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Osteogenesis by Dextran Coating on and among Fibers of a Polyvinyl Formal Sponge

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Abstract**—** A scaffold is necessary for tooth regeneration because of its three-dimensional geometry. For restoration of defect, it is necessary for the scaffold to be prepared in the shape of the defect. Sponges made from polyvinyl alcohol with formalin cross-linking (PVF sponge) have been used for scaffolds for bone formation *in vivo*. To induce osteogenesis within the sponge, methods of growing rat bone marrow cells (rBMCs) among the fiber structures in the sponge might be considered. Storage of rBMCs among the fibers in the sponge coated with dextran (10 kDa) was tried. After seeding of rBMCs to PVF sponge immersed in dextran solution at 2 g/dl concentration, osteogenesis was recognized in subcutaneously implanted PVF sponge as a scaffold *in vivo*. The level of osteocalcin was 25.28±5.71 ng/scaffold and that of Ca was 129.20±19.69 μg/scaffold. These values were significantly higher than those in sponges without dextran coating ($p<0.01$). Osteogenesis was induced in many spaces in the inner structure of the sponge with dextran coated fibers.

Keywords**—**Dextran, Polyvinyl formal sponge, Osteogenesis, Scaffold.

I. Introduction

IT is sure that regeneration of tooth is difficult because the tooth consists of ectodermic enamel, mesodermal dental pulp, dentin and cement. Restoration of partial defect of the tooth or reproduction of the whole tooth that had been missed for any reason should be a definitive goal of the regenerative therapy in the dentistry. Restoration of partial defect of the tooth or reproduction of the whole tooth that had been missed for any reason should be a definitive goal of the regenerative therapy in the dentistry.

It is well-known that bone consists of an apatite structure like dentine. Osteocalcin which should be synthesized by osteoblasts is present in dentine as calcium-binding protein [1, 2]. Then, regenerated bone might be used as dentine for the restoration of teeth. Because of the three dimensional structure, a scaffold for proliferation and differentiation of bone marrow stem cells and osteogenesis is required for tooth or bone regeneration. Polyvinyl alcohol (PVA) sponge with bone marrow cells was already used for bone regeneration in the defective part of bone [3]. In this study, commercially available polyvinyl formal (PVF) sponge was selected as a scaffold. However, the sponge may be inappropriate to keep cells because of the fibrous construction. One of the methods for seeded many cells to attach in the scaffold is coating of chemical substance with cell adhesive effects [4]. Dextran is a kind of polysaccharide existing in extracellular matrix and shows superior biocompatibility [5]. Dextran promotes adhesion between protein and cells [6].

The purpose of this experiment was to confirm effect of dextran to induce stem cell differentiation in dextran coated PVF sponge. To confirm the effects of dextran and the availability the PVF sponge as a scaffold, coating of a PVF sponge with dextran was accomplished by immersion in dextran solution and *in vivo* examinations were performed.

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II. Materials and Methods

A. Experimental animals

In this study, 6- and 7-week-old male Fischer 344 rats (CLEA Japan Inc., Tokyo, Japan) were used. The Animal Welfare Committee of Osaka Dental University approved the experimental procedures regarding use and care of animals in this study. This study was performed under the Guidelines for Animal Experimentation at Osaka Dental University.

B. Preparation of rat bone marrow cell suspension

Rat bone marrow cells (rBMCs) were obtained from the bone shaft of femora of six of 6-week-old male Fischer 344 rats after euthanasia by intraperitoneal overdose of sodium pentobarbital essentially according to the methods described in previous reports [7, 8]. Both ends of the femur were cut off at the epiphysis and bone marrow was flushed out with 10 ml of minimum essential medium (MEM: Nakalai Tesque Inc., Kyoto, Japan) expelled from a syringe through a 21-gauge needle. Primary culture of rBMCs was performed in MEM supplemented with 15% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) for 1 week. The cells were isolated from the bottom of T75 flask with trypsin-EDTA (0.5 mg/ml trypsin and 0.53 μmol EDTA) solution. The cells were re-suspended in culture medium at 1×10^7 cells/ml concentration.

C. Polyvinyl formal (PVF) sponge and rBMCs seeding

The PVF sponges with cubic configuration (5×5×5 mm) were obtained by cutting from a sheet. The diameter of pores was 130 μm in an average. The PVF sponges were sterilized in ethylene oxide gas before use.

Dextran with 10 kDa of molecular weight was purchased (Sigma-Aldich) and dissolved at 2 g/dl concentration in ultra

purified water and sterilized by filtration (0.22 μm). PVF sponges were immersed in the dextran solution for 24 hours. The sponges used as a control were immersed in ultra purified water. Micro structure of PVF sponges with and without immersion in dextran solution were observed with scanning electron microscopy.

Each of the PVF sponges with and without immersion in dextran solution were respectively seeded with 1×10^6 rBMCs in a 0.1ml cell suspension and were incubated for 3 hours in 5 % CO_2 and 95% relative humidity at 37°C to promote cell adhesion in the sponges.

D. Implantation of PVF sponge with rBMCs in subcutis

The dorsal skin of 7-week-old male Fischer 344 rats was incised close to the scapula on both sides across the vertebra at right angles and subcutaneous pockets were made using a mucosal raspatory. All rats were respectively implanted with four scaffolds in individual subcutaneous pockets alongside the vertebra. Two scaffolds without dextran coating but with rBMCs seeding were inserted in the left subcutaneous pocket of three rats and the other two with dextran coated and rBMCs seeding were inserted in the right side of the other three rats.

E. Histological examination of implanted PVF sponge

Implanted scaffolds were removed from the dorsal subcutis of the rats 4 weeks postoperatively. After decalcification, the scaffolds were embedded in paraffin and 6 μm serial sections were made. All sections were stained with hematoxylin-eosin and examined under an optical microscope.

F. Osteocalcin and calcium in implanted PVF sponge

The other remaining sponges removed from rat subcutis were frozen and crushed respectively. The crushed samples were respectively sonicated and centrifuged at 16,000×g. Quantity of osteocalcin in the supernatants were measured immunochemically by Rat Osteocalcin ELISA kit (DS Pharma Biomedical Co., Ltd., Osaka, Japan). Each precipitation was decalcified and calcium quantity was measured biochemically using Calcium-E test WAKO (Wako Pure Chemical Co. Inc., Osaka, Japan). Data were statistically analyzed using Tukey-Kramer's test ($p<0.01$).

III. Results

A. SEM examination of PVF sponge

The SEM image of the PVF sponge without immersion in dextran solution showed reticular configuration as in Fig. 1-a. Sizes of the fibers ranged from about 5 μm in the fine portion to 150 μm in the thick portion. The large nodes formed a spacious shelf and extended fibrous branches in every direction. The major axes were 150 to 250 μm.

The fibers of PVF sponge immersed in dextran solution were larger in diameter than those of sponges without immersion in dextran solution. Each fiber presented a thick plate with a width of 150-300 μm (Fig. 1-b). The fibers of the sponge seemed to be covered with viscous substance.

Sponge kept in MEM for 4 weeks after immersion in dextran solution was shown in Fig. 1-c. The possibility that the dextran coating on the fiber was preserved under rBMCs seeding and during subcutaneous tissue implantation was shown. The surface of the substance on the fiber of sponge just after immersion in dextran solution was smoother than the surface of the sponge stored in MEM.

B. Histological examination of implanted PVF sponge

PVF sponge as a scaffold after a 4-week implantation, there was no bone recognized in the sponge without immersion in dextran solution prior to seeding of rBMCs as shown in Fig. 2. Densely arranged fibrous connective tissue infiltrated the fibers of the sponge. In the sponge with immersion in dextran solution prior to rBMCs seeding, conspicuous osteogenesis was recognized on fibers accompanied by infiltration of connective tissue (Fig. 3).

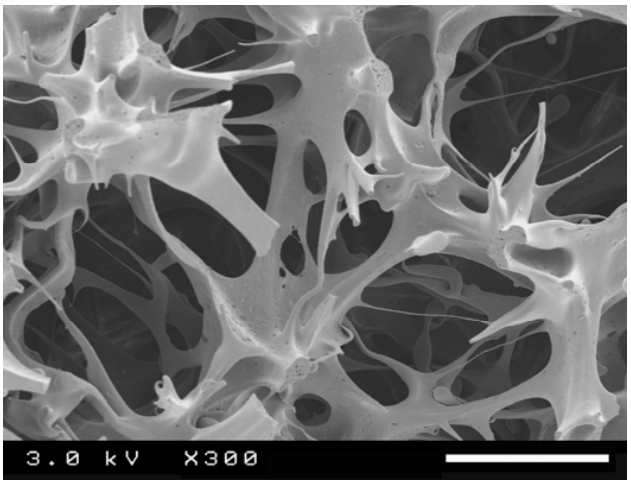

Fig. 1-a SEM image of PVF sponge without immersion in dextran solution
Fine fibers connected with each other were observed in the interior of the sponge. (Bar: 100μm)

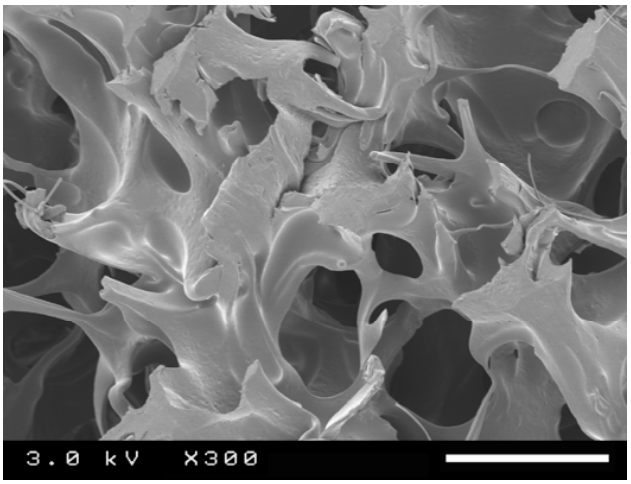

Fig. 1-b SEM image of PVF sponge immersed in dextran solution
The fibers were covered with viscous substance. (Bar: 100μm)

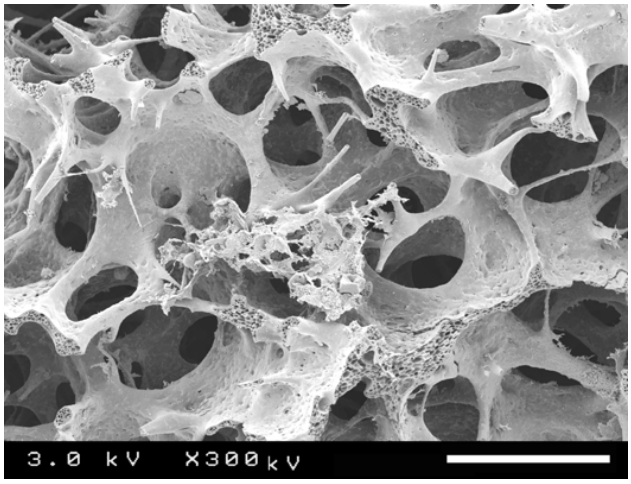

Fig. 1-c SEM image of PVF sponge immersed in dextran solution
After storage in MEM for 4 weeks following immersion in dextran solution (Bar: 100μm)

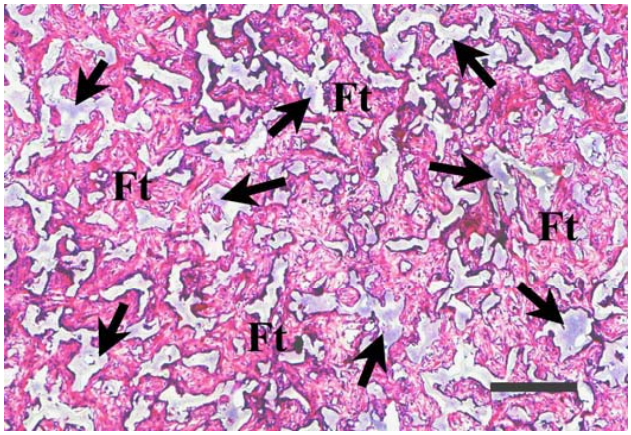

Fig. 2 Implanted PVF sponge without immersion in dextran solution before implantation
There was no apparent bone in the sponge. Between fibers of the sponge, densely arranged fibrous connective tissue was seen.
Ft: Fibrous tissue infiltrated in the sponge Arrow: Fiber of sponge (Bar: 500 μm)

C. Immunochemical quantitative analysis of osteocalcin in implanted sponge

In the sponge without immersion in dextran solution before rBMCs seeding and subcutaneous tissue implantation, 9.42 ± 5.67 ng/scaffold of osteocalcin was measured (Fig. 4). On the other hand, 25.28 ± 5.71 ng/scaffold of osteocalcin was detected in the sponge immersed in dextran solution. There was a significant difference between the sponges with and without immersion in dextran solution ($p<0.01$).

D. Biochemical quantitative analysis of calcium in implanted sponge

Quantity of calcium detected in the implanted sponge with immersion in dextran solution before rBMCs seeding was

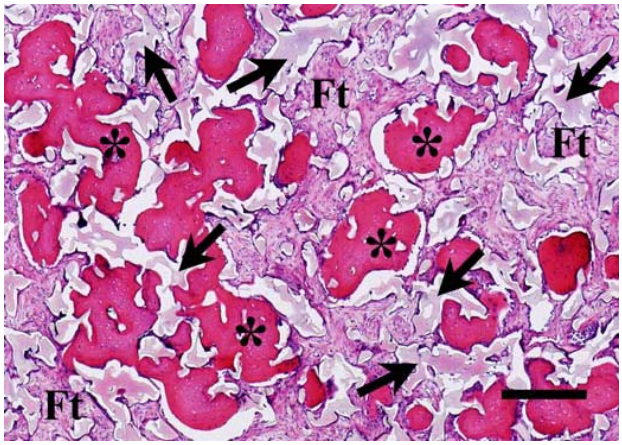

Fig. 3 Implanted PVF sponge immersed in dextran solution before implantation
Conspicuous osteogenesis was recognized among the fibers of sponge. Infiltrated fibrous connective tissue was also seen.
Ft: Fibrous tissue infiltrated in the sponge Arrow: Fiber of sponge * : Bone (Bar: 500 μm)

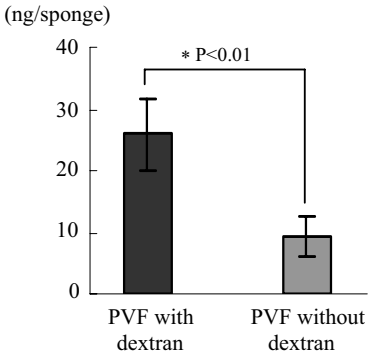

Fig. 4 Quantity of osteocalcin in implanted PVF sponge

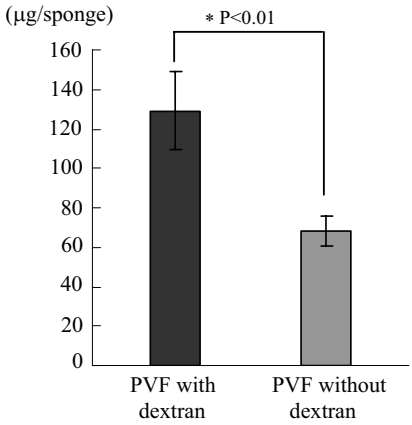

Fig. 5 Quantity of osteocalcin in implanted PVF sponge

129.20 ± 19.69 μg/scaffold (Fig. 5). In implanted sponge without immersion in dextran solution, detected quantity of calcium was 79.41 ± 8.69 μg/scaffold.

There was a significant difference between the implanted sponges with and without immersion in dextran solution followed by seeding of rBMCs. There was a significant difference between the sponges with and without immersion in dextran solution ($p<0.01$).

IV. Discussion

Tooth configurations vary, and there are differing origins of hard tissues and pulp that construct a tooth. Therefore, tooth regeneration is difficult for the complicated configuration [9].

For tooth regeneration, several steps will be required, such as restoration of the defect by regeneration of hard tissue, realization of endothelial and fibrous conjugation to a regenerated root, regeneration of the dentine-pulp complex, and then, complete tooth regeneration.

For restoration of a tooth defect, stem cells should be differentiated into odontoblasts from odontoblast precursor cells in a scaffold. Therefore, it would be desirable for the scaffold to be configurated according to the defect. PVF sponge is considered a desirable scaffold material because it can easily be shaped to the configuration of the defect. PVF scaffold has previously been used for three-dimensional *in vivo* osteogenic examination [10].

On SEM observation, pores measuring about 100-280μm in diameter were recognized in PVF sponge. It was reported that such a pore size is suitable for osteogenesis [11]. This finding suggests that osteogenesis in the PVF sponge is possible. However, there is a conspicuous difference in the internal structures of a hydroxyapatite (HA) scaffold and those of a PVF scaffold. Reticular structure of PVF sponge was shown by SEM findings. Stem cells seeded in PVF sponge may flow out of the sponge with the suspension, while HA scaffold, stem cells may be taken into the pores. The PVF sponge is an unfavorable substrate for adhesion of BMCs.

Therefore, for osteogenesis in PVF sponge by subcutaneous tissue implantation, the sponge should be modified so that seeded stem cells could adhere in the sponge. In fact, it was reported that modification of PVA by extracellular matrix is necessary for adhesion and proliferation of stem cells in the sponge [12].

In this *in vivo* study of seeding bone marrow cells in PVF sponge, there was no osteogenesis recognized in the sponge without dextran coating. This finding showed that rBMCs did not adhere to the construction in the PVF sponge. PVA having aldehyde groups and hydroxyl groups as functional groups reacts with formalin and produces acetal in the intramolecule of PVA.

As a result, it is thought that these functional groups were covered and lost reactivity. PVF sponge without functional group could not be used as a scaffold for osteogenesis by BMCs.

Intercellular adhesion involves direct binding by cell adhesion molecule in an extracellular matrix or cell membrane [12]. In addition, the cells are activated by stimulus from cytokine and adhesive high polymer and adhere to the surface of the scaffold [13]. By coating the inner structures of PVF with a highly cytotropic substance, adhesion of seeded stem cells is enabled.

It is known that dextran is a major extracellular matrix and it is a natural macromolecule polysaccharide. Furthermore, dextran shows excellent biocompatibility and adhesiveness to protein and cells [6]. It has also been reported that dextran promotes differentiation of BMCs to osteoblasts [14]. Therefore, rBMCs may adhere to PVF sponge through dextran. It is evident that stable adhesion may occur between PVF sponge and dextran [15].

Based on the SEM findings in this experiment, it seemed that the internal structures of the PVF sponge were covered with a layer of dextran after immersion in the solution. Osteogenesis was subsequently recognized in the PVF scaffold that had been immersed in dextran solution. These findings show that dextran adhered sufficiently to the internal structure of the sponge and facilitated the attachment of the rBMCs.

Greater osteogenesis was induced by 10kDa of dextran in PVF sponge than by higher molecular weight dextran. It is necessary to retain BMCs in the PVF sponge for effective osteogenesis. One method to achieve the purpose appears to be using low-molecular-weight dextran to coat the scaffold.

It was suggested in this study that coating PVF sponge with low-molecular-weight dextran (10kDa) effectively promoted osteogenesis in the sponge.

V. Conclusion

For regeneration of a tooth defect, in this study, PVF sponge was selected as the scaffold because it could be easily shaped and showed excellent biocompatibility.

In addition, it was considered that dextran was useful to promote adhesion of BMCs. *In vivo* osteogenesis by rBMCs in PVF sponge as a scaffold with dextran coating was examined histologically, biochemically and immunochemically.

The findings obtained from this study were as follows.

1. rBMCs did not adhere to untreated PVF sponge and no bone was formed.
2. The fiber in PVF sponge should be covered with a layer of the dextran by immersion in a solution of 10kDa dextran.
3. In PVF sponge immersed in dextran solution, the dextran layer coating the inner structure of the sponge persisted after 4 week immersion in MEM.
4. In PVF sponge coated with dextran, osteogenesis by rBMCs was promoted.

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