CONSTRUCTION OF CELL DENSITY-CONTROLLED 3D HIERARCHIC TISSUES USING CELL BEADS

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ABSTRACT

We developed a method to construct three-dimensional (3D) tissues that mimic in vivo-like hierarchic structures with different cell-densities using “cell-bead method” (Fig. 1). To construct the 3D structure, we prepared monodisperse collagen type-I gel beads by using an axisymmetric flow-focusing device (AFFD), and obtained two types of cell beads with different size: neonatal human epidermal keratinocyte (NHEK) beads and neonatal human dermal fibroblast (NHDF) beads. We then molded the NHDF beads and the NHEK beads subsequently into a polydimethylsiloxane (PDMS) chamber. After 24-hour incubation, we successfully obtained 3D tissues having in vivo-like hierarchic structure of the NHEK cells and the NHDF cells with different cell density. And tissue remodeling was observed in the reconstructed 3D tissue. We believe that the in vivo-like 3D skin tissue models are useful as the alternatives to animal testing in cosmetics, pharmacology and so on.

KEYWORDS: 3D tissue, 3D skin model, microfluidic gel bead formation, hierarchic structure, cell density

INTRODUCTION

Tissue engineering has been seeking a method to construct large-scale 3D tissue that mimic micro-sized complex tissue structure [1, 2]. Experimental systems that make it possible to study cells in a 3D-tissue architecture would be useful in generating realistic in vitro models in testing cosmetics and drugs. Although many approaches using hydrogels have been tried by several researchers [3], constructing micro-sized complex tissue structures with (i) different cell type, (ii) different extracellular matrix (ECM) type and (iii) different cell density have not been achieved. Our tissue fabricating process can reconstruct millimeter-thick macroscopic tissues with micro-sized complex structures by stacking a large number of collagen gel-based microtissue units called “cell beads”. The advantage of this method is able to reconstruct 3D tissues composed of different types of cells with controlled cell density and ECM type [4].

In this study, we performed reconstruction of a cell-density controlled 3D tissue that has in vivo-like layered tissue structure using NHEK and NHDF beads.

Figure 1: Conceptual strategy to construct hierarchic and controlled cell-density skin tissue structures using cell beads. Two types of cell beads: different cell types and different size, are molded subsequently to realize hierarchic 3D tissue structures. This method would be applicable to reconstruct engineered 3D tissue precisely mimicking structure and function of native tissues.
EXPERIMENTAL

We used the axisymmetric flow focusing device (AFFD) fabricated by stereolithography to produce collagen gel beads [5]. The AFFD consists of two concentric hollow cylinders to yield monodisperse droplets (Fig. 2a). The inner fluid is focused and broken into droplets at the orifice of the AFFD. We designed two types of the AFFD by changing the orifice size; the orifice is 250 μm or 550 μm in diameter. We plotted the size of droplets produced with each AFFD versus flow rate ratios of the inner fluid and the outer fluid. Flow-focusing the collagen solution with corn oil plus 2 w/w% lecithin as the outer fluid in the AFFD resulted in the formation of stable, monodisperse collagen droplets in the oil (Fig. 2b left). After gelation at 37 °C for 30 min, we extracted the collagen gel beads from the oil without breaking the beads and transferred them to the cell culture media by centrifugation (Fig. 2b right). NHDF beads were prepared by seeding NHDFs onto the 500 μm collagen gel beads and agitated 60 rpm for 17 h at 37 °C. In a similar manner, NHEK beads were prepared by seeding NHEKs onto the 100 μm collagen gel beads and agitated 60 rpm for 2 h at 37 °C. Prior to use in molding process, we fluorescently labeled each cell, NHEKs and NHDFs, using CellTracker™ Orange and Green, respectively. We then molded NHDF beads and NHEK beads subsequently into a PDMS chamber to construct 3D hierarchic tissues (Fig. 3e). Obtained tissues were fixed with 4 % paraformaldehyde, and cross sections were prepared for the histological analysis.

RESULTS AND DISCUSSION

We fabricated two types of AFFD having the different orifice size. Fig. 2(c-d) shows how the diameter of the droplet varies with the flow rate ratio (the outer flow rate/inner flow rate) for each diameter of orifice. When the size of orifice is 250 μm in diameter, we could produce 50~250 μm collagen droplets (Fig. 2c). On the other hand, when the size of orifice is 550 μm in diameter, we could produce 350~560 μm collagen droplets (Fig. 2d). Various diameters of the micro scale droplets can be formed by varying the flow rate ratio and diameter of the orifice, thus the AFFD should be useful for tissue assembly because we can chose the volume of collagen by changing the size of droplets. In addition, this droplet formation system allows for mass-production. Thus, We can obtain enough beads to construct the large scale tissue in a short amount of time. By using two types of AFFD, we obtained two types of droplets: diameter of droplets is 100 μm or 500 μm.

After 17 hours and 2 hours of culture of collagen beads with NHDFs and NHEKs, NHDF beads and NHEK beads were obtained, respectively (Fig. 3a-d). Both cell beads have shrunk because the cells pull on the collagen and contracted the gels.

Figure 2. Preparation of collagen gel beads using the AFFD. (a) Schematic diagram of the AFFD to produce monodisperse collagen droplets. (b) Microscopic views of the collagen droplets in oil and collagen gel beads collected in cell culture media. Plot of the size of collagen droplets in the corn oil versus the flow rate ratio using the AFFD which orifice is 250 μm (c) or 550 μm in diameter (d).

Figure 3. Fabrication of 3D hierarchic skin tissue. (a-d) Microscopic images of the NHEK beads and the NHDF beads prepared by seeding cells on the collagen gel beads of different sizes (100 μm or 500 μm in diameter). NHEK and NHDF are visualized with CellTracker™ Red after 17.5 hour incubation or CellTracker™ Green after 2.5hour incubation, respectively. (e) Schematic diagram of the molding process to construct 3D hierarchic tissues.
We obtained 3D skin that show in-vivo like hierarchic arrangement of the NHEK layer and the NHDF layer with different cell-density (Fig. 4a). The NHDF layer had collagen rich structures compared with the NHEK layer (Fig. 4b-d). Immunohistochemical analysis using a antibody specific for collagen type-IV revealed localization of the collagen type-IV, especially in the collagen gel beads (Fig. 5). No significant staining of other components of tissue could be detected. This image shows that NHEKs in the reconstructed tissues secreted their own ECM: collagen type-IV. The result suggests that the tissue remodeling had occurred in the reconstructed 3D tissue.

CONCLUSION
We reconstructed the 3D hierarchic tissues mimicking microstructures of living tissues using the cell-bead method. We believe that this method would enable reconstruction of functional 3D tissues with complex microstructures and microenvironments as living tissues.

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Figure 4. Histological cross section of the fabricated tissues. (a) Fluorescence microscopic image shows the fabricated tissues composed of the NHDF layer and the NHEK layer. (b-d) Analyses of hematoxylin-eosin-stained tissue cross section show that the NHDF layer has collagen-rich structures compared with the NHEK layer.

Figure 5. The remodeled tissue. Immuno-histochemical staining shows that NHEKs secrete their own ECM (collagen type-IV), suggesting tissue remodeling is occurred within the 3D hierarchic tissues.