INTRODUCTION

Various regenerative therapies, such as guided tissue regeneration and the use of enamel matrix derivative, are routinely utilized in clinical practice. However, the outcomes have been limited and associated with poor clinical predictability1,2). Recently, stem cell-based approaches for periodontal regeneration have been conducted to address these challenges3). Previous studies have demonstrated the application of cytotherapy using mesenchymal stromal cells (MSCs) to differentiate into periodontal ligament4,5) and the transplantation of MSCs to support periodontal regeneration4-6).

Cell sheet technology is a scaffold-free method for tissue reconstruction. A sheet-shaped scaffold would be suitable for the regeneration of periodontal membrane. We designed a stem cell sheet combining human mesenchymal stromal cells (hMSCs) and a 10-µm thick biological apatite (BAp) membrane fabricated with an ArF pulsed laser ablation for periodontal regeneration. X-ray diffraction showed that crystalline hydroxyapatite (HAp) was present in BAp and HAp membranes after post-annealing. Energy dispersive analysis of the BAp membrane revealed peaks of Na and Mg in addition to Ca and P. Approximately 3×10^4 hMSCs were cultured on BAp and HAP membranes for 7 and 14 days. From in vitro assays, hMSCs grew faster and had higher osteoblast differentiation when cultured on the BAp membrane than did the cell culture on the HAP membrane. Stem cell sheets combined with a BAp membrane may have potential applications in guided bone regeneration and osteoconductive scaffolds.

Keywords: Stem cell sheet, Human mesenchymal stem cell, Hydroxyapatite, Biological apatite membrane, Pulsed laser ablation

MATERIALS AND METHODS

Fabrication of BAp and HAp membranes

Figure 1 is a diagram of the PLD apparatus. The BAp and HAp bulk targets (commercially available from Eccera Corporation, Tokyo, Japan and Seikagaku Corporation, Tokyo, Japan, respectively) were placed in a vacuum chamber with background pressure of approximately 8×10^-7 Torr. Molecules, atoms, and ions consisting of the target surface were desorbed in the vacuum chamber by irradiation with an ArF excimer laser beam, phenomenon called laser ablation. The laser fluence was about 1 J/cm^2, and the pulse repetition rate was 10 Hz. The deposition rate was about 10 nm/min with atmospheric gas (O₂+H₂O, partial pressure of approximately 0.8 mTorr) and a 10-µm thick membrane prepared on the NaCl substrate. The membrane thickness was estimated based on the deposition rate.
Fig. 1 Diagram of the PLD apparatus.

Fig. 2 Diagram of the separation process of the BAp and HAp membranes from the NaCl substrate.

Figure 2 is a diagram of the separation process of BAp and HAp membranes from the NaCl substrate. The BAp- and HAp-deposited NaCl substrate was placed on a strainer over a Petri dish with the BAp- and HAp-coated surface facing downward. Then, the Petri dish was filled with purified water to completely immerse the NaCl crystal. After NaCl crystals were completely dissolved, the purified water was removed, and the BAp and HAp film remaining on the strainer was dried in air. By immersing the BAp and HAp membrane several times in purified water, NaCl was completely removed from the surface. Then, the BAp and HAp membrane were crystallized by a post-annealing process for 10 h in an electric furnace at 400°C in an O₂+H₂O gas atmosphere.

Characterization of BAp and HAp membranes
The phase composition of crystallized BAp and HAp membranes was analyzed using X-ray diffraction (XRD; Ultima IV; Rigaku Corp., Tokyo, Japan). Surface morphology, composition, and roughness of the membranes were analyzed by scanning electron microscopy coupled with an energy-dispersive spectrometer (EDS; KRA8800; Ametek Corp., Tokyo, Japan).

Seeding of cells on the membrane-type scaffolds
hMSCs were obtained from RIKEN Cell Bank (Tsukuba, Japan) and maintained by continuous culture at 37°C in a humidified atmosphere containing 5% CO₂. The hMSCs were expanded for 7–10 days in DMEM supplemented with 10% heat-inactivated FBS, 3 ng/mL fibroblast growth factor-2, and 1% antibiotic/antimycotic in 75-mm² flasks. After a sufficient number of hMSCs was obtained, they were seeded at a density of 3×10⁴ cells onto BAp and HAp membranes placed in the wells of 24-well tissue culture plates. The cells were incubated for 3 days in a CO₂ incubator at 37°C. Subsequently, the medium was removed from the wells, and the cells were cultured in an osteogenic differentiation medium (10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 µg/mL ascorbic acid) for 7 and 14 days.

DNA content analysis
DNA content was measured on days 7 and 14. The medium was then removed, and the cells were washed twice with PBS. Afterwards, 300 µL of 0.2% Triton X-100 was added to each well, and the cells were removed by a cell scraper (Becton–Dickinson, Franklin Lakes, NJ, USA) for DNA extraction. DNA content was measured by a Quant-iT™ PicoGreen® dsDNA Reagent and Kit (Invitrogen, Carlsbad, CA, USA). Fifty microliters of sample was mixed with a DNA-binding fluorescent dye solution (0.5 µL PicoGreen reagent in 100 µL 1×TE buffer), and the fluorescent intensity was measured by a microplate reader (Ex 450 nm/Em 510 nm; SpectraMax® M5; Molecular Devices, Sunnyvale, CA, USA).

Measurement of alkaline phosphatase activity
Alkaline phosphatase (ALP) activity was measured on days 7 and 14 using LabAssay™ ALP (Wako Pure Chemical Industries, Osaka, Japan). One-hundred microliters of a working assay solution (6.7 mM/L p-nitrophenylphosphate disodium) was added to 20 µL of the same sample used for DNA measurement, mixed thoroughly, and incubated at 37°C with 5% CO₂ for 15 min. After 80 µL of stop solution (0.2 M/L sodium hydroxide) was added to the mixture, the absorbance was measured at 405 nm with the SpectraMax® M5. The levels of ALP activity were normalized to the amount of total DNA in the cell lysates.

Quantitative real-time RT-PCR
Total RNA was isolated using a Mag Extractor (Toyobo, Osaka, Japan) according to the manufacturer's protocol, and single-stranded cDNA was synthesized using a High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA). Runx2 mRNA levels were analyzed by quantitative real-time PCR using a
TaqMan® Gene Expression Assay (Hs00231692 mL; Applied Biosystems) on a Step One Plus PCR system (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was co-amplified as an internal standard (Human GAPDH endogenous control; Applied Biosystems). Gene expression was measured using the ddCt method.\(^1\)

Osteocalcin measurement
The hMSCs were cultured under the same conditions described for the DNA content analysis, and the expression of osteocalcin (OCN) in both cell types was determined by a Gla-Type Osteocalcin EIA Kit (Takara Bio Inc., Shiga, Japan). On days 7 and 14 of culture, the medium was removed, cells were washed with PBS, and 300 µL of 10% formic acid was added to each well followed by the removal of cells using a cell scraper. Samples (100 µL) were added to each well of an anti-OCN antibody-coated microtiter plate and incubated at room temperature for 2 h. After three washes with PBS, 100 µL of a peroxidase-conjugated anti-OCN antibody solution was added to each well followed by incubation at room temperature for 1 h. After four washes with PBS, 100 µL of substrate solution (tetramethylbenzidine) was added to each well followed by incubation at room temperature for 15 min. After 100 µL of stop solution was added to each well, the absorbance was measured at 450 nm with the SpectraMax® M5.

Statistical analysis
All experiments were conducted in quintuplicate and repeated at least twice. All data are expressed as the mean and standard deviation. Differences were evaluated by Student’s \(t\)-test. Differences were considered significant at \(p<0.05\).

RESULTS
Images of the BAp and HAp membranes are presented in Fig. 3. The color of the square membranes was milky white. The XRD pattern of BAp and HAp membranes exactly matches the JCPDS pattern, with the same orientation of the HAp target, and indicates the phase pure composition of the coating, without any additional calcium phosphate phases within the detection level (Fig. 4). The very large broadening of the 211, 112, and 300 reflections at 32–34° and the relatively high intensity and sharpness of the 002 reflection at 26° were shown in Fig. 4. EDS showed peaks of Na and Mg apart from those of Ca and P on the BAp membrane (Fig. 5). The peaks of Ca and P were lower than those obtained on the BAp membrane, while those of C and O were higher.

The osteoblast differentiation ability of hMSCs cultured on BAp and HAp membranes was compared by using an osteoblastic differentiation marker. As an indication of cell proliferation, the DNA content of hMSCs cultured on the BAp membrane was significantly higher than that of hMSCs cultured on the HAp...
membrane on days 7 ($p<0.05$). However, no significant differences between the two membranes were observed on days 14 (Fig. 6 (a)). As a marker of early stage osteoblastic differentiation\textsuperscript{13}, the ALP activity of hMSCs cultured on the BAp membrane was slightly higher than that of hMSCs cultured on the HAp membrane on days 7 and 14. Nevertheless, no significant differences between the two membranes were observed ($p>0.05$; Fig. 6 (b)). Runx2 expression of hMSCs cultured on BAp membrane was significantly higher than that of hMSCs cultured on the HAp membrane on days 7 ($p<0.05$), but no significant difference between the two membranes was observed on days 14 (c). OCN expression of hMSCs cultured on BAp and HAp membranes was not observed on days 7. However, hMSCs cultured on BAp had higher OCN expressions than that of hMSCs cultured on HAp by days 14 (d).
DISCUSSION

We have described the construction of a freestanding BAp membrane that can be easily separated from a substrate of NaCl by its immersion in water. This BAp membrane provides rapid cell differentiation and is flexible and transparent. MSCs are an attractive cell source for tissue engineering approaches because they are readily available and practical. In this study, we combined MSCs with a BAp membrane and evaluated the osteoblast differentiation in vitro for periodontal regeneration.

We created thin membranes of BAp by using a PLD technique with an ArF excimer laser. Numerous deposition techniques exist for BAp membranes, such as plasma spray\(^{19}\) and sputter\(^{10}\). The advantage of the PLD technique is that the composition of the membrane and the target is similar, i.e., the source of prepared membrane. Since the chemical composition of the HAp membrane, particularly the ratio of Ca and P, is essential for biocompatibility, the PLD technique is suitable for maintaining the membrane's chemical composition\(^{17}\). Furthermore, artificial chemical doping can easily be examined by the PLD technique. These features are unique to the PLD technique. In this study, freestanding BAp membranes were prepared by the deposition of BAp membrane onto water-soluble NaCl crystals.

The BAp membrane is crystallized, as well as HAp membrane in the XRD pattern, as shown in Fig. 4. Thermal heating of the substrate is generally needed for the crystallization of apatite. However, the HAp membrane cracked on NaCl crystals when the NaCl substrate was heated\(^{10}\). The reasons for this are unknown, but one possible explanation is due to the difference in expansion coefficient between NaCl crystals and HAp\(^{10}\). Yet using this method, the prepared BAp membrane was amorphous. Thus, the BAp membrane was crystallized by a post-annealing process in the electric furnace at 400°C in an O\(_2\)+H\(_2\)O gas atmosphere.

In this study, cell proliferation was measured based on the DNA content of cultured hMSCs. We found that hMSCs proliferate more rapidly on BAp than on HAp. In general, HAp enhances cell attachment and proliferation\(^{18}\) due to the adsorption of high levels of fibronectin\(^{19,20}\). Therefore, the differences in cell proliferation on HAp and BAp membranes may be due to differential absorption of serum proteins. Our previous study\(^{21}\) showed that the protein adsorption on the BAp membrane was higher than that of the HAp membrane using QCM methods. Specifically, a higher adsorption of serum fibronectin may be responsible for the higher rate of hMSCs proliferation on the BAp membrane.

The hMSCs are multipotent and have been differentiated into several kinds of mesodermal tissues, including bone, fat, and cartilage in vitro. Thus, hMSCs may be useful for studying the effects of bone replacement biomaterials. These cells are known to synthesize ALP. Compared to cells cultured on the HAp membrane, cells cultured on the BAp membrane for 7 or 14 days produced higher levels of ALP. However, no significant differences were observed between two membranes. This difference in ALP production may become greater with extended culture periods during extracellular matrix mineralization. Real-time RT-PCR analysis showed that hMSCs cultured expressed the osteogenic marker runx2, which is known to be an early stage marker of osteogenesis. OCN is recognized as a marker of late stage osteoblastic differentiation\(^{19}\). In osteoblastic cells, transcription of the bone-specific OCN gene is principally regulated by the runx2 transcription factor\(^{22}\). Runx2 expression of cells cultured on the BAp membrane was higher than that of cells on the HAp membrane at 7 days. As a result, OCN protein expression of cells cultured on a BAp membrane was higher than that of the HAp membrane at 14 days.

Our results also suggest that the BAp membrane was effective at promoting osteoblast differentiation of hMSCs. This may be due to the release of ions that mimic magnesium ions. Smith et al.\(^{23}\) demonstrated that a magnesium deficiency significantly and progressively diminishes bone formation, leading to osteoporosis. In addition, spectroscopic analyses indicate that in the BAp used for the current studies, both OH\(^-\) and PO\(_4^{3-}\) sites are substituted by carbonate ions\(^{24}\). Although the levels of carbonate ions in bone mineral are small, they may play a significant role in the biochemistry of hard tissues\(^{25}\). Consequently, a number of studies have focused on the production of synthetic carbonate-substituted HAp ceramics for bone replacement\(^{25,26}\).

CONCLUSIONS

hMSCs are a promising cell source for tissue engineering. When hMSCs are used for bone tissue engineering, undifferentiated hMSCs can be induced to be osteogenic prior to transplantation. In recent times, cell sheet engineering for the repair and regeneration of periodontal tissues has gained popularity over the use of hMSC transplantation\(^{27,28}\). Here, stem cell sheets, in the form of a BAp membrane, induced in vitro osteogenic differentiation of hMSCs into osteoblast-like cells. Our previous study\(^{29}\) on materials implanted in canine mandibles showed that a BAp membrane coated onto the implant dissolved and activated bone remodeling immediately after implantation. The 10-µm thick BAp membrane fabricated in this study may have followed the same mechanism. These fabricated stem cell sheets may have potential applications for guided
bone regeneration and for the generation of bioactive and osteoconductive scaffolds. However, animal experiments and clinical studies are still needed to validate the material’s performance efficiency.

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