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Effects of Laminin For Osteogenesis in Porous Hydroxyapatite

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Summery: As a tooth is composed of hard tissue covering pulp, it may be suitable for tooth regeneration to use porous cylindrical hydroxyapatite (HA) scaffolds with a hollow center. Generally, *in vivo* examination, bone marrow cell suspension for osteogenesis in cell/HA composite scaffold without subculture is prepared at a density of 1×10^7 cells/ml or higher. In dentistry, stem cells would be obtained from tooth pulp. For dentine formation, a smaller number of stem cells must be used. In this study, a suspension of rat bone marrow cells at 1×10^6 cells/ml of density was prepared to estimate the adhesive effect of laminin. After immersion of HA scaffold in laminin solution, bone marrow cells were seeded in the pores of the HA scaffolds by immersion in the cell suspension for preparing the cell/HA composite scaffolds. The specimens were respectively implanted in the dorsal subcutis of 7-week-old male Fischer 344 rats for 4 weeks for histological examination. Comparing with the results of *in vivo* examination, alkaline phosphatase activity of bone marrow cells on laminin-coated plate with and without dexamethasone cultured for 2 weeks was measured *in vitro*. It was considered that laminin contributed to bone formation in pores of a scaffold.

Keywords: adhesion; bioengineering; bone marrow cells; laminin; scaffold

Introduction

Undifferentiated mesenchymal cells and odontoblasts are found in tooth pulp tissue. Since these cells have the capability of actually inducing secondary dentine,[1,2] they may be appropriate for regeneration of dentine. However, pulp has important functions in sustaining the tooth by providing nutrition supply and reparative dentine formation.[3] Thus, pulp extirpation from normal tooth to obtain autologous odontoblasts and/or pulp cells would be undesirable. Moreover, the number of cells provided from one permanent tooth is not sufficient for tooth regeneration. As a long time is necessary for the proliferation and differentiation of pulp cells at present,[4] it would be unsuitable to use pulp cells for actual bone or tooth regeneration. Then, to reconstruct a tooth, we must establish a method for obtaining a sufficient number of tooth pulp cells.

In dentistry, a scaffold would be acceptable for reconstruction of a tooth with three-dimensional structure and we considered that the use of a porous hydroxyapatite (HA) scaffold should be effective for regeneration of dentine or a dentine-pulp complex. Therefore, an HA scaffold with a hollow center similar to a tooth structure was devised and used. Since, it is difficult to obtain a sufficient number of tooth pulp cells for tooth regeneration, we considered that bone marrow mesenchymal

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stem cells may be applicable to tooth regeneration. Laminin was used in this study as a cofactor to promote cell differentiation and bone formation in pores of the scaffold. The present study investigated the potential for hard tissue formation using a small number of bone marrow cells in the pores of the HA scaffold pretreated by immersion in laminin solution. Laminin for bone marrow cell differentiation and bone formation *in vivo* and *in vitro* were estimated.

Materials and Methods

Cylindrical porous HA structures with a hollow center (Figure 1) were used as a scaffold. The scaffolds were 8 mm in diameter and 10 mm in height with 55% total porosity. The internal diameter of the hollow center was 4 mm. These structures were manufactured by PENTAX Corporation (Tokyo, Japan). The superficies (Figure 2, a) and internal construction (Figure 2, b) of the scaffold were observed under a scanning electron microscope (SEM: JSM 5400, JEOL Inc., Tokyo, Japan).

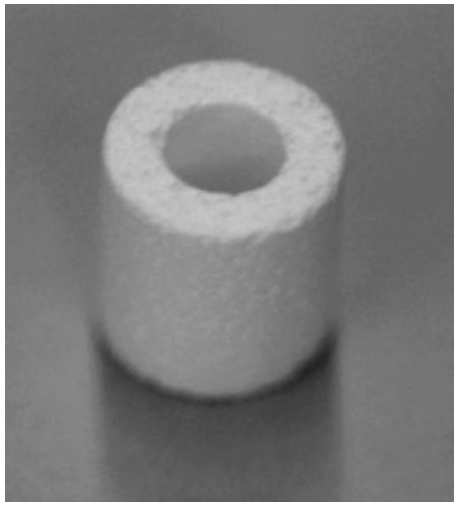

Figure 1.
Photograph of porous hydroxyapatite scaffold with a hollow center Apertures of pores were seen on the surface of the scaffold.

This study was performed under the Guidelines for Animal Experimentation at Osaka Dental University. Regarding use and care of the animals, the Animal Welfare Committee of Osaka Dental University approved the experimental procedures. Bone marrow cells were obtained from the bone shaft of the femora of 6-week-old male Fischer 344 rats after euthanasia by halothane suffocation. The cells were flushed out using 10 ml of culture medium expelled from a syringe with a 21-gauge needle. Primary culture for the cells was performed for 1 week in a cell culture flask (T75: BD Biosciences, MA, USA) containing minimum essential medium (MEM: Nakarai Tesque, Inc., Kyoto, Japan) supplemented with 15% fetal bovine serum (FBS:JRH Biosciences, KAN, USA) and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericine B, Sigma-Aldrich Co., MO, USA). The medium was changed 2 times. The bone marrow cells were released from their culture substratum using trypsin-EDTA solution (0.5 mg/ml trypsin, 0.53 μM/ml EDTA: Nakarai Tesque, Inc., Kyoto, Japan).

To evaluate the effect of laminin for osseous formation *in vivo*, scaffolds with a hollow center were used. The scaffolds were immersed in 0.5 mg/ml concentration of laminin (Mouse sarcoma origin: CHEMICON International, Inc., CA., USA) solution and dried by air. Scaffolds immersed in MEM were used as controls. The cell/HA composite scaffolds were soaked in bone marrow cell suspension at 1×10^6 cells/ml concentration. Specimens were kept in 5% CO_2 and 99% relative humidity for 2 hours at 37 °C in an incubator. After shaving and sterilization with povidone iodine, the dorsal skin of 7-week-old male Fischer 344 rats was incised under general anesthesia with intraperitoneal injection of sodium pentobarbital, and the scaffolds were respectively implanted in the subcutaneous tissue. Implanted scaffolds were removed 4 weeks postoperatively after euthanasia by halothane suffocation, decalcified in 10% formic acid solution,

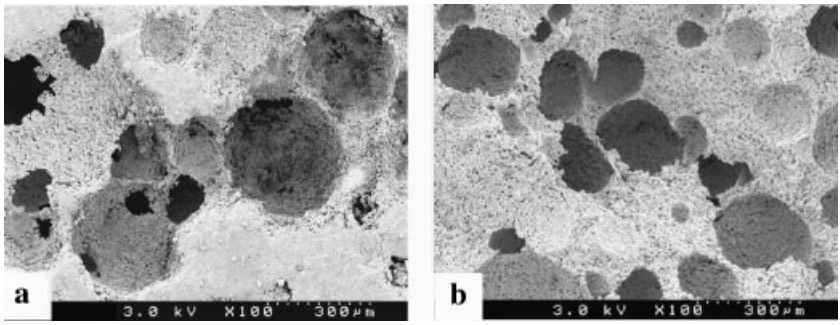

Figure 2.
SEM image of porous hydroxyapatite scaffold with a hollow center a: Surface of the scaffold. Apertures to pores of approximately 300 (m in diameter were seen. (Bar: 100 μm) b: Split surface of the scaffold. Interconnected pores of 150–300 μm in diameter were seen. (Bar: 100 μm)

embedded in paraffin, and 6 μm serial sections were made. The sections were stained with hematoxylin-eosin and histologically examined under an optical microscope.

Effect of laminin on osseous formation *in vitro* was evaluated in commercially available laminin-coated 6-multi well plates (Bio-coat^{TM}, Laminin coat 6-multi well plate; BD Biosciences). Uncoated 6-multi well plates were used as controls. Cell suspension at 1×10^5 cells/ml was prepared in MEM. The bone marrow cells of 2×10^5 cells contained in 2 ml of MEM were seeded in each well of both laminin-coated and uncoated 6-multi well plates. For 2 weeks, subculture was performed with MEM containing 15% FBS, 1 mM β-glycerophosphate (β-GP: EMD Biosciences, Inc., CA., USA), and antibiotics. The medium was renewed three times a week. To promote bone marrow cell differentiation and calcified nodule formation, 10 nM dexamethasone (Dex: ICN Pharmaceutical Inc., CA., USA) and 82 μg/ml ascorbic acid (Vc: Sigma-Aldrich Co., MO, USA) were added.

After 2 weeks, the cells were released from their culture substratum using trypsin-EDTA solution. In centrifuge tubes, the cells were washed three times with PBS (−). The cells in the buffer solution were homogenized and 0.1 ml of sonicated cell suspension was used for DNA measurement. Salmon sperm DNA was used as the standard. DNA measurements were obtained by fluorescence emission at 460 nm in the presence of 0.5mg/ml of Hoechst 33258 (ICN Pharmaceutical Inc.).[5] For measurement of alkaline phosphatase (ALP) activity, another sonicated cell suspension was used. The suspension was centrifuged at 16,000×g and *p*-nitrophenylphosphate (Zymed Laboratories, Inc., CA., USA) was added to 0.1ml of the supernatant as a substrate. The ALP activity was represented as μM of *p*-nitrophenol released after 30 minutes of incubation at 37 °C. The results are expressed as means ± standard error. Difference between each group was analyzed by unpaired two-tailed Student's t-test ($P < 0.005$).

Cultured bone marrow cell differentiation was estimated by ALP stain. Substrate solution composed of Naphtol-AS-MX phosphate sodium salt (MP Biomedicals, CA., USA) and Fast Red Violet LB salt (ICN Pharmaceutical Inc.) was added and incubated at room temperature for 5 minutes after fixation of the cells in the wells of a laminin-coated plate and an uncoated one for 12 hours by 4% paraformaldehyde.

Calcified product of the cells was stained by von Kossa's silver nitrate method. After fixation of the cells in the other laminin-coated plate and uncoated one, the wells were filled with acetone-ethanol (1:1) solution for 1 minute. Under sunlight, calcified product in the wells was soaked in 5% silver nitrate solution for 60 minutes.

Then, the product was reacted with 5% sodium thiosulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Moreover, the cells were counterstained with Kernechtrot solution for 5 minutes.

Results

After 4 weeks of implantation in the dorsal subcutaneous tissue of rats, it was found that bone was formed in most pores of the cell/HA composite scaffolds immersed in laminin solution before seeding of bone marrow cells (Figure 3, a). The connective tissue in the hollow center of the scaffold had become necrotic. In the cell/HA composite scaffold that had not been immersed in laminin solution before seeding of bone marrow cells, there was no bone detected in the pores (Figure 3, b). In the pores and the hollow center of the scaffold, vital connective tissue was present. In the pores of the scaffold immersed in laminin solution without seeding of bone marrow cells, connective tissue filled both the pores and the hollow center.

After 2 weeks of bone marrow cell subculture in MEM with Vc, β-GP and Dex, many macroscopic ALP-positive areas were observed in the culture plate with and without coating of laminin (Figure 4). Without the addition of Dex, ALP activity of cells with and without laminin coating on the culture plate remained very low in the culture. The results of ALP/DNA ratio were shown in Table 1. Between bone marrow cell culture in the plates with and without laminin coating, there was no significant difference.

It was found by von Kossa's stain that bone marrow cell culture in MEM with Vc, β-GP and Dex induced many mineralized areas on the well bottoms of culture plate with and without laminin-coating (Figure 5). Calcium deposition was also observed in the culture plate without laminin coating using MEM containing Vc, β-GP and Dex. Bone marrow cell culture on laminin-coated plate without Dex did not induce any calcium deposition.

Discussion

Multifunctional undifferentiated mesenchymal stem cells from bone marrow and periosteum[6] differentiate to chondroblasts, adipocytes or osteoblasts. Moreover, it was recently suggested that these cells show phenotypes of endothelial cells, neurocytes, smooth muscle cells and myocardial cells.[7] Mesenchymal bone marrow stem cells are used for osseous regeneration[8–10] and osseous healing.[11] Regeneration of tooth hard tissue or dentine-pulp complex may become practical by inducing osteogenesis in the pores of a porous

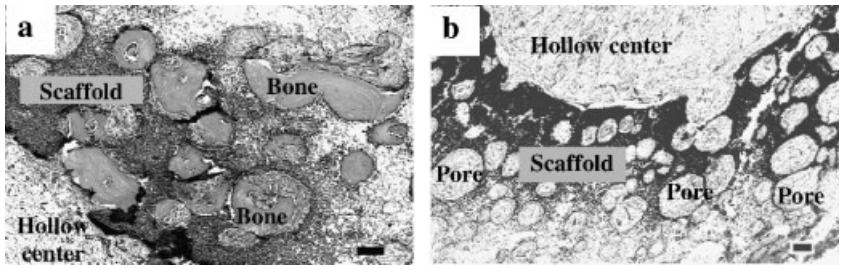

Figure 3.
Optical microscopic image of HA scaffold after 4 weeks of implantation in dorsal subcutaneous tissue a: HA scaffold immersed in laminin solution before seeding of bone marrow cells. Osteogenesis was recognized in the pores of the scaffold. Infiltrated connective tissue in the hollow center is necrotic. (Bar: 100 μm) b: HA scaffold without immersion in laminin solution before seeding of cells. There was no bone in the pores of the scaffold. Viable connective tissue was seen in the hollow center and pores in the scaffold. (Bar: 100 μm)

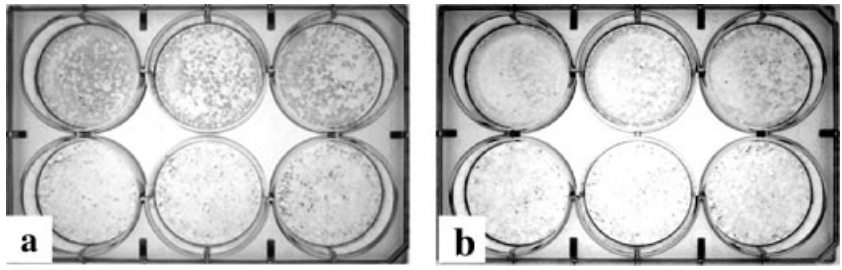

Figure 4.
ALP stain of subcultured bone marrow cells *in vitro*. a: Image of plate with laminin coating. The cells in the upper three wells were well stained. They were cultured in MEM containing Vc, β-GP and Dex. b: Image of plate without laminin coating. In the upper three wells, MEM containing Vc, β-GP and Dex was used.

scaffold. It is obvious that the use of undifferentiated mesenchymal cells in pulp tissue may be desirable for tooth regeneration. However, the number of human permanent teeth ranges from 28 to 32. Each human permanent tooth includes a small number of pulp cells. Moreover, there are not very many undifferentiated mesenchymal cells in pulp tissue. Pulp extirpation from a normal tooth may be disadvantageous for the tooth. There will need to be discussed before obtaining consensus on the application of cells for tooth regeneration. In this study, we attempted to regenerate the dentine-pulp complex using a small number of cells. It is considered that a circular cylindrical scaffold with a hollow center, resembling the dentine-pulp complex, is the most appropriate configuration for tooth regeneration. For subcutaneous implantation of a scaffold *in vivo*, cell suspension with a density of 1×10^7 cells/ml would commonly be used.[12] In this study, cell suspension with a density of 1×10^6 cells/ml was used. In this *in vivo* examination of osteogenesis in the cell/HA composite scaffold implanted heterotopy, no osteogenesis was recognized in the scaffold immersed in a suspension with a density of 1×10^6 cells/ml. However, osteogenesis was recognized in the pores of scaffolds immersed in a solution with the same cellular density after immersion in laminin solution.

It has been reported that laminin contributes to osteoblast-like cell propagation.[13] To induce osteogenesis *in vivo* by seeding of bone marrow cells in a porous HA scaffold, cell density of the suspension should be approximately 1×10^7 cells/ml or higher.[12] Based on the findings of this *in vivo* experiment, it was recognized that osteogenesis in a pore of an HA scaffold was induced by seeding with a bone marrow cell suspension of 1×10^6 cells/ml. It was clear that laminin promoted osteogenesis in cell/HA composite scaffolds. For cellular proliferation and differentiation, adhesion of cells to the substrate and cell-matrix bonding are required.[14] Before adhesion to the wall of the pores, many bone marrow cells seeded in the HA scaffold would be released from the pores by the flow of tissue fluid into the pores from the outside of the scaffold. However, many bone marrow stem cells would adhere to the pore wall

Table 1.
ALP activity of cells with and without laminin coating on the culture plate.

	Laminin coating plate		Non coating plate	
Dex in MEM	addition	non	addition	non
Mean (μmol/μg DNA)	0.174	0.051	0.169	0.062
Standard deviation	0.046	0.012	0.055	0.026

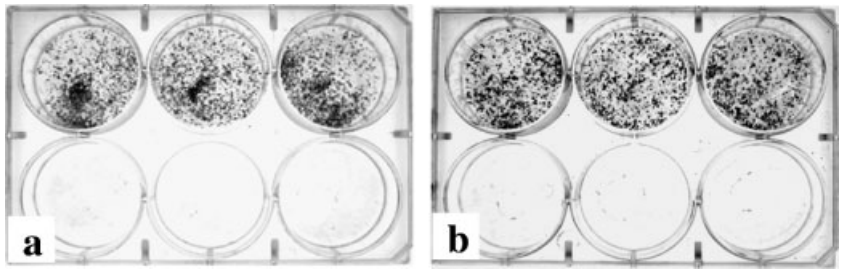

Figure 5.
von Kossa's stain of mineralized areas formed by bone marrow cell culture a: Image of plate with laminin coating. In the upper three wells, the cells were cultured in MEM with Vc, β-GP and Dex. There was no stain detected in the lower three wells. In the lower wells, the cells were cultured in MEM without Dex. b: Image of plate without laminin coating. Calcified product was only found in the upper three well. The cells were cultured in MEM with Vc, β-GP and Dex. In the lower wells, the cells were cultured in MEM without Dex.

of the scaffold due to the effects of laminin, and these would differentiate to osteoblast precursor cells or osteoblasts. It was shown in a previous study that laminin in bone marrow is an adhesion substrate for bone marrow stem cells and progenitor cells.[15] These studies also clearly showed ALP activity in a laminin-coated culture plate without the addition of Dex compared to that in a non-coated culture plate. A previous report [16] indicated that differentiation to osteogenic cells should be stimulated by human mesenchymal stem cells with laminin. The report suggested the possibility of high ALP activity of bone marrow cells by laminin. It is considered that laminin promotes differentiation of bone marrow cells to osteogenic cells and shows distinct ALP activity of the cells. Consequently, laminin would be considered an osteogenetic factor.

Conclusions

Effects of laminin as a factor for bone marrow cell differentiation and osteogenesis in pores of HA scaffold were examined in this study.

The osteogenesis in the pores of a cell/HA composite scaffold was dependent on soaking of the scaffold in laminin solution. The bone marrow cell differentiation and osteogenesis by Dex would be improved by laminin coating on the culture plate. In the culture of bone marrow cells on a laminin-coated culture plate, bone marrow cell differentiation and osteogenesis were improved by the addition of Dex to the culture medium.

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