In Vitro and In Vivo Evaluations of Bioactive Poly(ε-caprolactone)–Organosiloxane Nano-Hybrid Developed as a Novel Bone Substitute

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ABSTRACT

We evaluated the cellular behaviors of human bone marrow stromal cells (hBMSCs) on the surface of apatite pre-coated poly(ε-caprolactone)–organosiloxane nano-hybrid material (SiOPCL) and assessed the osteoconductivity of SiOPCL in the diaphyses of the rabbit tibiae. The attachment and proliferation of hBMSCs on both polycaprolactone (PCL) and apatite pre-coated SiOPCL (SiOPCL/HAp) was comparable to those observed on tissue culture plates (TCPs). The alkaline phosphatase activity of hBMSCs on both PCL and SiOPCL/HAp was significantly higher compared with those on the TCPs. There was newly formed bone, together with numerous osteoblasts, along the entire surface of the SiOPCL with in vivo testing. However, we observed severe inflammatory reactions and foreign body responses without evidence of new bone formation after the implantation of PCL. These results demonstrated that SiOPCL had compatible hBMSCs responses and excellent osteoconductivity in vivo.

Keywords: Polycaprolactone; Siloxane; Osteoconduction; Bioactivity; Bone substitute

1 INTRODUCTION

Recently, some nano-composites, which are composed of polymers and ceramics, have been developed through a sol-gel method as a new bone substitute [1-4]. However, the drawback of this bioactive and degradable organic/inorganic nano-composite is that it has the potential to leave behind residual products, such as silica particles, after the preferential degradation of the polymer phase in vivo, because there is no heat-treatment stage in the manufacturing process. This problem is a shortcoming, because it can induce inflammation in vivo.

In order to resolve the problem of the rapid release of silicon from the nano-composite system, we developed a novel poly(ε-caprolactone)–organosiloxane nano-hybrid material (SiOPCL), which was composed of poly(ε-caprolactone) (PCL) segments that were connected to each other with siloxane linkage segments that contained a silanol group and calcium [3, 5]. It provided an advantage in not releasing silicon ion at all, even after the preferential degradation of the PCL segment in vivo, while it also had an apatite-forming ability and mechanical properties that were comparable to those of human cancellous bone.

All bioactive materials form an apatite layer on their surfaces in the living body and they bond to living bone through this apatite layer [6]. Thus, cellular responses to the bioactive materials that are pre-coated with an apatite layer on their surfaces after the biomimetic process in vitro, may be more similar to the events that occur in vivo [5,7,8]. Especially, precoating the apatite layer before in vitro testing is desirable for bioactive materials, such as SiOPCL, that release calcium quickly and in large amount.

In this study, we evaluated the cellular behaviors of human bone marrow stromal cells (hBMSCs) on the surface of apatite pre-coated SiOPCL (SiOPCL/HAp) and assessed the osteoconductivity of SiOPCL in the diaphyses of the rabbit tibiae. PCL, which is backbone of its organic phase, was also evaluated as a control to confirm the advantages of a structural modification of SiOPCL.

2 MATERIALS AND METHODS

2.1 Preparation of Specimens

The SiOPCL was prepared by the sol-gel method we reported in a previous study [3]. Briefly, the polymer precursor, triethoxysilane end-capped PCL (Si-PCL) was prepared through a reaction with α,ω-hydroxyl PCL and 3-isocyanatopropyl triethoxysilane (IPTS) with 1,4-diazabicyclo [2,2,2] octane (DABCO) as a catalyst and dry toluene as a solvent. The molar ratios of the reactants were PCL 1, IPTS 3, and DABCO 2. The reaction was performed at 70 °C for 24 hours with constant stirring under dry Ar atmosphere. The synthesized Si-PCL was purified through repeated precipitation in cold methanol 3 times and then dried under a vacuum at room temperature for 24 hours. Its average molecular weight, which was measured by a gel permeation chromatography system was approximately 13,000 Da.

The Si-PCL was dissolved in ethanol with 10 wt % calcium nitrate tetrahydrate to the Si-PCL in dry Ar atmosphere at 70 °C. The HCl and water were added into the Si-PCL solution and reacted for 10 minutes. The molar ratios of the reactants were Si-PCL, 1; HCl, 0.01; and water, 6. The hydrolysis that occurred following the condensation reaction (gelation) continued for 1 week at 40 °C in a Teflon® mold that was covered with a Parafilm® with a few pinholes. After the gelation, the specimen was heat-treated.
at 60 °C for 2 days. Hereafter, this prepared specimen will be referred to as SiOPCL.

In order to prepare the SiOPCL that was pre-coated with a low crystalline hydroxy carbonate apatite layer (SiOPCL/HAp), the disk-shaped SiOPCL (14 mm in diameter and 2 mm in thickness) was polished with a #400 abrasive and incubated in a simulated body fluid (SBF) for 2 weeks at 36.5° C. For the in vivo testing, a cylindrical-shaped SiOPCL specimen (3.4 mm in diameter and 10 mm in height) was prepared. In addition, a pure PCL specimen (Mn 42,500, Aldrich Chem. Co. Inc. WI, U.S.A.) was prepared for use as a control by a conventional solvent casting method. All of the specimens were cleaned with distilled water, sterilized with 70% ethanol for 15 minutes, and then washed with phosphate-buffered saline (PBS) three times before they were used in the experiments.

2.2 Isolation and Culture of Human Bone Marrow Stromal Cells

This study was approved by our Institutional Review Board. We obtained fresh bone marrow samples from the iliac crests of patients during total hip arthroplasties, after receiving informed consents. The isolation and culture techniques for hBMSCs were derived from those described by Pittenger et al [9].

For the osteogenic differentiation, the hBMSCs were cultured in an osteogenic medium that consisted of DMEM-High Glucose (DMEM-HG, GibcoBRL®) that was supplemented with 0.1 μM of dexamethasone (Sigma Chemical Co. St. Louis, MO, U.S.A.), 10 mM of sodium β-glycerophosphate (Sigma Chemical Co.), 50 μg/ml of ascorbic acid-2-phosphate (Sigma Chemical Co.), and 10% FBS.

2.3 Cell Attachment and Proliferation

For the attachment assay, hBMSCs were seeded onto the PCL and SiOPCL/HAp disks at a density of 1.0×10⁶ cells/specimen and cultured for 3 hours at 37° C in a 5% CO₂ atmosphere. The number of attached cells was determined with a cell counting kit-8 (CCK-8, Dojindo, Tokyo, Japan).

For the proliferation assay, the hBMSCs were seeded onto the PCL and SiOPCL/HAp disks at a density of 3.0 × 10⁶ cells/specimen and cultured in osteogenic media. On days 1, 7, and 14, the proliferated cell numbers were determined with CCK-8. Tissue culture plates (TCPs) was used as control materials for the cell culture assay.

2.4 Alkaline Phosphatase Specific Activity

In order to evaluate the osteogenic differentiation of the hBMSCs that were cultured on the PCL and SiOPCL/HAp disks, alkaline phosphatase (ALP) activity was measured with an alkaline phosphatase diagnostic kit (#85, Sigma Chemical Co.) at days 14 and 21.

2.5 In Vivo Testing

Twelve 6-month old male New Zealand white rabbits that weighed approximately 3.0 Kg each, were used in this study upon proper approval of ethics committees and legal authorities of our hospital. The cylindrical-shaped specimens (3.4 mm in diameter, 10 mm in height) were sterilized with ethylene oxide gas.

Bone defects that were made in both tibias were randomly assigned and used for the two groups (n of the tibiae = 12 in each group). The bone defects were implanted with PCL and SiOPCL. A hole was made transversely through the diaphysis 2 cm apart from a tibial tuberosity using a 3.2 mm drill bit. After the gauze packing for 10 minutes to control the bleeding and washing with a sterile physiological saline solution, a cylindrical-shaped specimen was inserted into the hole transcortically and press-fitted in both cortical bones. Four rabbits (4 tibiae in each group) were sacrificed for optical microscopic examinations at 2, 4, and 8 weeks after the implantations.

2.6 Histologic Study

The bone specimens were dehydrated, embedded, and sectioned with a thickness of 30 um. The final thin sections were stained with Goldner’s modified Masson trichrome, and examined under an optical microscope.

2.7 Statistical Analysis

All data were reported as the mean ± standard deviation (SD) and were compared among three groups (TCPs, PCL, and SiOPCL/HAp) using nonparametric Kruskal-Wallis tests at the corresponding times. A parametric two-way ANOVA was performed to analyze the interaction between group and time, and a multiple comparison analysis was also conducted within three groups. P values < 0.05 were considered significant.

3 RESULTS

3.1 In Vitro Testing

Although the attached cell number was higher on the PCL than that on the SiOPCL/HAp, there was no statistically significant difference. The attachment of the hBMSCs on both the PCL and the SiOPCL/HAp was comparable to that on the TCPs (figure 1).

The hBMSCs cultured on the TCPs, PCL, and SiOPCL/HAp demonstrated increased cell numbers over the time during the osteogenic differentiation. The proliferation activities of the hBMSCs on both the PCL and the SiOPCL/HAp were also comparable to those on the TCPs and there were no statistically significant differences.

The alkaline phosphatase (ALP) activities of the hBMSCs during osteogenic differentiation were greater on
both the PCL and the SiOPCL/HAp than were those on the TCPs on day 14 but those differences were not significant statistically. There were significant differences on day 21. The ALP activity was decreased on the TCPs, whereas those activities did not decrease on the PCL and the SiOPCL/HAp. The differences in the PCL and the SiOPCL/HAp compared with the TCPs were statistically significant (p<0.05). There was no significant difference between the PCL and the SiOPCL/HAp on day 21 (figure 2).

3.2 In Vivo Testing

As expected, the SiOPCL specimens appeared to conduct new bone formation in the medullary canals of the tibiae, which were normally filled with fatty marrow. There were no signs of adverse effects, such as inflammation or foreign body reactions. There was osteoconduction along the entire surface of the SiOPCL with no histologically demonstrable intervening non-osseous tissue. Under high magnification, we clearly identified that the newly formed bone was lined with numerous cuboidal-shaped osteoblasts. There was close contact of the bone with the surface of the SiOPCL specimens.

However, the pure PCL did not conduct bone formation in the medullary canal of the rabbit tibiae. The histological findings at 8 weeks after the implantations demonstrated more severe inflammatory reactions and foreign body responses. Numerous inflammatory cells infiltrated, the vessels were dilated, and multinucleated giant cells began to appear around the PCL specimens. In particular, large cavities that developed from the degradation of the PCL appeared. These cavities were lined with multinucleated giant cells and filled with inflammatory fibrovascular tissue.

4 DISCUSSION

SiOPCL, which is a novel poly(ε-caprolactone)–organosiloxane nano-hybrid material, possesses distinguishing structural characteristics, which were reported previously [3]. In SiOPCL, the triethoxysilane end-capped polycaprolactones are connected directly to each other without a silica phase and new siloxane linkages are formed by a condensation reaction among the silanol groups, which originate from the silane coupling agent. In addition, SiOPCL has both silanol groups (which do not participate in the condensation reaction) and soluble calcium salt, which act as the nucleation sites and as an accelerator for the formation of the apatite crystals, respectively.

Recently, a polycaprolactone/hydroxyapatite (HA) composite material, in which the HA particles were simply added to the PCL matrix by a phase inversion and casting technique, was reported as a scaffold for bone tissue engineering [10]. The degree of the dispersion of the HA particles inside the PCL matrix is a major factor that affects the mechanical and biological characteristics of this composite material and it has the potential to be adversely affected by a phase-separation phenomenon.

Compared to this composite material, clustering or uneven dispersion of the inorganic phase and phase-separation are no longer concerns in SiOPCL, because all of the osteoconducting elements are incorporated chemically and distributed uniformly throughout its microstructure. For these structural characteristics, SiOPCL can induce the even formation of apatite crystals on its surface and stereotypical bioactivity can be expected. Indeed, in the present study, there was newly formed bone, together with numerous osteoblasts, along the entire surface of the SiOPCL that was implanted in the medullary canals of the rabbit tibiae and there was no evidence of adverse responses, such as inflammation or foreign body reactions, around the SiOPCL. This means that the new PCL–organosiloxane hybrid material has excellent osteoconductivity, even without a silica phase and it demonstrated good biocompatibility in vivo.

To the best of our knowledge, this is the first report to demonstrate the bioactivity and biocompatibility of a novel organic/inorganic nano-composite in vivo after the comparison with those of its back-bone polymer. During in vivo testing, there was newly formed bone together with
numerous osteoblasts along the entire surface of the SiOPCL in the medullary canal of the diaphyses of the rabbit tibiae. Furthermore, there was no evidence of adverse responses, such as inflammation or foreign body reactions, around it. In addition, the biomimetically modified SiOPCL (SiOPCL that is pre-coated with apatite layer, SiOPCL/HAp) also demonstrated appropriate attachment, compatible proliferation, and an enhanced osteoblastic differentiation of the hBMSCs. These results implied that this new nano-hybrid material had a potential to be used as a bone substitute or a material for bone tissue engineering.

REFERENCES