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Bone Formation in a Scaffold Composed of Cylindrical Hydroxyapatite and Tryptophan- or Lysine-Coated Sponge *in Vivo*

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Abstract

Because of the three-dimensional structure of bone or hard tissue such as a tooth, a scaffold is necessary for its regeneration by cellular engineering. Commonly, for *in vivo* examination, hydroxyapatite (HA) has been used as such a scaffold. Cylindrical HA with a hollow center, which included a columnar formalin-treated polyvinyl alcohol sponge, was used in this examination as a scaffold. The sponge had been coated with L-tryptophan or L-lysine before insertion into the hollow center of the HA. Rat bone marrow cells (rBMCs) derived from the femur were seeded in the sponge before insertion into the hollow center of HA. The number of rBMCs seeded in each sponge was 1.5×10^6. These scaffolds were implanted subcutaneously into the backs of Fischer 344 rats for 6 weeks. In the amino-acid-coated sponge in HA, osteogenesis was found histologically. An osteocalcin level of approximately 10 μg was measured in the scaffolds with L-tryptophan-coated formalized polyvinyl alcohol sponge containing rBMCs, 4 μg on average in the scaffolds with L-lysine-coated sponge containing the cells and about 2 μg in each scaffold with uncoated sponge containing the cells. The structure of the scaffolds used in this study was thought to be suitable for osteogenesis by rBMCs. It was concluded that tryptophan, as a factor for bone formation by stem cells, functioned by promoting cell adhesion and the differentiation of stem cells into osteoblasts.

Keywords

Scaffold, Hydroxyapatite, Bone Marrow Cells, L-Tryptophan, L-Lysine, Poly-Vinyl Formal Sponge

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1. Introduction

A scaffold is necessary for bone and hard tissue regeneration because of their three-dimensional structure [1]. Cylindrical porous hydroxyapatite (HA) might be suitable as a scaffold in which mesenchymal stem cells differentiate into hard tissue-forming cells for the regeneration of bone or tooth [2]. However, a considerable number of mesenchymal stem cells are necessary for producing a sufficient amount of bone or tooth. Unfortunately, it is difficult in dentistry to obtain a large number of stem cells for tooth regeneration. Therefore, a source for obtaining stem cells used for tooth regeneration is required. Buccal mucosa, cutis or dental pulp is expected as such a source. However, for various reasons, bone or tooth regeneration by the cells derived from these tissues has not been achieved. One of the reasons for this may be that the number of stem cells obtained from these sources is insufficient for the regeneration of tooth or bone. The proliferation of stem cells and prompt induction of their differentiation into osteoblasts or odontoblasts must thus be promoted. In addition, some chemicals may be necessary as factors to induce the growth or proliferation and differentiation of stem cells.

Chemicals applied to an HA scaffold might effectively induce a large amount of hard tissue via a small number of mesenchymal stem cells [3] [4]. In this regard, attention was focused on the potentialities of some amino acids. Previously, the effectiveness of L-leucine for osteogenesis by its coating of a sponge component in a scaffold was reported [5]. In the present study, L-tryptophan and L-lysine were selected as factors for bone formation and their effects were evaluated. L-tryptophan would be available as a factor for the proliferation or differentiation of stem cells to facilitate bone-like tissue formation with bone marrow cells [6]. L-lysine has been mentioned as having a desirable effect during the healing process for the improvement of bone fractures [7]. It was reported that L-lysine also affected the bone status in metabolic diseases such as primary and secondary osteoporosis, in which an imbalance between the anabolic activity of osteoblasts and bone resorption by osteoclasts is the cause of bone rarefaction [8].

Scaffolds with a sponge in the hollow center of a porous cylindrical HA structure were used in this study to evaluate the influences of amino acids on bone formation by bone marrow cells. Specifically, the purpose of this study was to estimate the effect of L-tryptophan or L-lysine coating on the sponge component with bone marrow cells in a cylindrical HA scaffold on bone formation.

2. Materials and Methods

2.1. Preparation of Bone Marrow-Derived Cell Suspensions

This study was performed under the Guidelines for Animal Experimentation at Osaka Dental University. Regarding the use and care of animals, the Animal Welfare Committee of Osaka Dental University approved the experimental procedures. The rats used in this study were kept in standard rat cages with free access to dry pellets and water with unrestricted movement at all times.

Bone marrow cells were obtained from 8 of 6-week-old male Fischer 344 rats (Clea Japan, Osaka, Japan). After euthanasia by intra-peritoneal injection of an excess dose of sodium pentobarbital (Dainippon-Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan), the femora were removed and the cells were flushed out from the bone shaft of each femur using 10 ml of culture medium expelled from a syringe with a 21-gauge needle. Primary culture of the cells was performed for 1 week in a cell culture flask (T-75; BD Biosciences, MA, USA) containing minimum essential medium (MEM; NacalaiTesque, Inc., Kyoto, Japan) supplemented with 15% fetal bovine serum (FBS; JRH Biosciences, KS, USA) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B; Sigma-Aldrich Co., MO, USA). The medium was changed 2 times a week.

After primary culture, bone marrow cells in the T-75 culture flask were washed three times using phosphate-buffered solution without Ca^{2+} and Mg^{2+} (PBS (-); NacalaiTesque Inc.) and were then isolated from the bottom of the T-75 flask with trypsin-EDTA solution (0.5 mg/ml trypsin and 0.53 μmol EDTA; NacalaiTesque Inc.) to prepare bone marrow cell suspension. Harvested cells were resuspended in culture medium at 1×10^7 cells/ml.

2.2. Preparation of Experimental Scaffolds Composed of a Sponge with L-Tryptophan or L-Lysine Coating and Cylindrical Hydroxyapatite for *in Vivo* Examination

L-tryptophan and L-lysine were purchased from the Peptide Institute, Inc. (Osaka, Japan), and each dissolved at 100 mM concentration in ultra-purified water. Each liquid was sterilized by filtration (Millex®: 0.22 μm; Milli-

pore Japan, Tokyo, Japan).

HA with the same properties as that used in this study is commercially available, but the configuration is disk-shaped (Hoya Technosurgical Company, Tokyo, Japan). According to our design, cylindrical HA structures with a hollow center were custom-made by Hoya Technosurgical Company. The lot of the HA structures used in this study was the same as that described by Yabuuchi *et al.* [4]. The HA structures were 8 mm in diameter and 10 mm in height, with a hollow center measuring 5 mm in diameter. Total porosity of the structures was 55%. The diameter of each aperture on the surface of the HA structure was approximately 100 - 300 μm. The aperture diameter between interconnected pores was approximately 50 - 300 μm. They were sterilized in ethylene oxide gas.

Commercially available sponges made of formalin-treated polyvinyl alcohol (PVF sponge) were used as a component of the scaffolds used in this study. A sheet of PVF sponge was generously donated by Ione Co., Ltd. (Osaka, Japan). PVF sponges were cut cylindrically from the PVF sponge sheet. The cylindrical PVF sponge used as a scaffold in this study was 5 mm in diameter and 8 mm in height. Pore size was 130 μm on average. The prepared cylindrical sponges were sterilized in ethylene oxide gas before immersion in an amino acid solution. The PVF sponges were immersed in L-tryptophan or L-lysine solution for 24 hours. The ones used as a control were immersed in ultra-purified water. After being air-dried under radiation with ultraviolet light in a clean bench, each of the PVF sponges with or without immersion in L-tryptophan or L-lysine solution was inserted into the hollow center of the HA structures to prepare a scaffold (**Figure 1**).

An rBMC suspension of 150 μl at 1×10^7 cells/ml concentration was placed on the PVF sponge in the cylindrical HA component of the scaffold. In each sponge, rBMCs were seeded at 1.5×10^6 cells.

2.3. Implantation of the Scaffolds Composed of HA and L-Tryptophan- or L-lysine-Coated Sponge

2.3.1. Histological Examination of Implanted Scaffolds

This *in vivo* study was performed using 7-week-old male Fischer 344 rats under general anesthesia by intra-peritoneal injection of sodium pentobarbital. The backs of the rats were shaved and disinfected with povi-

Figure 1. An image of the scaffold showing the process of PVF sponge insertion into the hollow center of cylindrical porous hydroxyapatite.

done iodine solution (ISODINE® solution 10%; Meiji Seika Pharma Co., Ltd., Tokyo, Japan). On the back, along the spinal column, subcutaneous pockets were made on each side. The scaffold composed of L-tryptophan-coated sponge and porous cylindrical HA structure was inserted into the left subcutaneous pocket on the scapula side and the scaffold with uncoated sponge including the cells was put into that on the tail side. The scaffold with L-lysine-coated sponge including rBMCs was inserted into the right pocket. The numbers of each type of implanted scaffold were 6.

Incised wounds on the back were sutured and then shielded with adhesive (Aron Alpha®; Toagosei Co., Ltd., Tokyo, Japan). The scaffolds were removed from the backs of animals 6 weeks postoperatively after euthanasia by intra-peritoneal injection of an excess dose of sodium pentobarbital. In 10% buffered formalin solution, 3 of 6 scaffolds were fixed. Then, they were decalcified in 10% formic acid solution for 3 weeks followed by dehydration in absolute ethanol. They were permeated with xylene for embedding in paraffin. The paraffin-embedded specimens were serially cut into 9-μm-thick sections. These serial sections were stained with toluidine blue or hematoxylin-eosin alternately for histological examination under an optical microscope.

2.3.2. Quantitative Analysis of Alkaline Phosphatase Biochemically and Osteocalcin Immunochemically in Implanted Scaffolds

The other 3 scaffolds were used for measurement of the alkaline phosphatase (ALP) level biochemically and quantitative analysis of osteocalcin immunochemically. The scaffolds were immediately frozen in liquid nitrogen after removal from the dorsal subcutaneous tissue of rats. Then, they were crushed and homogenized in 1 ml of a 10-fold concentration of TNE buffer solution (pH 7.4; 10 mMTris-HCl, 1 mM EDTA, 100 mMNaCl) using a Mixer Mill® (MM301; Verder Scientific Co., Ltd., Tokyo, Japan). The homogenized samples were sonicated (BIORUPTOR UCW-201; Tosho Denki Co., Ltd., Yokohama, Japan) for 30 seconds at 3˚C. The emulsified sample was passed through a column (PD-10 desalting column; GE Healthcare UK Ltd., Buckinghamshire, UK) to collect the osteocalcin adhered to HA. Each sample was centrifuged at 16,000 × g for 1 minute. The quantity of ALP in the scaffolds was measured biochemically.

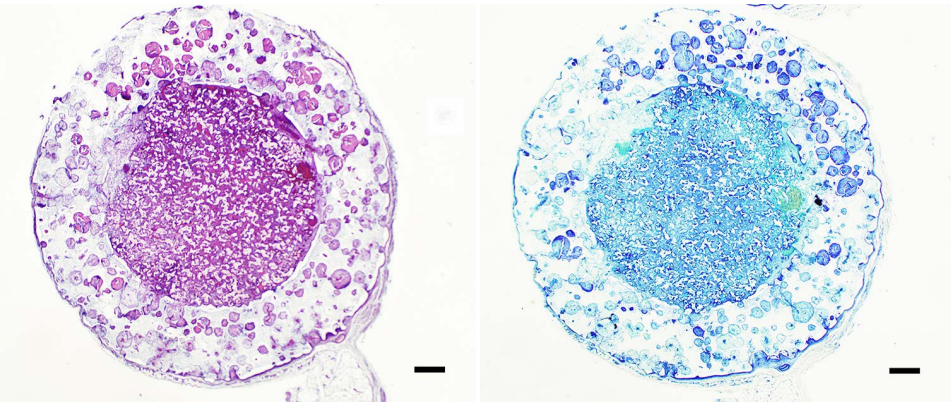
The supernatant was reacted at 37˚C for 30 minutes with *p*-nitro-phenyl phosphate (Zymed Laboratories Inc., CA, USA) as a substrate. Absorbance of the reactant was measured at 405 nm. The value of ALP activity is presented per scaffold as μM of *p*-nitro phenol released after 30 minutes of incubation at 37˚C. For quantitative analysis of osteocalcin, the supernatant was also passed through a PD-10 desalting column to collect the osteocalcin from HA to which osteocalcin might have adhered. The measurement of the quantity of osteocalcin was performed using a kit (Rat Osteocalcin ELISA kit DS®; DS Pharma Biomedical Co., Ltd.). In brief, the procedures were as follows. The desalted supernatants were poured into each well of a 96-well micro-plate. The bottom of each well was coated with anti-rat osteocalcin antibody. Peroxidase-conjugated anti-rat osteocalcin polyclonal antibody was added to each well. An equivalent mixture of peroxidase substrate and hydrogen peroxide water was added, followed by incubation in the dark. Absorbance at 450 nm was measured using a spectrophotometer (SpectraMax M5; Molecular Devices Corporation Japan, Tokyo, Japan).

Data are presented as the mean ± standard deviation. Statistical comparisons between the mean values in implanted scaffolds were performed using two-way unrepeated ANOVA followed by post hoc analysis using the Tukey-Kramer's test. Differences of $p < 0.01$ were considered significant.

3. Results

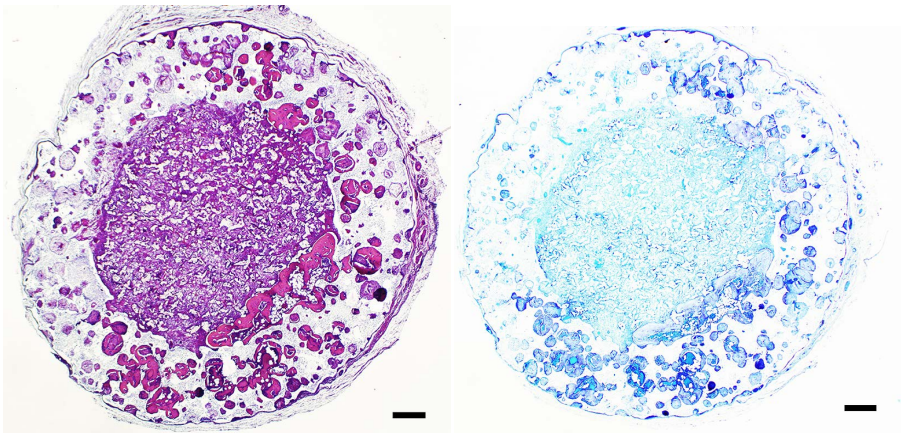
3.1. Histological Examination of Implanted Scaffolds

After 6 weeks of subcutaneous implantation, serial sections of the scaffolds were histologically examined under an optical microscope. Substantial amounts of bone formed in the scaffold composed of cylindrical porous HA and L-tryptophan-coated sponge with rBMC seeding before implantation (**Figure 2(a)**, **Figure 2(b)**). Many pores in HA composed of the outer layer of the scaffold contained newly formed bone. In the sponge that was surrounded by HA component, bone formation was found in a large area. Much bone was also found in the scaffold composed of cylindrical porous HA. In L-lysine-coated sponge with rBMC seeding before implantation, newly formed bone was observed in many pores in the HA structure (**Figure 3(a)**, **Figure 3(b)**). In the scaffold with non-amino-acid-coated sponge with rBMC seeding before implantation and HA structure, newly formed bone was observed. However, the amount of bone found in the sponge region was small. The number of pores with bone was smaller than that in the scaffolds containing an amino-acid-coated sponge.



(a) (b)

Figure 2. Micro-photographic image of subcutaneously implanted scaffold composed of L-tryptophan-coated sponge and porous cylindrical HA structure. In the PVF sponge structure, rBMCs were seeded before subcutaneous implantation. (a): Many pores with new bone in the HA structure and in the PVF sponge are observed. Hematoxylinand eosin staining (Bar: 500 μm). (b): Metachromasia indicating bone is recognized in the HA structure and in the PVF sponge. Toluidine blue staining (Bar: 500 μm).



(a) (b)

Figure 3. Micro-photographic images of theimplanted scaffold composed ofL-lysine-coated sponge including rBMCs and porous cylindrical HA structure. (a): Osteogenesis is found in the border where the sponge contacts with HA.However, no bone is present in the center of the sponge. Hematoxylinand eosin staining (Bar: 500 μm). (b): Metachromasia is indicating newly formed bone included in more than half of the pores. Toluidine blue staining (Bar: 500 μm).

3.2. Quantitative Analysis of Alkaline Phosphatase and Osteocalcin in Implanted Scaffolds

The quantity of ALP measured in the scaffold with L-tryptophan-coated PVF sponge containing rBMCs was the lowest among the three scaffolds (**Figure 4(a)**). The quantity of ALP in the scaffold with non-amino-acid-coated sponge containing the cells was significantly higher. The details are as follows. The level of ALP was 100.88 ± 31.25 μM/scaffold in the scaffold with L-tryptophan-coated sponge containing the cells and 827.54 ± 111.73 μM/scaffold in the scaffold with uncoated sponge containing the cells.

The quantity of osteocalcin measured in each scaffold is indicated in **Figure 4(b)**. The quantity of osteocalcin in the scaffold with L-tryptophan-coated PVF sponge containing rBMCs was significantly high. The scaffolds that showed the lowest quantity of osteocalcin were uncoated sponges that included rBMCs. An osteocalcin level of approximately 10 μg was measured in the scaffolds with L-tryptophan-coated PVF sponge containing

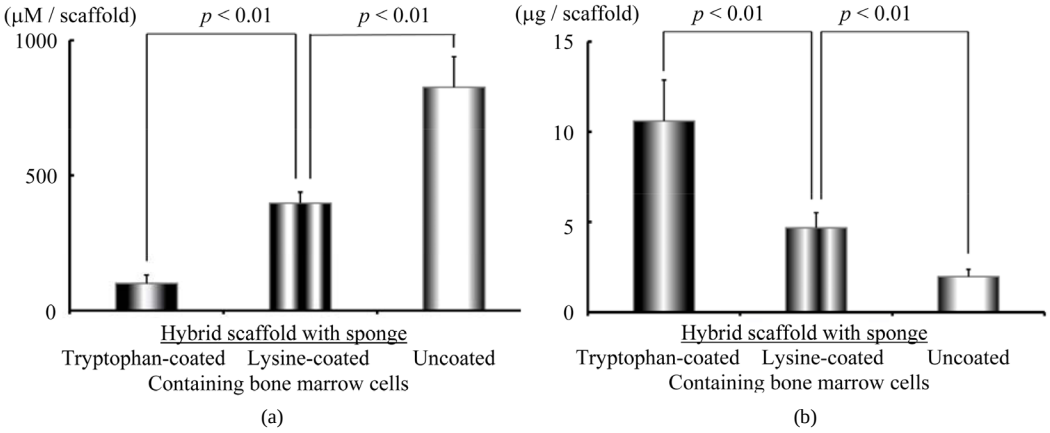


Figure 4. (a) Quantity of alkaline phosphatase in the scaffolds 6 weeks afterimplantation. The scaffold with L-tryptophan-coated sponge showed a significant difference from the scaffold containing a formalized polyvinyl alcohol sponge with L-lysine-coated fiber structure ($p < 0.01$). (b) Quantity of osteocalcin in the scaffolds 6 weeks after implantation. The average quantity of osteocalcin in the scaffold with formalin treated L-tryptophan-coated formalized polyvinyl alcohol sponge containing rat bone marrow cells was the highest ($p < 0.01$).

rBMCs, 4 μg on average in the scaffolds with L-lysine-coated sponge containing the cells and about 2 μg in each scaffold with uncoated sponge containing the cells.

4. Discussion

In orthopedics, regenerated bone has been applied clinically. However, the clinical application of regenerated tooth has not yet been realized, despite tooth regeneration having been attempted by numerous researchers [9] [10]. The possibility that tooth germ could develop in a mature tooth was demonstrated [11]. However, regeneration of a whole tooth appeared to be difficult. Several factors are assumed to complicate the regeneration of teeth. One of them may be the structural complexity associated with tooth development. It is known that a tooth consists of ectoderm and mesoderm [12] [13]. Though tooth regeneration by cellular engineering would be extremely difficult because these two fetal tissues must be connected by a mixed culture, the regeneration has been reported lately [14]. The second reason for the difficulty of tooth regeneration is the multiplicity of the configurations of the teeth in the oral cavity. The anatomical configuration of the teeth in each person varies depending on the kind of tooth. The number of human teeth is usually from 28 to 32. They are classified in 8 kinds: central incisors, lateral incisors, canines, two kinds of premolar and three kinds of molar. Regarding homonymous teeth, the configuration is considerably different between a maxillary tooth and a mandibular tooth. Each pair of homonymous tooth on the left and right sides presents a symmetrical configuration, and they have characteristics that reveal the side to which they belong. Because the configuration differs in each tooth in a person. Owing to these difficult problems associated with tooth development and configurations, the regeneration of teeth by cellular engineering has not been achieved.

If a portion of regenerated teeth could be substituted by a scaffold, the reconstruction of teeth by cellular engineering may be possible. A porous HA scaffold with or without modification has been utilized for bone regeneration [15] [16]. The application of a porous HA scaffold for the regeneration of teeth having an apatite structure similar to bone, was considered to be rational. In the pores, the stem cells proliferate and then differentiate into osteoblasts. Subsequently, bone would be formed by the cells. However, the obtainment of stem cells would be necessary in order to realize tooth regeneration by cell engineering in dentistry. In orthopedics, osteogenic stem cells for regeneration of the articular head are generally obtained from bone marrow cells in iliac bone [17]. Surgery may constitute a considerable load to the patient. Therefore, in dentistry, it would be difficult to obtain stem cells from the iliac bone of patients. The establishment of a source of stem cells is one of the most important problems for tooth regeneration in dentistry, as mentioned by Yoshikawa *et al.* [4]. In the oral cavity, a source to obtain stem cells could be dental pulp [18], tooth germ [9] [11] or oral mucosa [19]. However, the number of stem cells obtained from these sources might be much lower than for bone marrow stem cells obtained from the iliac bone. Extirpation of tooth germ from the jaw of a child might present a severe physical

burden, so it may be easier to obtain oral mucosa-derived stem cells [20] because, in resection of the oral mucosa, the surgical stress may be slight. The number of stem cells obtained from intraoral tissues is also a small [4]. In addition, a considerable period of stem cell culture for proliferation would be necessary to obtain a large number of stem cells from dental pulp or oral mucosa. It was thus thought that improvement of the cell culture method would be necessary to obtain a large number of stem cells for tooth regeneration.

Growth factors would be suitable for the prompt proliferation of cells in culture in order to obtain a large number of stem cells. The selection of an effective supplement is desirable for the promotion of stem cell proliferation in culture. Yoshikawa *et al.* reported that some amino acids had the possibility of promoting bone formation by bone marrow cells in a porous HA scaffold [21]. Thus, amino acids were evaluated in terms of their ability to promote bone formation using rBMCs as a supplement by coating on the materials in the scaffold in this study. Moreover, Yoshikawa *et al.* were convinced that the function of a hollow center in porous HA might be a pathway for nutritional supply to the bone marrow cells [2] [22]. In this study, the effect of promoting bone formation due to coating of the amino acids on a sponge in the scaffold was evaluated. A PVF sponge in the scaffolds was coated by using L-tryptophan or L-lysine solution. It was shown that two amino acids, which were estimated in this study, contributed to the proliferation and differentiation of bone marrow stem cells. In addition, it was also found that the amino acids might support the promotion of bone formation in a scaffold. It was mentioned previously that bone formation in PVF was difficult because rBMCs did not adhere to the fiber structure of a PVF sponge [5] [23]. However, in this study, bone was clearly induced in the scaffold including a sponge coated with L-tryptophan or L-lysine in the cylindrical HA structure. In a previous study, the potential for the differentiation of osteoblast-like cells from bone marrow-derived mesenchymal stem cells was evaluated in peptide-modified scaffolds [24]. In addition, increases of the adhesion and proliferation of rBMCs were shown in the scaffold modified with amino acid. Considering the construction of a porous scaffold, adhesion is essential for rBMCs to proliferate and differentiate into osteoblasts for bone formation in the pores. It is shown by the results of this study and a previous report [25] that coating with dextran or L-lysine of a fine construction of sponge might improve the adhesive ability of the sponge for rBMCs.

The fine mesh structures of these sponges were coated with an amino acid. From the histologic findings and quantitative analysis of osteocalcin in this study, it was confirmed that amino acid coating on a PVF sponge might contribute to increase bone formation in the scaffold. In the L-lysine-coated sponge, which constituted the inner portion of the scaffold, bone formation was also found histologically. The chemical reaction of L-lysine residues to the fine structure inside the PVF sponge might involve ionic bonding. It was reported that cells adhered well to an L-lysine-coated culture plate [26]. Seeded stem cells would adhere to the L-lysine-coated internal structure of the sponge by ionic bonding, and bone might be formed conspicuously by those cells. However, in the scaffold, immunochemical analysis of osteocalcin showed a significantly larger quantity in L-tryptophan-coated sponge than in L-lysine-coated sponge. Specifically, the quantity of osteocalcin in the scaffold L-tryptophan-coated sponge more than doubled that in the scaffold with L-lysine coated sponge. The lower levels of ALP and the high level of osteocalcin demonstrated in L-tryptophan coated scaffold might indicate that the scaffold contributed to the bone formation due to the activation of stem cells at an early stage. It is known that L-tryptophan influences protein synthesis [27]. Moreover, it was reported that bone loss in both femoral metaphysis and diaphysis, which was caused by an impairment of bone turnover with bone resorption exceeding bone formation, was repaired by the effects of tryptophan [28].

In a previous report, it was also described that L-tryptophan, the precursor of serotonin biosynthesis, plays a role in the reconstruction of bone defects [29]. However, the direct participation of L-tryptophan in bone formation was not proved in this study. Moreover, it was shown in this study that bone formation was induced actively by L-tryptophan coating of the sponge in the scaffold. It was considered from the results of this study that stem cells included in the bone marrow cells might be stimulated by L-tryptophan and would differentiate into osteoblasts. Therefore, L-tryptophan coating should be useful for the adhesion of bone marrow cells to the fine structure of the sponge of the scaffold. L-tryptophan would act as a factor for bone morphogenetic encouragement due to stem cells in the scaffold. Lysine also seems to be associated with the formation of bones [30]. The quantity of formed bone was significantly smaller in lysine-coated sponge than in tryptophan-coated one. The results of the *in vivo* study using the scaffold containing an amino-acid-coated sponge showed that the effects of L-tryptophan on stem cells in rBMCs were superior to those of L-lysine. L-tryptophan coating on PVF sponge in a hollow center of HA induced substantial bone formation by rBMCs.

The structure of the scaffolds used in this study was thought to be suitable for osteogenesis by rBMCs. It was concluded that tryptophan, as a factor for bone formation by stem cells, functioned by promoting cell adhesion and the differentiation of stem cells into osteoblasts. The biphasic scaffold of HA and the PVF sponge used in this study was reproduction of a dentine-pulp complex. A scaffold is required for regeneration of dental pulp. However, the sponge in the scaffold used in this study was non-absorbable. It may be desirable for the component of bi-phasic scaffold to be absorbed after regeneration of dental pulp. Furthermore, bone regeneration is not necessary in the sponge to induce dental pulp tissue. Fibroblasts, neurocytes and vascular endothelial cells are seeded in the sponge, and regeneration of the tissue similar to pulp must be realized in the hollow center of HA scaffold. Moreover, it was considered that modification of the size and the configuration of the scaffold might be important for practical use. A scaffold coinciding to the configuration of tooth root is produced and should be tested *in vivo*.

5. Conclusion

It is concluded that L-tryptophan acted effectively as a factor for osteogenesis by the stem cells in the scaffold. From the osteogenesis found in the scaffold in this study, the scaffold must contribute to the regeneration of teeth by cellular engineering. In the scaffold, it was shown that an amino acid with excellent tissue compatibility was suitable as a factor to promote bone formation.

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