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Effect of hydroxyapatite-coated nanofibrous membrane on the responses of human periodontal ligament fibroblast

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In this study, hydroxyapatite-coated biopolymer nanofibrous membrane was synthesized by mineralizing the electrospun polycaprolactone nanofiber. The effect of the hydroxyapatite-coated nanofibrous membrane was mainly investigated on the proliferation and differentiation of human periodontal ligament fibroblast, which is necessary for periodontal tissue regeneration. Scanning electron microscopy revealed favorable cell attachment and spreading appearance on 1 d and MTS assay showed increased cell proliferation on the hydroxyapatite-coated nanofiber membrane during 14 d. From 7 to 14 d, alkaline phosphatase activity of the hydroxyapatite-coated nanofiber was significantly increased when compared to that of control group. Hydroxyapatite-coated nanofibrous membrane showed prominent mineral formation on day 14. As a result, it was clarified that the developed hydroxyapatite-coated nanofibrous membrane had favorable effects on the proliferation and differentiation of human periodontal ligament fibroblast and might be a good candidate material for periodontal tissue regeneration.

Key-words : Biomaterial, Hydroxyapatite, Membrane, Periodontal ligament fibroblast, Periodontal tissue regeneration

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1. Introduction

Periodontitis is a kind of highly prevalent disease which causes detachment of the periodontal ligament (PDL) from the tooth-surrounding alveolar bone and eventually leads to tooth loss. The periodontal wound healing is a series of interactions between gingival epithelial cells and the cells of the tooth attachment apparatus; i.e., gingival connective tissue, alveolar bone, cementum, and PDL. Among this cell population, PDL fibroblast has been regarded to be utmost important, as it has been asserted that PDL fibroblast differentiated to osteoblast for restoring lost alveolar bone.[1)]

Ultimate goal of periodontal therapy is to regenerate periodontal tissues. In order to achieve this clinical outcome, specific tissue growing concept which was based on preventing the apical downgrowth of the gingival epithelium into a bony defect and creating a secluded space that can be repopulated by PDL fibroblasts was proposed.[2)] Early developed medical device for this purpose was mainly non-resorbable membrane which required a second surgery to be removed. Therefore, resorbable membranes have been preferred for periodontal regeneration therapy.

Whilst this procedure gained clinical acceptance, these membranes often showed inconsistent clinical results.[3),4)] This might be due to the fact that the membranes seldom provided PDL cell promoting effect. Therefore, development of active material for PDL cell promotion seems to be reasonable to solve this dilemma. Polycaprolactone (PCL) has been reported to have a level of biocompatibility for bone-marrow stromal cell,[5)] still needs additional material to enhance its mechanical and biological properties. Hydroxyapatite was added for this purpose and showed enhanced osteoblast function and growth as well as mechanical properties, including elastic modulus and tensile strength.[6)]

Although the histological and clinical outcomes of periodontal regeneration therapy have been well documented,[7),8)] the biological effects of membranes remain a question yet. Few studies have been conducted, such as cell proliferation and differentiation, by which membranes influence the cells adjacent to the periodontium.

The purpose of this study is to develop a hydroxyapatite-coated membrane by mineralizing the electrospun polycaprolactone nanofiber for the PDL fibroblast promotion. The effect of the hydroxyapatite-coated nanofiber was mainly investigated on the proliferation and differentiation of human periodontal ligament fibroblast, which is necessary for periodontal tissue regeneration.

2. Experimental procedures

2.1 Synthesis of nanofibrous hydroxyapatite PCL membrane

Poly(ε-caprolactone) (Sigma, St. Louis, USA) was dissolved at 10% w/v in a mixture of organic solvent (dichloromethane : ethanol = 4 : 1). The PCL solution was loaded into a syringe and injected under a high DC voltage through a laboratory-made multinozzle onto a metal collector for the electrospinning process which was carried out to generate the solution into a nanofibrous form. The electrospinning condition was as follows: a voltage of 10 kV, a distance of 10 cm, and an injection rate of 0.5 ml/h. The electrospun product was collected with a thickness of approximately 250

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μm. Nanofibrous web was dipped into the NaOH solution and incubated at 24°C for 12 h while gentle stirring (80 rpm). Then, this product was taken out from the NaOH solution to be washed with distilled water and followed by an alternate soaking into Ca- and P-individual solutions composed of 150 mM of $CaCl_2$ and Na_2HPO_4. Finally, nanofibrous web was incubated into a simulated body (Na^+ 142 mM, K^+ 5 mM, Mg^{2+} 1.5 mM, Ca^{2+} 2.5 mM, Cl^- 147.8 mM, HCO_3^- 4.2 mM, HPO_4^{2-} 1.0 mM, and SO_4^{2-} 0.5 mM), which was designed to contain ionic concentrations similar to those of human body plasma. After dipping into mineral solution for 7 days, the membranes were dried and sterilized for the cell assays.[9)]

2.2 Human PDL cell culture

Human PDL fibroblast cell line (ScienCell, San Diego, USA) was used. Cell pellets were suspended and plated in flasks in 15 ml of α-minimum essential medium (Gibco, Grand Island, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin. The flasks were maintained in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C. When confluent, the cells were washed with phosphate buffered saline and, then, enzymatically lifted from the flask using 0.125% trypsin in 4 mM of EDTA (Gibco, Grand Island, USA). The cells were concentrated by centrifugation at 1200 g for 3 min.

2.3 Cell loading

Hydroxyapatite-coated nanofibrous membranes were soaked in the medium to allow immersion for 2 days before the cells were seeded. Samples of human PDL fibroblast with predetermined cell density were prepared and loaded on the membranes and then were incubated for 2 h to allow them to attach on the surface of membranes. After 2 h of incubation, the cell culture medium supplemented with 50 μg /ml ascorbic acid, 10 mM β-glycerolphosphate and 10^{-7} M dexamethasone was added on each well. The incubation period was lasted for up to 14 d. Culturing was set at 37°C in a humidified atmosphere of 95% air and 5% CO^2. The medium was changed every 2–3 d. The passage of the cell line used was 6–8.

2.4 Cell adhesion study by scanning electron microscopy

Hydroxyapatite-coated nanofibrous membranes with 7×7 mm dimension were prepared in 48-well culture plates (Nunc, Rochester, USA) and seeded with 1×10^4 human PDL fibroblast. Each sample was washed twice in 0.1 M phosphate buffered saline and fixed with 2.5% glutaraldehyde for at least 10 minutes. The complexes were then incubated for 10 minutes in a postfixative of 1% aqueous OsO_4 and subsequently washed twice with phosphate buffer for 10 minutes. Samples were dehydrated through ascending ethanol (70, 90, 95 and 100%), allowed to air dry overnight and shadowed with 20 nm Au/Pd. The morphology of cells attached to membranes was observed by a scanning electron microscope (S-4700, Hitachi, Tokyo, Japan) after 1 day and 7 d.

2.5 Cell proliferation assay

To measure the cell proliferation, mitochondrial dehydrogenase activity of human PDL fibroblast cells was determined by using water soluble enzyme substrate, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Madison, USA), which was converted to blue water-soluble product formazan accumulated in the cytoplasm of viable cells. 1×10^4 cells of human PDL fibroblast were inoculated on the hydroxyapatite-coated nanofibrous membranes samples with 7×7 mm dimension. The surface of polystyrene culture dish (Nunc, Rochester, USA) was regarded as a control group. 100 μl of MTS solution was added into each well containing 500 μl of medium and incubated at 37°C for 3 h. The absorbance of formazan produced was measured at 490 nm with a microplate reader (Thermomax, Molecular Devices, Sunnyvale, USA). Cell proliferation was measured on day 1, 4, 7 and 14.

2.6 Alkaline phosphatase activity test

For the analysis of cell differentiation, the alkaline phosphatase (ALP) activity was investigated. Hydroxyapatite-PCL nanofibrous membranes of 14×14 mm dimension were prepared in 12-well culture plates (Nunc, Rochester, USA) and seeded with human PDL fibroblast (4×10^4 cells/well). The surface of polystyrene culture dish (Nunc, Rochester, USA) without membrane regarded as a control group. 1.5 M of alkaline buffer was added on each sample. Thawed cell suspensions were sonicated for 10 seconds in ice and centrifuged at 12,000 rpm at 4°C for 15 min in order to extract the enzyme. The protein content was determined by the Bradford method using 1 mg/ml of bovine serum albumin as a standard. P-nitrophenyl phosphate was used as a substrate and the reaction was made in 1.5 M of alkaline buffer for 1 hour incubation. The ALP activity was measured by absorbance at 405 nm on day 7 and 14. The incremental ALP activity ratio was calculated as follows:

$$\text{ALP activity ratio} = \text{optical density of day 14} / \text{optical density of day 7}$$

2.7 Mineralization assay

For mineralization assay, hydroxyapatite-PCL nanofibrous membrane, of which dimension was 7×7 mm, was seeded with 1×10^4 cells in 48-well microculture plates. The surface of polystyrene culture dish (Nunc, Rochester, USA) was regarded as a control group. After 14 d passed, each sample was washed with phosphate buffered saline and fixed with 70% ethanol for 10 minutes. It was washed with phosphate buffered saline twice and then stained for 40 minutes with 40 mM of Alizarin red S stain (Sigma, St. Louis, USA). Sample was then washed again with phosphate buffered saline at least 4 times to remove remnant staining solution, and examined under light microscope (JP/BX50, Olympus, Osaka, Japan).

3. Results and discussion

It is crucial that for a membrane to be successfully useful, especially in the tissue regeneration applications, both the structural/mechanical properties of the material and its biological activities should be equally considered because both parameters play a critical role in the cell adhesion, proliferation, and new tissue formation. Herein, we used a novel biomaterial with a nanofibrous structure as a potential periodontal tissue regeneration membrane. In particular, the surface of the polymeric nanofiber matrix was coated with bioactive hydroxyapatite by the mineralization method in

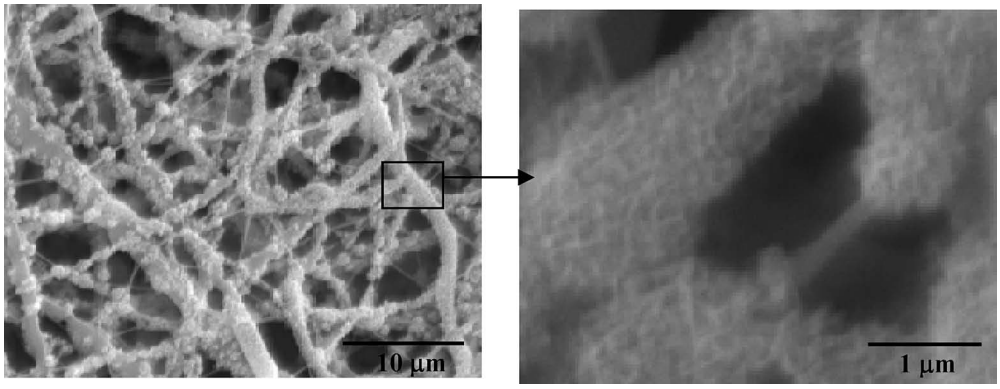

Fig. 1. SEM photograph of hydroxyapatite-coated PCL nanofiber.

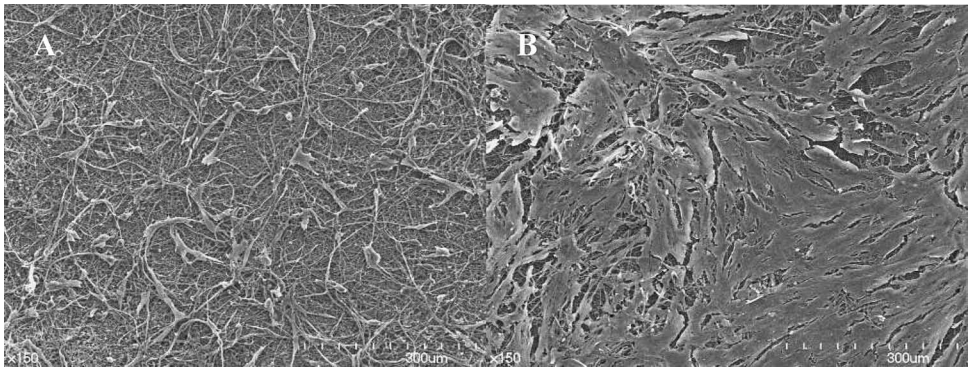

Fig. 2. PDL fibroblast attached to hydroxyapatite-coated PCL nanofibrous membrane on day 1 (A) and day 7 (B). (150×).

order to improve the initial PDL fibroblast responses and consequent bone tissue formation. Our current study has unique implications, since there has been little work on the biological responses of periodontal ligament cells to the nanofibrous substrate with bioactive material.

Figure 1 shows the surface morphology of the hydroxyapatite-coated PCL nanofibrous membrane, obtained by the mineralization method. A nanofibrous structure of PCL, as obtained by the electrospinning technique, was successfully preserved in the mineralized membrane with hydroxyapatite coverings. Higher magnification shows the nanofibrous mesh was covered with nanocrystallines, which were proven to be an apatite phase.[9] The weight of the nanofibrous membrane after the hydroxyapatite mineralization process was observed to increase approximately 30% of the initial PCL membrane due to the covering of hydroxyapatite phase. In practice, PCL, as one of the synthetic biopolymers, is known to have poor initial cell affinity due to the hydrophobic property and being lack of cell-adhesive ligand.[10] Because cells can recognize only the surface of a biomaterial, there have been paramount efforts to modify the surface of synthetic polymers including PCL.[11),12] Herein, we developed nanofibrous membrane in which hydroxyapatite layer covering the surface of the PCL synthetic polymer in order to generate beneficial role in the regulation of PDL fibroblast responses.

Figure 2 shows human PDL fibroblast attachment on the hydroxyapatite-coated PCL nanofibrous membrane. On day 1, the PDL fibroblasts were round-to-spindle–shaped with elongated cytoplasmic extensions which proved to be well attached to the membrane (Fig. 2A). This appearance was not normally observed in typical PCL surface. The cell population on day 7 was significantly increased to present more prominent cell spreading and aggregating appearance on the surface of hydroxyapatite-coated nanofibrous PCL membrane (Fig. 2B). It was previously reported that PDL fibroblasts synthesize osteonectin, a molecule described as a major bone matrix glycoprotein, having the ability to bind to hydroxyapatite. Therefore it was clear that hydroxyapatite may stimulate the PDL fibroblast attachment and spread.[13]

The cell proliferation data assessed by an MTS assay was summarized in **Fig. 3**. The human PDL fibroblast showed an on-going increase in the PDL fibroblast proliferation during the time course of 14 d. Control group (polystyrene cell culture plate surface) revealed the highest optical density value, which were 2.155 ± 0.394 on day 1, 3.801 ± 0.100 on day 4, 4.327 ± 0.222 on day 7 and 4.773 ± 0.061 on day 14. Hydroxyapatite-coated PCL nanofiber showed optical density value of 2.129 ± 0.033, 4.491 ± 0.059, 3.987 ± 0.151, 3.030 ± 0.252 respectively, which were slightly lower values without any significant differences compared to those of control group at the designated time point ($p > 0.05$). This finding coincides with other previous report in which cultured osteoblast-like cells on differently cross-linked collagen membranes showed the highest value of cell number on the polystyrene culture dish surface.[14] The surface of poly-

styrene culture dish has been shown to produce reliable cellular attachment and rapid cellular confluence. Hence the hydroxyapatite-PCL nanofibrous membrane could be considered to display prominent PDL fibroblast adhesion performance.

The ALP activity of the hydroxyapatite–coated PCL nanofiber on day 14 was significantly increased when compared with that of day 7. In **Fig. 4**, the incremental cell differentiation was presented by the ratio of the ALP activity on day 14 with respect to that of day 7. Data showed the incremental ALP activity ratio of hydroxyapatite-coated PCL nanofiber was nearly 2.5 times higher (2.611) than that of the control group (1.080). This result also suggests that the hydroxyapatite coating can stimulate PDL fibroblast differentiation. The findings obtained from present experiment correspond with our previous report in which the gelatin-hydroxyapatite nanocomposites improved the functional activity of the bone-derived cells and that the hydroxyapatite-containing composites had significantly higher ALP levels on day 14.[15)]

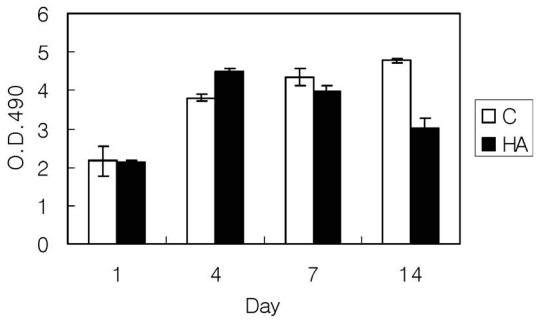

Fig. 3. MTS assay of hydroxyapatite-coated PCL nanofiber on human PDL fibroblast proliferation (O.D. 490 = optical density at 490 nm, C = Control, HA = hydroxyapatite-coated PCL nanofiber).

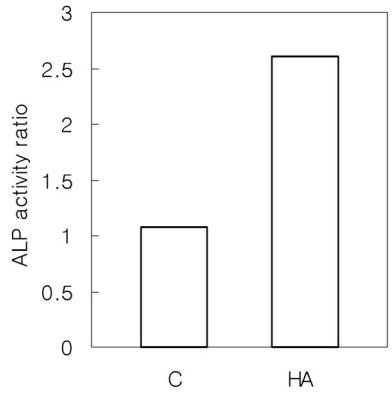

Fig. 4. Incremental ALP activity ratio of hydroxyapatite-coated PCL nanofiber. (C = Control, HA = hydroxyapatite-coated PCL nanofiber).

Differentiation of human PDL fibroblast into osteoblastic cell was crucially monitored by histochemical staining of mineralization foci as presented in **Fig. 5**. Significant amount of calcified tissue deposition occurred on the surface of hydroxyapatite-coated PCL nanofiber on day 14 (Fig. 5A). No mineral formation was observed in control group (Fig. 5B). From these data, we can deduce that the nanofibrous hydroxyapatite–coated PCL membrane had a stimulatory effect on the recruitment of human PDL fibroblast to elicit osteogenic potential.

Consequently, a new type of a biomaterial with a nanofibrous structure, developed by means of an electrospinning technique with an additional hydroxyapatite surface coating process, was shown to induce favorable PDL fibroblast modulation in the initial cell adhesion and the subsequent bone differentiation for periodontal tissue regeneration. Further studies including the long-term biological tests and *in vivo* animal work are needed to identify clinical efficacy.

4. Conclusions

In this study, we investigated the biological effect of a novel biomaterial made of hydroxyapatite-coated PCL nanofibrous membrane on the human PDL fibroblast proliferation and differentiation. Hydroxyapatite-coated PCL nanofibrous membrane promoted human PDL fibroblast attachment at day 1 and increased cell proliferation during 14 days. Regarding cell differentiation, incremental ALP activity of the hydroxyapatite-coated PCL nanofiber was 2.5 times higher than that of control group. Moreover, hydroxyapatite-coated PCL nanofibrous membrane showed significant mineral formation on day 14, whilst no mineral foci were observed in the control group. Within the limitation of this study, we can conclude that hydroxyapatite-coated polycaprolactone nanofibrous membrane has favorable

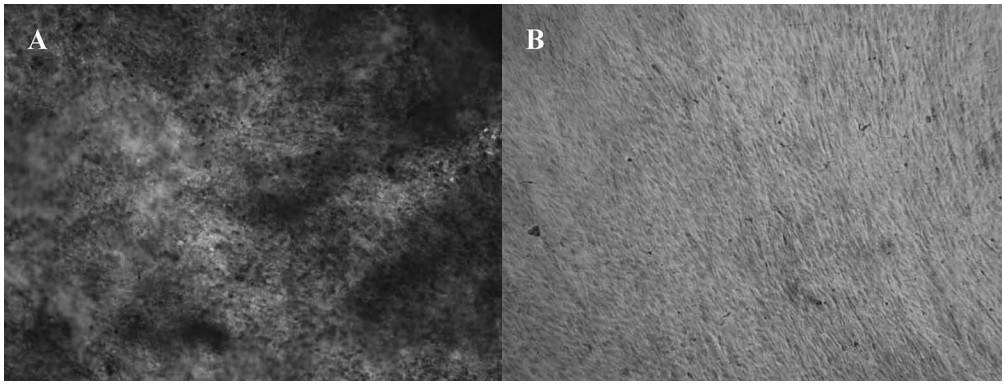

Fig. 5. Mineralization assay of hydroxyapatite-coated PCL nanofiber (A) and control (B) on day 14 (100×).

effects on the proliferation and differentiation of human periodontal ligament fibroblast and might be a good candidate material for periodontal tissue regeneration.

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