The preparation of PLLA/calcium phosphate hybrid composite and its evaluation of biocompatibility

Yoshinosuke HAMADA^{1,2,5}, Wataru FUJITANI³, Naomasa KAWAGUCHI^{1,4}, Kiyoshi DAITO², Tsutsu NIIDO¹, Ayako UCHINAKA¹, Seiji MORI¹, Yoshihisa KOJIMA¹, Masashi MANABE⁵, Kiheiji NISHIDA⁵, Kenji ARITA², Takayoshi NAKANO³ and Nariaki MATSUURA¹

¹Department of Molecular Pathology, Osaka University Graduate School of Medicine and Health Science, Osaka 565-0871, Japan

²Department of Pediatric Dentistry, Osaka Dental University, Osaka 573-1121, Japan

³Department of Materials Science and Engineering, Graduate School of Engineering, Osaka University, Osaka 565-0871, Japan

⁴Department of Cardiovascular Pathology, Osaka University Graduate School of Medicine and Health Science, Osaka 565-0871, Japan

⁵Department of Medical Economics and Management, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

Corresponding author, Yoshinosuke HAMADA; E-mail: y-hamada@sahs.med.osaka-u.ac.jp

In tissue engineering, biodegradable polymer materials with both high biocompatibility and high strength are very important as scaffolds for long term use. Therefore, in this research, we tried to prepare the three types of poly(L-lactic acid) (PLLA)/calcium phosphate (CP) hybrid composite for a scaffold biomaterial. The effects of addition of different CP on both biocompatibility and mechanical properties were evaluated. CP powders and voids were three-dimensionally and uniformly distributed in the solid samples and porous composite samples. These compositions of CP and PLLA greatly improved the cellular adhesiveness, which increased as the volume fraction of CP in the composite increased. For the porous samples, cells migrated into the pores. This study demonstrated that a composite of PLLA and CP is an effective new scaffold material that results in better osteoconductivity, bone regeneration, and mineralization and has moderately high strength.

Keywords: PLLA/OCP composite, Biocompatibility, Tissue engineering, Bone regeneration

INTRODUCTION

Bone tissue consists mainly of inorganic hydroxyapatite (HAp), which principally gives bone strength, and organic collagen fibers, which contribute to flexibility. Bone tissue has a complex microscopic structure¹⁾. Bone replacement materials have been widely used to treat bone diseases and defects, and to quickly restore bone structure and functions. Materials to be implanted in a living body must have sufficient strength and be highly biochemically stable. Metallic materials such as Ti and stainless steel, which are widely used as structural materials, and ceramic materials are frequently used. Porous implant biomaterials have been used as scaffold materials because they are osteoinductive and osteoconductive as a result of their pores, and therefore they attach strongly to the surrounding tissues¹⁾. In addition, because the apparent Young's modulus of a porous metallic implant can be reduced, decreases in bone mass, such as bone volume and bone mineral density changes, caused by stress shielding effects resulting from differences between the Young's modulus of bone and the metallic implant, can be mitigated.

When porous materials are implanted as scaffold materials in living bodies, biocompatibility is an extremely important factor. Cells must attach, proliferate, and be grafted onto the surfaces of implant materials and scaffold materials, and undesirable

tissues, including fibrous membranes, should not form at the interfaces between the surfaces of materials and newly formed tissues. The pore size of the porous material is an extremely important parameter. To accommodate invasion by the cells responsible for bone metabolism and bone tissue formation, a pore diameter larger than 100 μm is required^{2,3)}. However, when Ti, stainless steel, and ceramic materials are used as implants, they are not biodegraded and remain unchanged, that is, they cannot be transformed into living bone.

A biodegradable polymer, poly(L-lactic acid) (PLLA), has been used as a fracture fixation material because of its comparatively good strength and biocompatibility. With regard to the use of artificial bone, some researchers have tried to synthesize a composite of HAp, one of the main components of bone, and biodegradable PLLA⁴⁻⁸⁾. A new ideal pattern of bone tissue regeneration is expected in which, when this hybrid material is implanted, PLLA will be degraded and resorbed with time, and will eventually be replaced by new bone. A porous composite of a biodegradable polymer and vaterite, which is a form of calcium carbonate that is more water soluble than HAp, is under development⁹⁾. Such biodegradable polymer composites of PLLA and HAp or other bone-analog apatites not only supply Ca for bone formation, and control inhomogeneous hydrolysis of PLLA, but may also allow long-term maintenance of PLLA strength as a result of composite reinforcement. Furthermore, porous PLLA is considered to strongly influence new bone formation by degradation and absorption of PLLA.

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In other words, it is expected that porous PLLA would promote new bone formation and osteoinduction as a result of invasion of pores by cells responsible for bone metabolism, and would also reduce the occurrence of a specific fracture phenomenon caused by the differences between degradation behavior of the surface and the interior, which is a problem that frequently arises with biodegradable polymers. The PLLA porous material with CP powders on its surface is therefore expected to show strength similar to bone and better biocompatibility than solo PLLA.

Although addition of a minute amount of HAp or α -tricalcium phosphate (α -TCP) to PLLA in order to maintain long-term strength and to improve biocompatibility has been tried, making a composite with a precursor of HAp, namely Ca-deficient HAp (CDHAp) or octacalcium phosphate (OCP), seems to be more effective, considering solubility, osteoconductivity, bone regeneration ability, and calcification ability of these materials in a living body.

In this study, three types of hybrid material were produced: PLLA particles coated with pure HAp; PLLA particles coated with Mn-HAp¹⁰⁾ (HAp containing a minute amount of Mn), in which the cellular adhesiveness and growth potential are promoted by Mn addition; and PLLA particles coated with fine particles of calcium phosphate (CP) such as OCP, which is a precursor of HAp. The biocompatibility and potential for use as scaffold biomaterials of the three materials were then evaluated.

MATERIALS AND METHODS

Preparation and morphological observation of PLLA particles and CP powder

To fabricate the compound sample, PLLA particles and three types of CP fine powders, pure hydroxyapatite (pure HAp), Mn added HAp (Mn-HAp) and octacalcium phosphate (OCP) were selected as raw materials. PLLA particles were fabricated by the gas atomizing method. Pure hydroxyapatite (pure HAp) and Mn added HAp (Mn-HAp) were synthesized by the wet process^{10,11)}. Pure HAp was synthesized by dropping 100 mM calcium acetate and 60 mM ammonium dihydrogen phosphate at the same rate into stirred 1.3 M ammonium acetate at 80°C. Aqueous ammonia was also dropped into the system to maintain the pH of the solution at 7.4±0.1. Mn-HAp was also synthesized by the same method after 100 mM manganese acetate was mixed into calcium acetate because Mn added HAp powder (Mn-HAp) which has improved biocompatibility by the addition of small amount of Mn¹⁰⁾. The obtained pure-HAp and Mn-HAp powders were washed and collected followed by drying. OCP fine powder used in this research was purchased from Taihei-kagaku Co. Ltd. and used as-received.

Morphology of PLLA particles and CP powder was observed with a light microscope (Olympus BX-60, Tokyo, Japan) and a scanning electron microscope (SEM; JEOL Ltd. JSM-840A, Tokyo, Japan) after performing golden vapor deposition. The crystal structures of CP samples

were determined by X-ray diffraction (XRD; Rigaku Corporation X-ray diffractometer XRD-30, Tokyo, Japan) and Fourier transform infrared spectroscopy (FTIR; Shimadzu FTIR-8700, Tokyo, Japan). After being mixed with potassium bromide (KBr) powder, FTIR spectra for the samples was taken in the range of 500–4000cm⁻¹. The chemical composition of the samples was analyzed by atomic absorption spectrophotometry (Shimadzu AA-6800, Japan). The size and size distribution of PLLA particles were calculated from arithmetic average section length by the linear intercepting method based on the SEM image.

Preparation of a PLLA/calcium phosphate compound samples and evaluation of the mechanical properties

To prepare the PLLA/calcium phosphate composite samples, PLLA particles were mixed with 0, 1, 3 and 5 volume% CP fine powder. Mixed powder was compacted into the carbon dies with a diameter of 6 mm. Porous and solid composite were obtained by sintering the green compact at 171°C with and without pressure, respectively. Samples were cut to evaluate the mechanical properties by compression test (diameter: 6 mm, height: 10 mm) ($n=3$), to perform cell culture and to observe the microstructure. The compression test was performed at room temperature in the air atmosphere and their initial strain rate was 1.7×10⁻³s⁻¹. The sample used for the compression test was cylinder whose diameter was 6 mm and height was 10 mm.

Evaluation of bio-adaptability

1. Cells and culture conditions

Osteoblast-like cell (MC3T3E1 cells) were grown in Dulbecco's modified Eagle medium (DMEM, Japan Pharmaceutical Manufacturers Association [JPMA], Japan) containing 10% fetal bovine serum (FBS, Dainippon Sumitomo Pharma Co., Ltd. Osaka, Japan) and 50 units/mL penicillin-50 µg/mL streptomycin (GIBCO, NY, USA) at 37 °C in a 5% CO₂ atmosphere.

2. Cell adhesion assay

The solid samples (diameter: 6 mm, thickness: 1 mm) were placed on the bottom of each well of a 96-well multiplate ($n=12$). 150 µL of FBS free-DMEM solution containing cells (MC3T3E1 cells: 5×10⁵cells/mL) were added to each well. Cells were incubated at 37°C in a 5% CO₂/95% air atmosphere for 2.5 h. They were stained with 150 µL of 0.04% crystal violet at room temperature for 10 min after remove of non-adherent cells. After washing with PBS three times, the cells were solubilized with 30 µL of DMSO at room temperature for 10 min. After adding 120 µL of distilled water to the well, absorbance was measured at 550 nm and 415 nm (reference) with an MTP-32 microplate reader (Corona Electric, Ibaraki, Japan).

3. Cell proliferation assay

The solid samples (diameter: 6 mm, thickness: 1 mm) were placed on the bottom of each well of a 96-well multiplate ($n=12$) with 150 µL of 10% FBS-DMEM solution containing cells (MC3T3E1 cells: 5×10⁴cells/mL). Cells were incubated at 37°C in a 5% CO₂/95%

air atmosphere. Cell proliferation was determined by WST assay using Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan).

After incubation for 4, 24 and 48 h, 15 μ L of CCK-8 solution were added to each well, and the multiplate was incubated at 37°C in a 5% CO₂/95% air atmosphere for an additional 3 h. Absorbance was measured at 415 nm and 630 nm (reference) with a microplate reader.

Animal experiments and microstructure observation

All animal experiments in this study strictly followed a protocol approved by the Animal Research Subjects Committee of Osaka University Graduate School of Dentistry (19-055). Male eight-week-old Sprague-Dawley rats (Charles River Laboratories Japan, Tokyo, Japan) were used ($n=3$). The rats were anesthetized with diethyl ether and an intraperitoneal injection (0.1 mL per 100 g body weight) of 5% pentobarbital sodium (Nembutal: Dainippon Sumitomo Pharmaceutical, Osaka, Japan). After shaving of the calvarial region, a sagittal incision was made, with the subcutaneous connective tissue and the pericranial periosteum carefully elevated to expose

the parietal bones. A round defect (1×1.5×0.5mm) was formed in the center of the calvaria (on the sagittal suture) using a dental round burr. During drilling, the site was irrigated with sterilized physiological saline to prevent overheating. After the defect was completed, a PLLA-only porous sample and a PLLA porous composite sample with 5% OCP powder (PLLA-5% OCP porous sample) were put into the defects. These porous samples (1×1.5×1 mm) were cut by diamond foil saw and ground by the emery paper. After the defect was covered with the pericranial periosteum, the flap was closed with silk sutures. The rats were thereafter housed with free access to water and food.

Rats were sacrificed at 4 weeks after the operation, and the calvariae were extracted en bloc (5×3×1.5 mm). The specimens were immersed in 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for one night. After decalcification with 0.5 M EDTA for 3 days, the specimens were embedded in paraffin. For hematoxylin and eosin (HE) staining, paraffin sections were stained in Mayer's hematoxylin solution (Wako Pure Chemical Industries, Ltd.).

Fig. 1 SEM observation of both solid and porous composites samples surface. a) PLLA porous sample in side. b) PLLA solid sample in a section. c) PLLA porous sample in a section. d) PLLA+5%OCP solid sample in a section. e) PLLA+5%OCP porous sample in a section. f) PLLA+5%OCP porous sample in side.

Fig. 2 Average size and distribution of PLLA particles and void.

Specimen slides were evaluated using light microscopy with digital imaging system (BX51 and DP71: Olympus, Tokyo, Japan).

Statistical analysis

Experimental results were plotted in the graphs as mean±standard deviation (S.D.). Significant difference between the two groups was analyzed by using Student's *t*-test ($P<0.05$).

RESULTS

Production and morphological analysis of PLLA particles and CP fine powders

The PLLA particles used for making the composite samples were non-uniform and irregular in shape (Fig. 1a). The mean intercept length was measured using the linear intercept method, giving a mean particle size of 240 μm . The PLLA particle samples, however, had a particle size distribution because they were prepared using a gas atomization method. Measurement of the particle size distribution showed an almost normal distribution with a mean value of 240 μm (Fig. 2a). Figures 2a, b show the distribution of PLLA and void size respectively.

In contrast, the CP fine powders, that is, pure HAp, Mn-HAp, and OCP powders, used for coating the surfaces of the PLLA particles were nanosized, fine, needle-like particles of diameter much less than 1 μm ; they formed secondary particles of size approximately 20 μm^{10} . The Mn concentration in Mn-HAp was 0.26 mass% (4.469 at%). X-ray diffraction measurements of the powder samples demonstrated that the samples of pure HAp and Mn-HAp had sharp peaks mainly attributable to a hexagonal crystal system, whereas the OCP powder

Fig. 3 Typical XRD profiles of CP.

sample showed weak broad peaks (Fig. 3). In addition, in Fourier-transform infrared spectroscopy (FTIR), with absorbance as the ordinate and wave number as the abscissa, PO_4 peaks were observed in all samples, and the HPO_4 peaks in Mn-HAp were larger than those in pure HAp and OCP. The OH peaks in OCP were small. The peaks reflected the respective molecular structures (Fig. 4).

Production of composite samples and evaluation of mechanical properties

Next, solid or porous composite samples were prepared from PLLA particles and three types of CP fine powder.

A cylindrical carbon container of diameter 6 mm was filled with PLLA particles, heated at a temperature just above the melting point, and compressed with a billet of diameter 6 mm. Scanning electron microscopy (SEM) observations of the solid sample obtained showed that the surface was smooth with almost no voids (Fig. 1b). In contrast, a sample prepared by filling and heating (without compression) yielded a porous material in which PLLA particles were partly attached to each other, and many voids were formed (Fig. 1c). The mean intercept

Fig. 4 Typical FTIR profiles of CP.

Fig. 5 Effect of CP volume fraction on optical density showing MC3T3E1 cell adhesion behavior on the PLLA/CP hybrid composite solid pellets.

length of the voids in a section of the porous sample was approximately 80 μm (Fig. 2b). After rupturing the porous sample in which PLLA particles were linked and many voids were formed, SEM observations of the interior was performed, and revealed that the voids were connected to the sample interior (Fig. 1a).

Similarly, solid or porous composite samples were prepared from mixed powders of PLLA powder particle and three types of CP fine powder; the CP volume fractions were 1%, 3%, and 5%.

SEM observations of a solid composite sample of PLLA particles and an OCP powder volume fraction of 5%, showed a smooth PLLA surface with OCP powder in places (Fig. 1d). In contrast, SEM observation of a porous composite sample of PLLA particles with OCP powder showed PLLA particles coated with fine OCP powder (Fig. 1e). SEM observations of the sample interior after rupturing revealed that the all PLLA particle surfaces

Fig. 6 Effect of CP volume fraction on optical density showing MC3T3E1 cell growth behavior on the PLLA/OCP hybrid composite solid pellets.

Fig. 7 The compressive strength of the solid and porous composites.

were covered with OCP powder and there were voids connected to the sample interior (Fig. 1f). In this way, solid or porous composite samples in which CP fine powder and voids were distributed three-dimensionally and uniformly in bulk PLLA were produced.

The mechanical properties of the samples were evaluated using a compression test. The compressive strengths of the solid samples were higher than those of the porous samples. When the dependence of the 0.2% proof stress on the volume fraction of OCP powder in PLLA was investigated, although the offset yield points of the porous samples were almost constant, the yield points of the solid samples decreased slightly with increasing volume fraction of OCP powder (Fig. 7).

Fig. 8 SEM micrograph of the inside of the samples.
a) PLLA porous sample in side. b) PLLA+5%OCP porous sample in side. c) Expansion of a white frame in a). d) Expansion of a white frame in b).

Evaluation of biocompatibility

Cellular experiments were performed using osteoblastic MC3T3E1 cells to explore the possibility of using solid or porous composites of PLLA coated with nanosized CP fine powders as scaffold biomaterials.

1. Cellular adhesiveness experiments

Cellular adhesiveness experiments were conducted to obtain information about the initial adhesion of MC3T3E1 on the sample surfaces. The surface areas of the samples have to be uniform in the cellular adhesiveness experiments, so MC3T3E1 cells were seeded on the surfaces of solid samples of diameter 6 mm and thickness approximately 1 mm; the optical density was measured 2.5 h after seeding. The resulting optical density values were represented with the optical density of the sample as the ordinate and the volume fraction of CP fine powder as the abscissa (Fig. 5). With regard to the optical density, all the PLLA-CP composite samples had statistically significantly higher values than those of the PLLA-only samples. In addition, a dependence of optical density on the volume fraction of CP fine powder was observed in all samples. The highest cellular adhesiveness was observed when OCP was used to make the composites, followed by Mn-HAp, and then pure HAp. When the volume fraction of CP was 5%, a significant difference was observed between the optical density of the pure HAp composite sample and that of

the OCP composite sample.

2. Cell growth potential experiments

Cell growth potential experiments were performed using the PLLA solid composite samples with OCP powder; these showed the best initial adhesion properties in the cellular adhesiveness experiments. The results of the experiment at 37°C for up to 48 h demonstrated that growth potential increased with time, regardless of the volume fraction of OCP powder contained in the PLLA solid composite samples, and also that cell growth potential was significantly enhanced by addition of only 1% of OCP powder. A dependence on the volume fraction of OCP powder was also observed at 48 h, and the cell growth potentials of the PLLA-OCP composite samples were better than those of the PLLA-only samples (Fig. 6).

Animal experiments and tissue observations

A PLLA-only porous sample and a PLLA porous composite sample with 5% OCP powder (PLLA-5% OCP porous sample) were implanted in skull fractures of eight-week-old Sprague-Dawley (SD) rats. Four weeks after implantation, the behavior of the cells responsible for bone metabolism was observed using hematoxylin-eosin (HE) staining. Good invasion of pores by the cells was observed in both the samples, although the conditions were different. In the case of the PLLA-

Fig. 9 HE staining of the samples: a,c) PLLA sample, b,d) PLLA-5% OCP sample implanted into rat skull.

a) PLLA porous sample in a section. b) PLLA+5%OCP porous sample in a section. c) Expansion of a white frame in a). d) Expansion of a white frame in b).

only porous sample, however, few blood vessels, many mononuclear lymphoid cells, and chronic inflammation were observed. In the case of the PLLA-5% OCP porous sample, on the other hand, many blood vessels and many multinucleated osteoclastic cells were observed, and many new bone areas were also observed (Fig. 9).

DISCUSSION

It is known that the crystal structure of HAp is a close-packed hexagonal lattice¹²⁻¹⁴, and the longitudinal directions of fine needle-like pure crystals of HAp and Mn-HAp are almost parallel to the c-axis of a hexagonal crystal¹⁵⁻²⁴. X-ray diffraction analysis of pure HAp and Mn-HAp powders synthesized by the wet method used in this study revealed that these powders consisted of highly crystalline single-phase HAp, because sharp peaks attributed to a hexagonal crystal were observed (Fig. 3). It is possible that Mn-HAp is CDHAp, a precursor of

HAp in which the Ca sites of a hexagonal structure are partly replaced by Mn atoms. In contrast, OCP, which is also a precursor of HAp, was an orthorhombic crystal and had a different peak pattern from that of HAp. The OCP peaks were broader than those of pure HAp and Mn-HAp, suggesting low crystallinity. Such imperfect crystallinity is expected to strongly affect the sample solubility in culture solutions.

The absorbance changes were investigated by examining the intensities of the PO_4^{3-} , HPO_4^{2-} , and OH^- peaks (Fig. 4). Peaks attributed to PO_4^{3-} were observed in all samples of pure HAp, Mn-HAp, and OCP. However, the Mn-HAp sample had the strongest HPO_4^{2-} peak, and the OCP sample did not show any peaks attributable to OH^- . These results strongly suggest the possibility that pure HAp is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, Mn-HAp is $\text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5\text{OH}$, and OCP is $\text{Ca}_8(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$. The increase in solubility caused by the decrease in the Ca/P ratio may therefore result in improved biocompatibility.

In this study, solid or porous composite samples in which CP fine powder and voids were distributed three-dimensionally and uniformly in PLLA particles were successfully produced. The mean intercept length of the voids in a porous sample was 80 μm . This value was similar to the value reported in a study by Predecki *et al.*²⁾, in which, when Al_2O_3 samples with different-sized pores were implanted in dog femurs, new bone was generated in pores of diameter 95 μm or larger. Moreover, the pore size was expected to increase as a result of progressive degradation of the PLLA matrix. Ideally, a porous implant would have sufficient strength in the early stages after implantation, would degrade gradually as new bone was generated, and would finally be completely replaced by new bone. In fact, the size distribution of the generated voids was broad, and voids of diameter approximately 300 μm were also found. It has also been reported that, in the case of larger voids, implants with through pores yielded good osteoconductive and bone regeneration properties²⁵⁾.

When a material is used as a bone substitute material and implanted in a bone defect, the strength of the material is extremely important. In the case of porous samples, the dependence of the 0.2% proof stress on the volume fraction of OCP powder in PLLA was not noticeable. This could be because the strengths of the porous samples originated from a connected PLLA matrix. In contrast, the strengths of the solid samples slowly decreased with increasing volume fraction of OCP powder. When the volume fraction of OCP powder was 5%, the 0.2% proof stress of a porous sample was 20 MPa, and that of a solid sample was 50 MPa (Fig. 7). These deformation stress were roughly comparable to the values obtained when living bone was heated at 600°C to remove organic matter such as collagen, and when the bone was further sintered at 1200°C to make it more compact. Consequently, it was considered that the strengths of the solid and porous samples obtained in this study were adequate for their use as scaffold biomaterials.

When a material is used as a bone substitute material and implanted in a bone defect, it is important for the material to have an optimum Young's modulus, as well as sufficient strength. The Young's modulus of living bone is low, ranging between 15 GPa and 20 GP. In the case of metallic implants, the Young's modulus of Ti or Ti alloys is 100–120 GPa and that of a Co-Cr alloy is 200 GPa; these are far higher than that of living bone. When metallic implants are implanted in living bone, decreases in bone mass, such as changes in bone volume and bone mineral density, caused by stress shielding effects resulting from differences between the Young's modulus of bone and the metallic implants, is a major problem. In the case of synthetic polymers, however, the Young's modulus of an implant material can be optimized by controlling the chemical structure of the polymer, and an implant material with the desired purity and composition can be prepared. Accordingly, because the Young's modulus of PLLA materials can be easily adjusted to that of living bone, better bone

soundness will be maintained and bone regeneration will be achieved by using PLLA materials with physical properties, which have been optimized for use as implant materials.

When biomaterials are implanted in living bodies, biocompatibility is an extremely important factor. The addition of only 1 vol% of CP fine powder to PLLA when making a composite allows the composite to enhance cellular adhesiveness significantly (Fig. 5). Furthermore, the cellular adhesiveness drastically increases with increasing volume fraction of CP fine powder, that is, a significant dependence on the volume fraction is observed. Comparisons among the three types of CP fine powder, using the same volume fraction, showed that the highest cellular adhesiveness was observed when OCP was used to make the composites, followed by Mn-HAp, and then pure HAp. These differences in cellular adhesiveness among the three types of CP fine powder are considered to be caused by differences in the amounts of Ca and Mn eluted from the composite as a result of the differences in Ca/P ratios, and crystallinity imperfections. It is widely known that adhesion of cells to an extracellular matrix is generally mediated by integrin. It is supposed in this study that divalent Ca and Mn cations, which were eluted from the composite samples, adsorbed to divalent-cation-binding domains in extracellular integrin α -chain sites, leading to activation of integrin, which deeply affects cell adhesion, resulting in an increase in cellular adhesiveness^{26–28)}. Naturally, it is expected that the higher the volume fraction, the larger the amount of elution. Moreover, CP powders adhere on only surface of the PLLA particles; CP powders was confirmed to be localized in the case of the solid material used in the present study. Cells, however, homogeneously adhered overall specimen surface. It seems unlikely that cells adhered on the trace of dissolved CP powder. Cell growth potential experiments also showed the statistical significance of making PLLA composites with CP fine powders (Fig. 6). Such remarkable improvements in the biocompatibilities of PLLA-CP solid samples strongly suggest the possibility of using porous samples as scaffold biomaterials. If porous samples are used as scaffold biomaterials, the cells responsible for bone metabolism have to enter into the interior of the materials through voids. Cellular adhesiveness experiments were therefore performed using porous samples. Cells were seeded on the surfaces of PLLA-only porous samples and PLLA-CP porous composite samples of diameter 6 mm and thickness approximately 10 mm, and incubated in an atmosphere of 95% air and 5% carbon dioxide for 2.5 h. The samples were then cut at a height of 3 mm from the surface and the interior was observed. In the cases of both PLLA-only samples and PLLA-5% OCP samples, SEM observations demonstrated that cells responsible for bone metabolism entered the sample interiors and adhered well (Fig. 8). In the case of the PLLA-only porous samples, cells seeded on the surface are considered to enter into the interior through voids. An enlarged image of the boxed area in Fig. 8a showed that the cells extended well and attached well to the interior

of the material. In contrast, in the case of the PLLA-5% OCP porous samples, an enlarged image (Fig. 8d) showed cells extending well on the interior of the sample together with secondary particles of undissolved OCP.

The effect of addition of a minute amount of OCP to PLLA porous samples was also clearly observed in animal experiments using the cells responsible for bone metabolism. A PLLA-only porous sample and a PLLA-5% OCP porous sample were implanted in skull fractures of eight-week-old SD rats. Four weeks after implantation, the behavior of cells responsible for bone metabolism was observed using HE staining ($n=3$). A typical example is shown, good invasion of pores by the cells was observed in both samples, although the cellular tissue conditions were different. In the case of the PLLA-only porous sample, however, few blood vessels, many mononuclear lymphoid cells, and chronic inflammation were observed. In the case of the PLLA-5% OCP porous sample, on the other hand, many blood vessels (indicated by white arrows in Fig. 9d) and many multinucleated osteoclastic cells (indicated by black arrows in Fig. 9d) were observed, and many new bone areas were also observed (Fig. 9).

One of the reasons why new bone formation was promoted is thought to be that Ca and P, which are the raw materials of bone, were present in the OCP powder covering the surface of the PLLA particles in the composite, so Ca and P were supplied to the osteoblasts, leading to promotion of bone formation. Another reason is thought to be that Ca was eluted from the porous composite sample as Ca^{2+} ; the Ca^{2+} acted on the calcineurin/NFATc1 signal transduction pathway, dephosphorylation was promoted, and osteoclast activity was enhanced²⁹). Accordingly, the balance between bone formation promoted by supply of Ca and P from biomaterials to osteoblasts and bone resorption promoted by activated osteoclasts was improved, resulting in bone remodeling, and, finally, highly osteoinductive bone regeneration was achieved. The PLLA porous material with CP powders on its surface was therefore bone out to show strength similar to bone and better biocompatibility than solo PLLA. These results indicate the potential of PLLA porous composite samples with a minute amount of OCP for use as bone regenerative scaffold biomaterials.

CONCLUSION

Composite materials of PLLA particles, the surfaces of which were coated with fine powders of pure HAp, Mn-HAp (HAp containing a minute amount of Mn), or OCP (a precursor of HAp) were produced and the biocompatibilities of solid and porous samples prepared from the composite materials were evaluated. The results were as follows.

1. PLLA solid samples and PLLA porous samples were prepared from composite materials of PLLA particles and CP fine powders; the CP volume fractions ranged from 0% to 5%.
2. Among the PLLA solid samples, the sample

consisting of PLLA and 5% OCP showed the best biocompatibility.

3. Cellular adhesiveness experiments with osteoblastic MC3T3E1 cells demonstrated that MC3T3E1 cells adhered well to the sample, even at the center and at a depth of 3 mm from the surface of a sample of thickness 6 mm.
4. In living bodies, cells responsible for bone metabolism were activated more in PLLA-5% OCP porous samples than in PLLA-only porous samples. It is thought that improved bone metabolic capacity leads to an increase in osteoinductive capacity and good bone regenerative capacity.

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