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Proliferation of Periodontal Ligament Cells on Biodegradable Honeycomb Film Scaffold with Unified Micropore Organization*

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Abstract

Tissue-engineered grafts using a scaffold can be used in treating periodontal disease; however, previous scaffolds for the cultivations of the periodontal ligament cells have been structurally incompatible with the morphological requirements of human periodontal tissue. Here, we describe a self-organized honeycomb-patterned film (honeycomb film) that acted as an appropriate scaffold for periodontal tissue regeneration. The honeycomb films were prepared from biodegradable poly(ε-caprolactone) with highly regular three-dimensional micropatterned surface topography by casting a polymer solution of water-immiscible solvent under humid conditions. To evaluate its performance in activating the proliferation and organizing of cells, we have demonstrated specific behaviors of the cultured periodontal ligament cells on the self-organized honeycomb structures *in vitro*. Fibroblast-like cells derived from the periodontal ligament of extracted human molar teeth were cultivated on three types of honeycomb films with 5-, 10-, and 15-μm pore sizes for 4 h to 42 d. Morphological observation of the cultured tissues at 4–72 h revealed that the pseudopodiums of cell bodies were attached to the pillars in the honeycomb structure. A certain number of cells shifted their cell bodies into the honeycomb structural lumen through the oscula of 10- and 15-μm pores. After 28 and 42 d, the cells were observed to have formed multiple layers; further, each cell had penetrated through the 10- and 15-μm pores in the honeycomb film. The morphological examination of the honeycomb film along with the pillar structures revealed that the scaffold was clusteringly arrayed with interconnected structures, remarkably enhanced proliferation, and extension of the cultured cells. We consider that the film can be applied in periodontal therapy for use as a scaffold for periodontal tissue regeneration.

Key words: Self-organization, Biomaterials, Scaffold, Periodontal Ligament, Cell Adhesion, Tissue Regeneration

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1. Introduction

Human tissues are capable of autogenous healing for minor damages from injury and sickness. When tissues suffer damage on a scale that exceeds autogenous recovery capabilities, graft treatment with transplantation of tissue is one of the options for aiding recovery. The periodontal ligament is a supportive tissue of teeth; it does not possess exclusive autogenous capabilities that would enable complete oral rehabilitation and healthy conditions. In the case of the periodontal tissue of gingival and alveolar bone, morphological and functional recoveries could be satisfactorily achieved by autograft surgery using grafts from other areas of the oral cavity. However, autograft surgery simultaneously creates a pair of invasive sites, i.e., recipient and donor sites, in the oral cavity. The condition of autograft treatment should be restricted the quantity of the transplant organ or tissue so that the autograft procedure might be limited to the effect on the recovery of the recipient site. Explanted tissue-engineered grafts in which cells are grown *in vitro* from a tiny tissue resource of the donor site may be useful in supporting the regeneration of periodontal tissue[(1)]. A technique that ensures a minimal wound area would be preferred by patients; therefore, tissue-engineered grafts are considered to be preferable to autografts. The method for the proliferation of cells on a three-dimensional (3-D) scaffold could be applied for creating organized cell layers. However, previous 3-D scaffolds for the regeneration of the periodontal ligament have been structurally incompatible with cell proliferation and organization into a sheath shape that surrounds the root surface of the tooth. A membrane-style scaffold with a unified porous array that is two-dimensionally organized for matching the spatial anisotropy of periodontal cell structure may be applicable in regenerating the periodontal ligament.

Honeycomb films can be prepared by using biodegradable polymers[(2)–(9)]. Honeycomb films are characterized by the distribution of equal porous sizes in microns. This enables all the cells inoculated onto the film to be situated on unified circumstances of the topographic condition. Such a honeycomb scaffold with a single porous layer modeled in the shape of a flat film includes a horizontal micropore network modified to resemble the intercellular matrix[(2)–(9)]. In this study, we have demonstrated specific behaviors of cultured cells on fabricated honeycomb structures *in vitro*.

2. Materials and Methods

These experiments were approved by the Ethics Committee of the Dental Faculty of Tohoku University, Japan.

2.1 Film Preparation

Poly(ε-caprolactone) (PCL; Wako, Japan) and an amphiphilic copolymer (CAP) of N-dodecylacrylamide and ω-carboxyhexylacrylamide (Fig. 1) were used to fabricate both the flat and honeycomb films. PCL had molecular weights between 70 and 100 kDa. CAP powder was synthesized as previously reported[(10)]. The role of CAP in pattern formation is to prevent the fusion of water droplets[(3)].

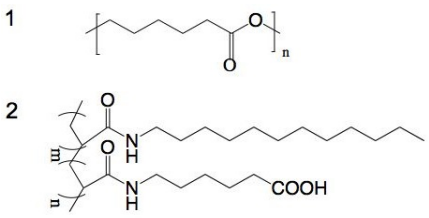


Figure 1: Chemical structures of poly(ε-caprolactone) (PCL) (1) and CAP (2).

Self-organized production of the honeycomb films (Fig. 2) from PCL and CAP were processed to produce a highly regular porous structure by casting in a humid atmosphere. The size distribution of each pore was less than 1 μm. The honeycomb films were fabricated on a flat glass substrate by casting a chloroform solution (10:1 ratio (w/w) of PCL and CAP, respectively) at a concentration of 5 g/L[3],[11],[12]. The flat films were prepared by spin-coating 150 μl of the PCL chloroform solution at a concentration of 40 g/L onto a glass substrate at approximately 30% humidity and 21 ± 1 °C using a commercially available spin-coater (1H-7D; Mikasa, Japan). Fabrication by this casting method would compose a bilayer membrane with oscula of 3 to 20 μm in both membranes. Further, the residual volume ratio of the matrix of the membrane was significantly decreased, so that the matrix of the honeycomb film was represented by numerous pillar frames that emulated the framework of the intercellular matrix. This bilayer structure with parallel dual membranes with pillars could unite into a film to produce a precision array of honeycomb-style lumens. Honeycomb film and flat film were clipped to 14 mm disc to fit a chamber of 24-well cell culture plate[3]. These films were washed with ethanol and 2-propanol before using for cell culture.

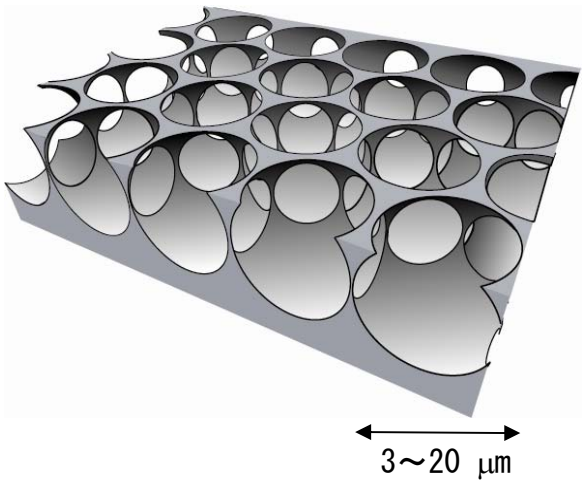


Figure 2: Schematic representation of the 3-D structure of the PCL honeycomb film with pillar structures using a template of water droplets.

2.2 Preparation of Cells

Fibroblast-like cells were derived from the periodontal ligament of human third molar teeth extracted from healthy individuals (age, 18–22 years) with no clinical signs of chronic periodontal disease after obtaining informed consent. Periodontal ligament tissues were dissected as small pieces from the mid-portion of the root with a sharp blade[13]. The pieces were cultivated in tissue culture dishes with a medium composed of α-Minimum Essential Medium (α-MEM)(Gibco 12571, Invitrogen) with 10% heat-inactivated fetal bovine serum (Gibco 26140-079, Invitrogen) and a component of antibiotic solution (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B) until confluent cell monolayers were formed. After confluence, the cells were washed with phosphate buffer solution (PBS) and resuspended with 0.075 g/L protease and 0.1 g/ L EDTA for cell passage. Following the initial culture derived from the human periodontal ligament samples, the cells were plated onto the honeycomb and flat PCL films on cover glasses at a density of 1×10^4 cells/cm^2 after the third and fifth passages in the experiment. The cells belonging to the experimental group were cultivated on three types of honeycomb films with 5-, 10-, and

15-μm pore sizes for 4 h to 42 d. Control group cells were cultivated on the flat PCL film without pores, and glass substrate.

2.3 Microscopic Observation

For morphological assessment of cell proliferation on the honeycomb film, the cells were observed by field emission scanning electron microscopy (FE-SEM; S-5200; Hitachi, Japan) according to the methods described previously[2],[4]. The cultured cells were fixed in 2.5% glutaraldehyde in PBS and incubated overnight at 4 °C. The cells were then washed three times with PBS and then with pure water. The samples were dehydrated by washing with increasing concentrations of ethanol and then air-dried. They were transferred to microporous specimen capsules and then dried using a critical point dryer (ES-2030; Hitachi, Japan). The dried samples were mounted on aluminum stages by using double-sided sticky tape and coated by using an ion sputter coater (HPC-1SW; Vacuum Device Inc.) All samples were observed by FE-SEM.

The morphologies of cultured cells were observed by a fluorescence microscope (IX71; Olympus, Japan) and a confocal laser scanning microscope (CLSM; FV-1000; Olympus, Japan). To visualize the cell proliferation and adhesion to the materials, staining was performed for vinculin, actin fibers, and cell nuclei. After washing with PBS, the cells were fixed with 4% paraformaldehyde (Wako) for 10 min at 37 °C and washed twice with PBS. For decontamination, the cells were permeated three times with 1% Triton X-100 (MP Biomedicals, Solon, OH) in PBS solution for 10 min at 20 °C, and then suspended in 0.05% Tween-PBS for 5 min. They were blocked by using 10% goat serum (Invitrogen) in 0.05% Tween-PBS for 15 min, and the samples were then washed with Tween-PBS. Vinculin was stained using primary antibodies (mouse anti-vinculin monoclonal antibody; Chemicon, Temecula, CA) and fluorescence-labeled secondary antibodies (Alexa Fluor 546 goat anti-mouse IgG; Invitrogen Molecular Probes, Carlsbad, CA). Actin fibers were stained with Alexa Fluor 488 phalloidin (Invitrogen Molecular Probes). The stained cells were rinsed three times with PBS, and subsequently immersed for 10 min after the fourth rinse. All the specimens were placed on glass slides and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen Molecular Probes) and covered with glass coverslips. The specimens were imaged using FM and CLSM.

3. Results

Outgrowth of human periodontal ligament cells from dissected tissue segments was observed within 7 d after being placed into plastic tissue-culture dishes. This process did not appear to be dependent on differences between individual volunteers. Precultivation of cells for inoculation on the target materials was carried out for 3–4 weeks.

In the control group, each cell adhered to the flat PCL film and glass substrates. The applanation shape was obtained at 24 h (Fig. 3a). Ordinary spindle cell shapes appeared at 72 h of cultivation, indicating the beginning of self-organization (Fig. 4a).

In the experimental group, histological observation of the cultured tissues at 4–72 h revealed the pseudopodia of the cell bodies to be attached to the pillars of the honeycomb structure. After 4 h cultivation, the cells were located on the pillars of the upper layer of the film. Some number of the cells appeared spindle shapes produced pseudopodia. At 24 h, the number of the cells on the pillars had increased. A numbers of cells extended along the ridge of the pillar frame in all the honeycomb structures. On the 5-μm pored honeycomb film, the cell bodies were horizontally spread with branching the pseudopodia to the pillar on the top roof of the film (Fig. 3b), whereas the other cells on the 10-(Fig. 3c) and 15-μm pores honeycombs were shifted their bodies into the honeycomb structural lumen through the

oscula of pores. The cell bodies were spindle-shaped, resembling fibroblasts.

After 72 h, the increase in the cell number and cell density was accelerated. On the film with 5-μm pores, the colonies appeared to be spheroids that had stays suspending them from the pillars (Fig. 4b). On the 10- (Fig. 4c) and 15-μm pore films, multiple cells had started to organize into plexuses, which reflected the pillar components in the honeycomb structure.

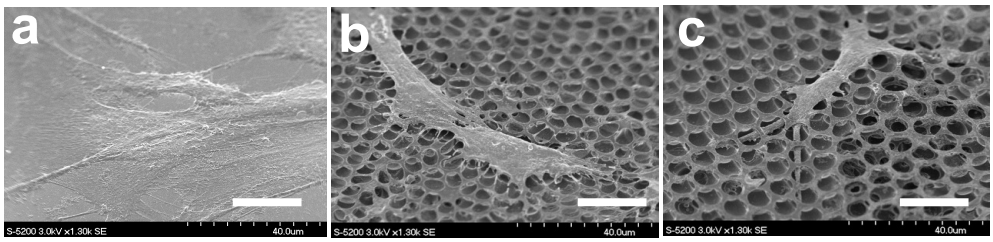


Figure 3: FE-SEM images of the fibroblast-like cells derived from the periodontal ligament of extracted human third molar teeth cultured for 24 h on glass as control (a), 5-μm (b), and 10-μm (c) pored honeycomb films as experimental cells. Control cultured cells were observed to migrate with applanation over the surface of the substrate (a). Experimental cultured cells were observed to bind to the pillars of the honeycomb frame (b) and migrated into the lumen of the honeycomb film (c) while maintaining 3-D configurations (Bar: 20 μm).

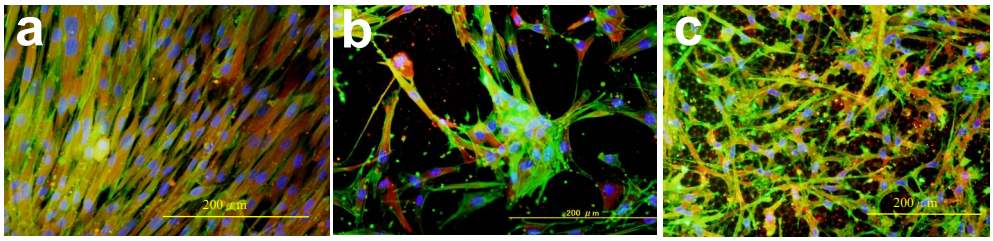
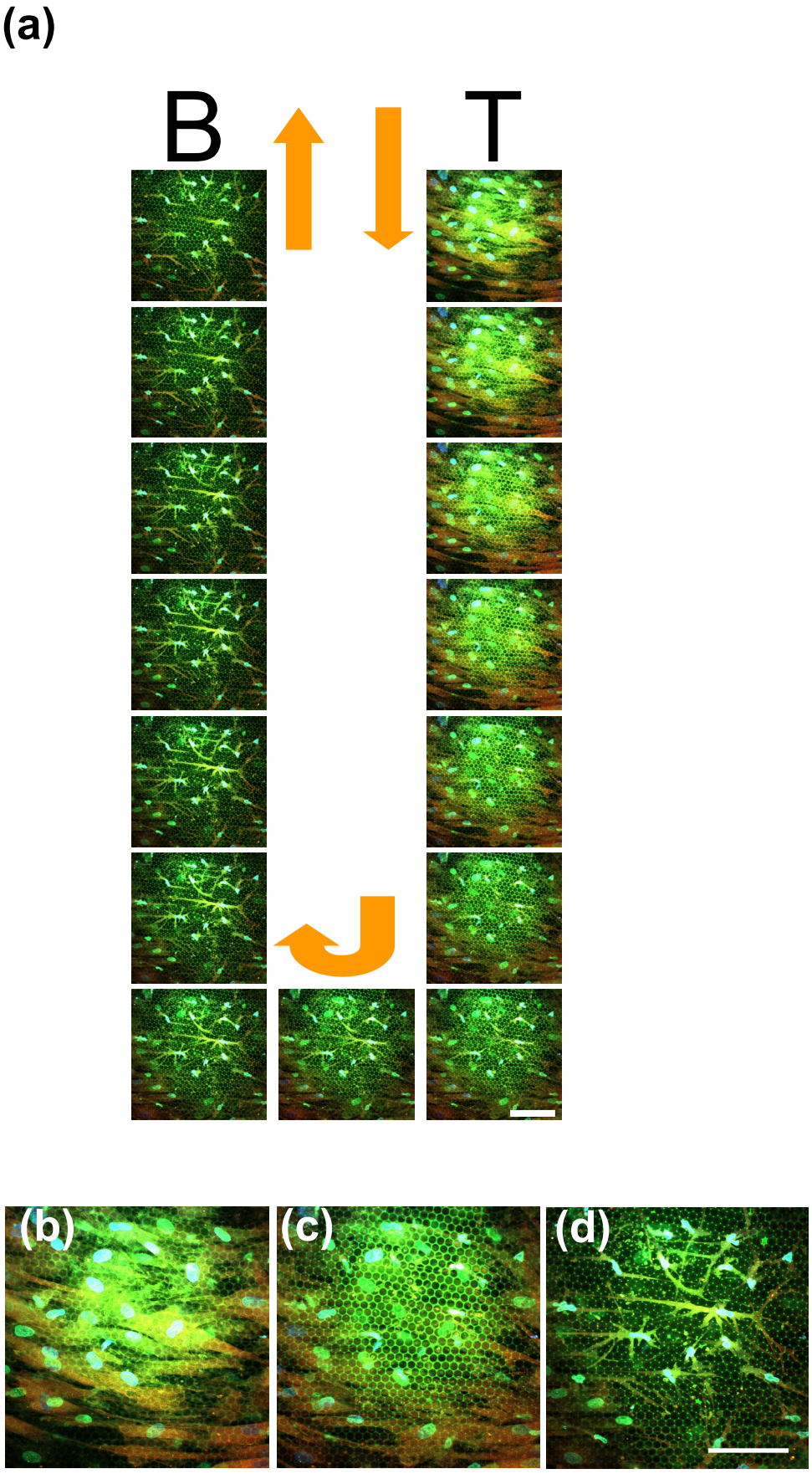


Figure 4: Fluorescent antibody chromatic images of periodontal ligament cells cultured for 72 h on flat film as control (a), 5-μm (b), and 10-μm (c) pored honeycomb films as experimental cells. Comprehensive assessment of the proliferation of the tissue, such as the number of multiplying cells as compared between the control and experimental groups seems an equation of property for the cultivation of the periodontal ligament cells. The cells on the flat film were uniformly organized with spindle shapes and unified directional orientation with closed intercellular clearance (a). On the 5-μm honeycomb, the condition of the cells was almost as homogenous as the control; however, in some spots, cells congregated to establish minor spheroids (b). On the 10-μm honeycomb, the condition of distributions of cells was heterogeneous and the cells were organized to accomplish the formation of a plexus with intercellular apertures. (Green: actin filaments. Blue: nucleus. Orange: vinculin. Bar 200 μm).

The cells cultivated for 28 and 42 d of both groups were observed to be organized in a sheet-like configuration after confluence. However, in the PCL flat film, detachments occurred in some areas of the organized cells.

The experimental cells on the honeycomb film with 10- and 15-μm pore structures formed multilayers through the structure (Fig. 5). CLSM observation revealed that the cells proliferated in the lumens of 10- and 15-μm pore films had horizontally spread their cell bodies in approximate range of the fifth next to other cells of the honeycomb; urther, their shapes had altered to dendrite forms. The degradation and collapse of the honeycomb films were not observed at the completion of periods on every cell cultures.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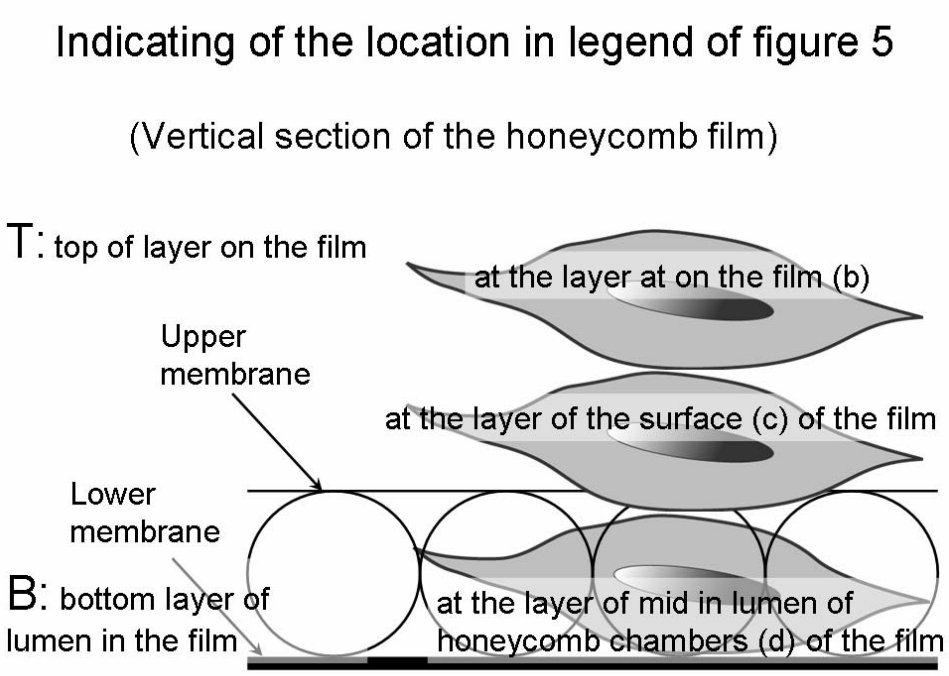

Figure 5: Serial confocal stacked (a) in 2-μm steps and sectional (b–d) images of periodontal ligament cells cultured for 28 d through a vertical thickness section (T: top of layer on the film, B: bottom layer of lumen in the film). At the layer on top of the film (b), at the layer on the surface (c), and at the layer in the mid-lumen of the honeycomb chambers (d) of the film. (Bar 100μm). A cell-rich zone was observed in the outer layer on the film rather than in the inner layer of the lumen of the film (b). On the other hand, in the lumen of film, dendriform cells were observed to migrate, penetrating through the multiunit chambers (d) (Bar: 100 μm).

4. Discussion

During cultivation, cells can modify their proliferative configuration depending on the microtopography of the substratum[14]. This is reflected in the interface between the recipient site and a biocompatible material, such as titanium implant osteosurgically embedded in human alveolar bone[15]. The migration of gingival fibroblasts on the grooved surface of the titanium demonstrated that the orientation of cultured cells could be controlled for horizontal expansion[16]. This process in which cells assume the orientation of the microfabricated scaffold and migrate along the microtopographic edges and grooves on the surface of the material is defined as contact guidance[17]. The microstructure of the surface of biocompatible materials cannot be disregarded while designing innovative technologies for fabrication of biomaterials[18].

The structure of the periodontal ligament enables the binding of the tooth's root surface to the alveolar socket with multiple fiber suspensions that confer flexibility in order to protect against physical stresses, such as impacts, to the tooth[19]. The distinguished facilities of the periodontal ligament were enhanced by the spatial diversities on the proliferation and the multifunctional differentiations to connect between proximate tissues such as the gingival fibroblast. We expect that when periodontal ligament cells grown to sufficient numbers on a teleologically designed scaffold[20] are transferred to the recipient site along with the appropriate carrier, they would contribute significantly to optimum grafting of recombinant tooth to alveolar bone as well as in dental implants. So far, our

understanding of the effects of biomaterials as carriers and scaffolds and their contribution to healing and regeneration in the periodontal surgery remains limited.

In the organization of the honeycomb structure in the polymer solution, the uniformly sized water droplets would act as a template for the honeycomb impressions when numerous droplets were arrayed on the surface of the polymer solution. The size of each droplet might be suitable as omnidirectional anchors for the attachment of cells. The shape of each pore elements of the honeycomb film could be precisely uniformed, so that the diameter of apertures to the hollow inside of the honeycomb structure would be actually constant[21]. A minimum gauge of the aperture that cells of a human biotissue could pass through to migrate, was defined to 6-μm[22]. It seemed that the human periodontal ligament cells assumed to be accommodated to the topographic conditions of circumferential matrices. On the experimental group, migration properties of the cells could be modified depending on the gauge of holes at the entrance of material surfaces, whether the cells could pass through into the honeycomb hollow or not. Because of the restriction into the honeycomb hollow through the surface pores, the cells inoculated on 5-μm pored honeycomb film have horizontally migrated and spread twining with honeycomb pillars on the roof of the film. On the other hand 10- and 15-μm pored honeycomb films could conduct the cells into their housing of the hollow spaces through the pores of the surfaces. The cells in the honeycomb hollows ought to configure an orientation of cell bodies and to accomplish a morphological conversion for the cavity-to-cavity migration in the faveolate space. Each circumference around the cells by the topographic situations between to be outside and inside of the honeycomb structure might appear the differences of morphologies of the cells.

In the process of proliferation, cell bodies tend to create adhesions to the extracellular matrix while initiating preparations to achieve the original cell shape and cell functions. The morphological examination of the honeycomb film along with the pillar structures revealed that the scaffold was clusteringly arrayed with interconnected structures, remarkably enhanced proliferation, and extension of the cultured cells. We consider that the film can be applied in periodontal therapy for use as a scaffold for periodontal tissue regeneration.

5. Conclusions

We have demonstrated specific behaviors of fibroblast-like cells derived from the periodontal membrane of extracted human molar teeth on honeycomb films with different pore sizes *in vitro*. Morphological observations of the cultured tissues for 4–72 h revealed the pseudopodia of cell bodies to be attached to the pillars in the honeycomb structure. The cells cultivated for 28 and 42 d were organized into a multilayered structure penetrating through the 10-μm pore film. Our results suggest the suitability of 3-D honeycomb films as a scaffold for potential *in vivo* periodontal tissue regeneration.

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