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Spheroid Array incorporated in Hydrogel as a Tissue-engineered Construct.

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It is noted that three dimensional cellular aggregates (= spheroid) have the advantage to maintain the differentiation function due to their microenvironment nearer to in vivo tissue. In this study, we demonstrate the micropatterning of spheroids on a nonfouling substrate, followed by their transcription into poly(ethylene glycol) (PEG) hydrogel from the substrate. The spheroid-containing gel was intensively studied in terms of physicochemical and biological view points. PEG hydrogel was size selective of permeation due to the difference of crosslinking density, which was clearly ensured by swelling properties. Note that the spheroids maintained their function even after transcription, which may have the high utility to reconstruct large size organization (organoid) for tissue engineering.

Key words: poly ethylene glycol, spheroid array, hydrogel, tissue engineering

1. INTRODUCTION

While two-dimensional culture can provide an adequate representation of cellular behavior in many instances, other cell types respond favorably to a 3-D physical microenvironment by alterations in cell shape, gene expression, and resemblance to in vivo responses. Also, the development of cell microarray platforms that retain cells in 3-D culture, rather than on rigid 2-D substrates, represents an important step toward tissue engineering and cell-based assays that accurately predict in vivo behavior. Thus, many researchers have explored the merits of culturing cells in three dimensions (3-D), rather than in the flat-dish's two dimensions, because the 3-D culture is much closer to their behaviour in vivo. Biology also find that the cells maintain their differentiation function much more in 3-D than in 2-D. However, culturing cells in 3-D needs the complex and time-consuming procedures. In addition, from the viewpoint of material transmission such as the oxygen and nutrition, it had a crucial disadvantage such that a center of spheroid (=3-D cell aggregates) necrotizes when too big (1). We have recently established the 3-D spheroid culture in arrayed structure easily (2). Microscale approaches may provide templates to induce the reaggregation of cells in a reproducible manner. We use a photolithography of nonadhesive PEG gel, providing templates for the formation of aggregates of various cell types, including hepatocyte and ES cells. This approach aims to overcome the spheroid culture raised in the past disadvantages by providing control over the size, shape, and other features of the cellular assembly in a scalable manner.

In this study, we further developed to transfer the spheroid microarray on the substrate to the PEG hydrogels, as an implantable material, for tissue engineering (Fig. 1). Hydrogels are increasingly popular biomaterials for 3-D cell culture because their high water content and mechanical properties resemble those of tissues in the body. Additionally, many hydrogels can be formed in the presence of cells by photocrosslinking. Poly(ethylene glycol) (PEG)-based hydrogels are of particular interest because of their biocompatibility, hydrophilicity, and ability to be customized by varying chain length or chemically adding biological molecules. These materials have been used to immobilize various types of cells that can attach, proliferate, and produce matrix within hydrogels. The PEG hydrogel presented here can restrain upsizing the incorporated spheroids by the union between them. As a result, an established spheroid culture may mimic the physiological in vivo functions, which have the quite possibility to provide the large-scale tissue-organization (Organoid Engineering).

2. EXPERIMENTAL

Cell culture

The mouse osteoblastic cell lines, MC3T3-E1 cells, were cultured with alpha-minimal essential medium (α-MEM) that was supplemented with 10%(v/v) fetal bovine serum, 2%(v/v) penicillin, and streptomycin at 37℃ under humidified atmosphere with 5% CO_2. Under these culture conditions, they reached confluent within 3-4days. After confluent, cultured cells were trypsinized with 0.25% trypsin-EDTA, centrifuged, and resuspended. Then concentration of cell suspension was modulated to 1×10^6cells/ml.

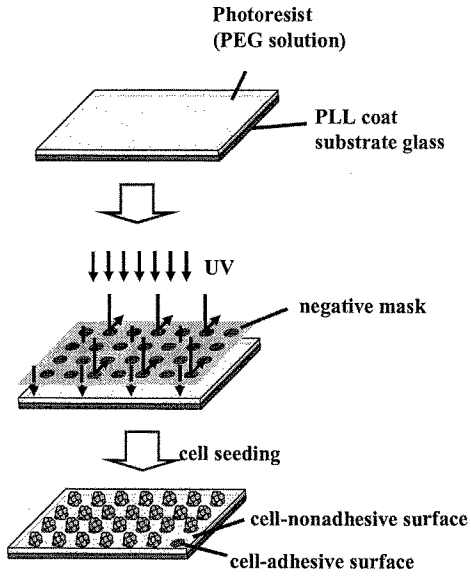

Fig.1 Manufacturing method of the substrate glass patterned with PEG.

Formation of spheroid array

The spheroid array was formed by seeding cells to the glass substrate micropatterned with PEG (Fig.1); the glass substrate fit into 12well plate, and 1×10^6cells were seeded into well.

Spheroid array encapsul ation in PEG hydrogel

10%(w/v) of PEG hydrogel was prepared by polymerizing the multi-arm PEG acrylate (divergence n=8,MW=40,000) in α-MEM. The detailed procedure is shown as follows; photoinitiator, Irgacure 2959 was add to the hydrogel solution and mixed thoroughly to make a final concentration of 0.05% (w/v) (3). 150 μl hydrogel solution was injected to the spheroid array (18days after seeding), and exposed to UV light (365nm, 25mW/cm^2) for 35sec. The hydrogel was then removed from the substrate, and transferred to 12-well culture plates filled with culture medium (Fig.2).

Cell viability assay

The number of viable cells was evaluated by the cell counting kit-8, which measures the ability of mitochondrial dehydrogenase emzymes to convert the wst-8 into soluble orange formazan, and the absorbance of the resultant solution was measured using microplate reader at 450nm.

ALP activity assay

ALP activity was measured according to a method of lab assay ALP kit. 20 μl of cell lysis solution in 1 %(w/v) SDS, and 100 μl of substrate solution (0.5mM p-nitrophenylphosphate) were added to a 96-well plate and incubated for 15min at 37℃ (4). After incubation, the kinase reaction was stopped by adding 80 μl of 0.2M NaOH to each well. The absorbance of each well was measured using microplate reader at 405nm. ALP activity was calculated by concentration of p –nitrophenol using the number of estimated viable cells.

MTT staining

Spheroids in PEG hydrogel were stained with 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to observe viable cells. Two milliliters of MTT solution (0.5mg/ml in α-MEM supplemented with 10% FBS) was first added to the gel. After incubation for 4h, the MTT solution was removed and the gel was rinsed twice with PBS to observe cell viability (5).

3. RESULTS AND DISCUSSION

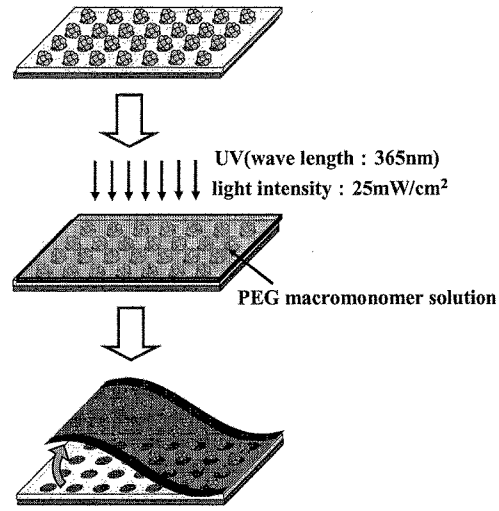

Fig.2 Mechanism of transference spheroids to PEG hydrogel.

ALP activity of spheroids on the substrate

Micropatterned PEG substrates with two-dimensional arrays of circular domains (Φ= 100 μm) were prepared by spin-coating of photoreactive PEG macromonomer on PLL substrate, followed by photolithography through a photo-mask pattern with circular holes.

MC3T3-E1 cells at passage 13 were then seeded onto the patterned surface with Φ =100 μm circular domains, and cultured at 37℃ in a 10% fetal bovine serum medium. Obviously, MC3T3-E1 cells adhered and formed spheroids only onto the circular domains, exposing PLL substrate.

MC3T3-E1 cells express ALP activity when cells differentiate. Spheroid culture expressed high ALP activity compared with mono-layer culture, demonstrating the enhanced differentiation function much more in 3-D than in 2-D (Fig.3). ALP activity of spheroid increases steeply in 12-17day, and then reached plateau level of ALP activity. Note that long-term maintenance was certainly confirmed in this ALP activity.

Spheroid array encapsulated in PEG hydrogel

PEG have been applied for biomedical applications and is easily modified with photoreactive and crosslinkable endgroups. Cells usually photoencapsulated in hydrogels

formulated from PEG macromers maintain their viability, and this environment is thought to promote the formation of an extracelluar matrix (6). The closslinking reaction of the photopolymerized PEG gels occurs through a radical initiated chain propagation that produces kinetic chains with covalent closslinks, which facilitate the entrapments of spheroids.

The encapsulation of spheroids from the array was accomplished at 18days after cell seeding. This day was when spheroids had a high ALP activity (Fig.3). After encapsulation, spheroid arrays kept their ALP activity (Fig.4).

After 2 weeks of incubation in PEG hydrogel, viable spheroids were stained with MTT reagent. The condition of viable cells was observed by the MTT staining, which stains the cell employing the ability of mitochondrial dehydrogenase emzymes to convert the yellow MTT into insoluble purple formazan. As shown in Fig.5 most spheroids were stained, indicating that spheroids were alive even in gels.

Considering from the results above obtained, this method of encapsulation could make spheroid arrays live and keep ALP activity. Accordingly, we presented the useful and easy methods for creating living cell arrays that are encapsulated within a poly(ethylene glycol)-based hydrogel to create a local 3-D microenvironment. Along with emerging hydrogel biomaterials with defined chemistry and physical properties, these tools for cell organization within a 3-D microenvironment may improve the performance of future tissue engineering.

4. CONCLUSION

MC3T3-E1 spheroid arrays were encapsulated in PEG hydrogels by using the biocompatible multi-arm PEG acrylate. ALP activity of spheroids in hydrogel was kept 7 days after encapsulation, and spheroid arrays in hydrogel were alive 2weeks after encapsulation. As a result of these experiments of encapsulation, a simple and convenient method to use two dimensionally alligned spheroids as an implantable construct was certainly established.

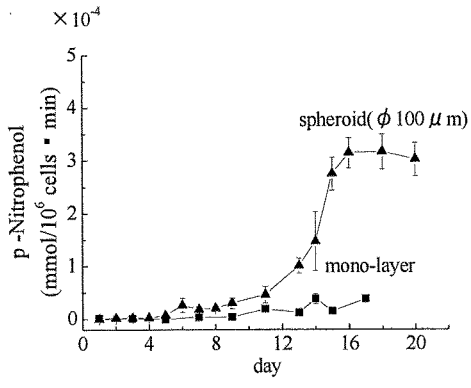

Fig.3 ALP activity of spheroid array culture (▲) and mono-layer culture (■). (n=3、error bar:SEM)

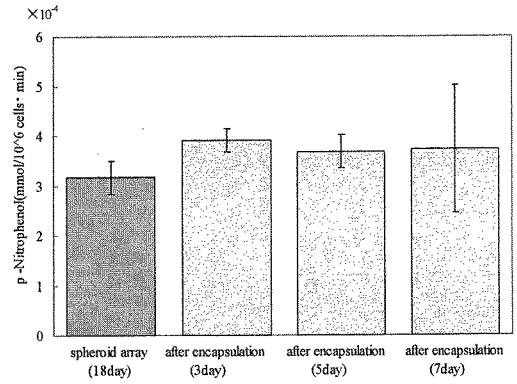

Fig.4 Comparing ALP activity of spheroid arrays of spheroids on glass substrate, 18days and in PEG hydrogels.

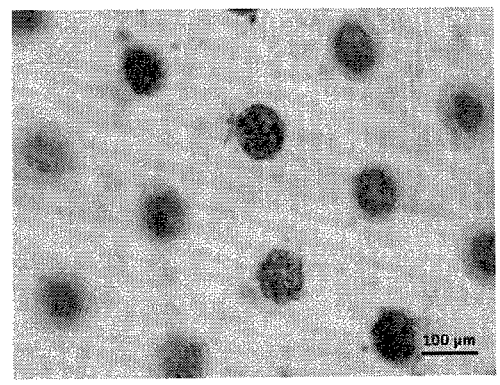

Fig.5 MTT staining of PEG hydrogels 2weeks after encapsulation of spheroid arrays.

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