sandwich. Therefore, we concluded that layering cell sheet on porous polymer sheet could overcome the thickness limitation of cell sheet thus could have a broad impact on future development of tissue engineering patch.

1. Introduction

Surgical repair by inserting layer of patch is highly required to treat many types of congenital heart defect (CHD), which affect around 3000 babies every year in US. Tissue engineering is one of the most promising method that alter the future treatment of CHD by providing engineered patch whose ability to grow without sacrificing its biocompatibility. In tissue engineering approach, the presence of seeded cells is a critical part to regenerate new tissue, whereas the polymer scaffold is necessary to support and guide seeded cells in shape and is expected to degrade completely without foreign materials as the new tissue forms [1]. In conventional method, however, a major procedure to obtain single cell is by trypsinization, which work by damaging cell-to-cell adhesion and separating the cells, hence over-trypsinization can digest the cells, lead to the decreasing of cell viability drastically [2].

Recently, innovative approach of harvesting cells by avoiding the need of proteolytic enzyme such as trypsin has been developed called as cell sheet technology [3]. Facilitated by temperature-responsive dishes, confluent cells are allowed to harvest as a layer of cells, thus the critical components of extracellular matrix such as growth factor protein, adhesion protein and cell-to cell junction protein are remained intact.

Considering the benefit of cell sheet, in this study, we developed a novel hybrid structure using cell sheet and porous polymer sheet for tissue engineered patch. Polymer sheet - made of copolymer of poly lactide and caprolactone (PLCL)- was fabricated using solid-liquid phase separation method followed by freeze drying method. MSCs cell sheet, prepared by temperature-responsive dish, was layered on polymer sheet to construct hybrid graft. The variational behavior of mechanical strength was investigated. The cell proliferation, and cell spreading behavior were observed during *in vitro* study.

2. Materials and Methods

2.1. Fabrication of polymer sheet

Porous polymer sheet composed of copolymer of lactide and ε-caprolactone (PLCL) at 75:25 ratio (Gunze Ltd., Kyoto, Japan) was fabricated by phase separation method (Mo et.al, 2006) followed by freeze-drying under vacuum. The polymer granules were dissolved in

1.4-dioxane solvent with final concentration of 6% (w/v). A Teflon tube of 10 mm in diameter, taken from -80°C was vertically dipped into polymer solution and pulled out at a constant rate (100 mm/min). This sample was frozen again at -80° C for at least 1 hour before it was freeze-dried at -50°C for 24 hours to remove solvent. Finally, the graft was pulled out from the Teflon tube and cut into sheet, as shown in **Figure 1**.

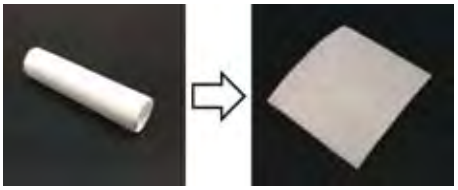

Figure 1. PLCL tube and sheet

2.2. Fabrication of Hybrid Graft

Mesenchymal Stem Cells (MSCs) (UE6E7TE, Riken BRC, Tsukuba, Japan) of passage 4-5 were seeded on 24-multi well temperature-responsive dish (*Upcell* ®, CellSeed Inc., Tokyo, Japan) at high density (5 x 10^4 cells/well) and cultured in cell growth medium containing α -MEM (Wako Chem.,Tokyo, Japan), 10% FBS, and 1% penicillin streptomycin in humidified atmosphere with 5% CO_2 at 37° C for 4 days. The confluent cells were detached spontaneously when exposed in room temperature within 20 minutes. To construct a hybrid graft, using a pipette, cell sheet was transferred on the polymer sheet which had been sterilized by ethanol 70% and ultraviolet. After removing media, the hybrid graft was incubated for 1 hour to promote adhesion between cell sheet and polymer sheet (10 mm x 10 mm). Finally, the hybrid graft was incubated in 2 mL of cell growth medium and cultured for 2 weeks.

2.3. Characterization of Hybrid Graft

The tensile test was performed using Shimadzu Compact Tabletop Testing Machine with 10 N load cell and a crosshead speed of 1 mm/min to investigate the variation behavior of mechanical properties during *in vitro* study. The microstructural properties were observed using FE-SEM (Hitachi Ltd., S-4100). The cell proliferation assay was performed using Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan). The histological images was obtained by microscope digital camera after staining the cross section of hybrid graft using Hematoxylin and Eosin.

3. Results and Discussions

To verify macro-mechanical properties of both polymer sheet and hybrid graft, the tensile tests were performed. Typically, hybrid graft exhibited steeper stress-strain curves compared to polymer sheet as shown in **Figure 2a**. Introducing MSCs sheet on polymer sheet obviously increased its tensile strength (**Figure 2b)**. In 4 and 7 days of culture, the tensile strength of the hybrid graft was significantly 85 kPa higher than polymer sheet. However in 14 days of culture, both tensile strength of hybrid graft and polymer sheet were tend to decrease. In addition, the elastic modulus was increased almost 2 times higher when MSCs sheet was applied on the polymer sheet on the initial culture days even though the pattern of decreasing elastic moduli was also found at 14 days of culture. Since we used biodegradable polymer to fabricate the graft, thus decreasing behavior of its tensile strength and elastic moduli was closely related with the degradation process of polymer. This is supported by weight loss data of polymer tube during *in vitro* culture (**Figure 3**).

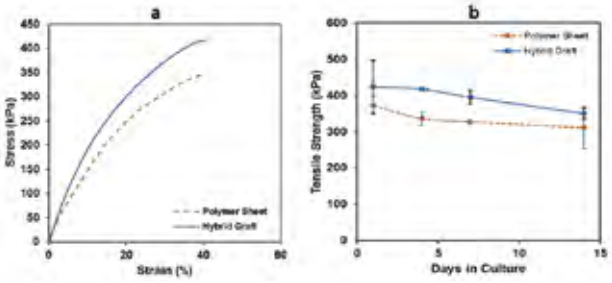

Figure 2. Mechanical evaluation of polymer sheet and hybrid graft. (**a**) Typical stress-strain curve at 1 day culture. (**b**) Comparison of averaged tensile strength (mean ± SD, n=3, * P<0.05).

Cell proliferation (**Figure 4**) was examined quantitatively using cell counting kit which contains water soluble tetrazolium salt, generating a yellow color from only viable cells. The number of live cells continuously increased during 30 days of culture. This result indicated that polymer sheet is compatible for cells growing.

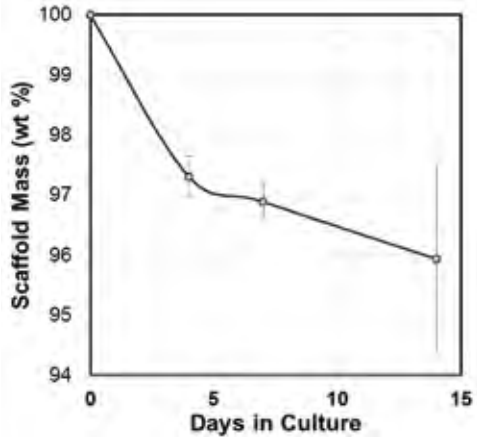

Figure 3. Weight loss data of polymer sheet in for 2 weeks.

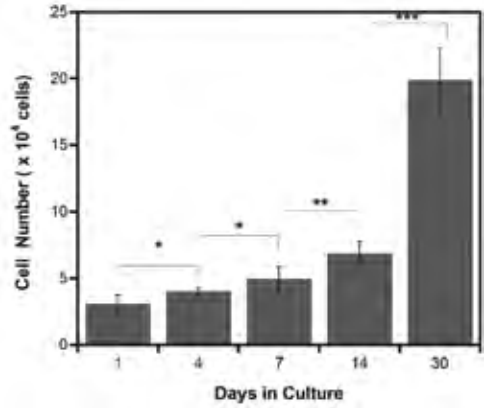

Figure 4. Cell proliferation assay. Each data represented mean ± SD, n=3, *P<0.05, **P< 0.001,

To observe the behavior of cell sheet spreading on the polymer sheet, SEM imaging of 7 days culture was performed. The cell sheet, recognized in denser images (**Figure 5b**), was completely covered the polymer sheet, thus the pore structure of polymer sheet could not be observed. However, cell sheet enables to proliferate actively to the entire surface of polymer

Figure 5. Cell spreading behavior on the hybrid graft in 14 days of culture. (**a**) The cell sheet covered the surface of polymer sheet and (**b**) proliferate horizontally by creating a connecting network.

sheet by creating a network structure of extracellular matrix (**Figure 5a**), as it was agreed with the increasing number of cells on proliferation assay.

In addition, histological staining revealed that at one day culture (**Figure 6a**), the cell sheet had already penetrated into the pores. The cells continued to proliferate deeper through the connecting pores and in 7 days of culture, we observed a new layer-like cell sheet on the bottom site of polymer sheet, resulting a unique structure like sandwich (**Figure 6b).**

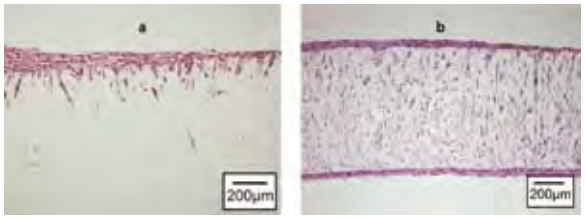

Figure 6. Histological staining of hybrid graft in cross section at 1 day (**a**) and 7 days of culture (**b**).

Previous study reported that in cell sheet engineering, high-cell density of cell sheet-more than 40 µm- without blood supply was unable to survive *in vivo* [4]. In contrast, our experiment shows that cell sheet can maintain their proliferation and reach a thickness more than 50 µm without indicating damaged cells. The porous tubular graft may play an important role in maintaining of cell sheet proliferation, as it can supply the nutrient while removing the metabolism waste [5].

4. Conclusion

In this present study, we successfully fabricated a unique graft made of biodegradable polymer sheet and MSCs sheet for tissue engineered patch. The variational behavior of tensile strength of hybrid graft were affected by cell sheet spreading behavior and degradation of polymer sheet. When cell sheet had been proliferated into the entire pores and formed a new layers in the opposite surface of polymer sheet, the hybrid graft exhibited significantly higher tensile strength. However, at 14 days of culture, the effect of degradation of polymer sheet was higher that cell sheet proliferation, therefore the tensile strength of hybrid graft decreased. In addition, this hybrid graft been successfully overcome classic problem of cell sheet related with thickness limitation.

Acknowledgment

This study was supported by Advanced Graduate Program in Global Strategy for Green Asia.

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