Dental Materials Journal 2010; 29(6): 673–681

Effectiveness of scaffolds with pre-seeded mesenchymal stem cells in bone regeneration —Assessment of osteogenic ability of scaffolds implanted under the periosteum of the cranial bone of rats—

Shunsuke BABA[1], Takeomi INOUE[1], Yoshiya HASHIMOTO[2], Daisuke KIMURA[3], Masatoshi UEDA[4], Kana SAKAI[5], Naoyuki MATSUMOTO[6], Chiaki HIWA[7], Taiji ADACHI[8] and Masaki HOJO[8]

[1]*Department of Regenerative Medicine, Foundation for Biomedical Research and Innovation, 2-2 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan*
[2]*Department of Biomaterials, Osaka Dental University, 8-1 Kuzuhahanazono-cho Hirakata, Osaka 573-1121, Japan*
[3]*PhD Course, Graduate School of Dentistry (Periodontology), Osaka Dental University, Department of Periodontology, Osaka Dental University, 8-1 Kuzuhahanazono-cho Hirakata , Osaka 573-1121, Japan*
[4]*Department of Periodontology, Osaka Dental University, 8-1 Kuzuhahanazono-cho Hirakata, Osaka 573-1121, Japan*
[5]*PhD Course, Graduate School of Dentistry (Orthodontics), Osaka Dental University, Department of Orthodontics, Osaka Dental University, 8-1 Kuzuhahanazono-cho Hirakata , Osaka 573-1121, Japan*
[6]*Department of Orthodontics, Osaka Dental University, 8-1 Kuzuhahanazono-cho Hirakata, Osaka 573-1121, Japan*
[7]*Core Research Team, Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan*
[8]*Department of Mechanical Engineering and Science, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*
Corresponding author, Yoshiya HASHIMOTO; E-mail: yoshiya@cc.osaka-dent.ac.jp

To date, there has been no study on the development of novel regimens based on the following tissue engineering principles: seeding and culturing mesenchymal stem cells (MSCs) on a scaffold before surgery or injecting cultured MSCs into a scaffold during surgery. The purpose of this study was to assess the *in vivo* osteogenic ability of scaffold/MSCs implanted beneath the periosteum of the cranial bone of rats in three different sample groups: one in which MSCs were pre-seeded and cultured on a scaffold to produce the 3-D woven fabric scaffold/MSC composite using osteo-lineage induction medium, one in which cultured MSCs produced by osteo-lineage induction in cell cultivation flasks were injected into a scaffold during surgery and a control group, in which only the 3-D woven fabric scaffold was implanted. The results indicate that pre-seeding MSCs on a scaffold leads to a higher osteogenic ability than injecting cultured MSCs into a scaffold during surgery.

Keywords: Scaffold, MSC, Bone regeneration, Poly-L-lactic acid fibers

INTRODUCTION

In dental implant treatments, oral surgeons often encounter an insufficiency of alveolar bone at implantation sites. Despite the increasing need for bone augmentation to facilitate implant placement, effective bone substitutes remain few on the market. Although highly invasive for patients, autogenous bone grafts still remain the gold standard in clinical practice. Guided Tissue Generation[1)] and Emdogain gel[2)] are used for regenerative therapy of advanced periodontal diseases involving vertical bone defects. Neither of these therapies, however, is indicated for cases associated with horizontal bone defects. Thus, the development of additional treatment methods for advanced periodontal diseases is needed.

In recent years, tissue engineering to enhance regeneration has been attracting attention. Tissue engineering is the process of harvesting cells from the tissue to be regenerated, culturing them in an *in vitro* environment, and implanting the cultured cells in the body to increase the efficiency of tissue regeneration. Tissue engineering, therefore, requires the cells from the tissue to be regenerated, a scaffold to retain the cells, and growth factors to induce cellular proliferation and differentiation[3)].

For alveolar reconstruction during dental implant treatment, the authors used tissue engineering to develop a bone regeneration procedure by which mesenchymal stem cells (MSCs), harvested from the patient's bone marrow and platelet-rich plasma (PRP) prepared from autogenous blood, are implanted. The usefulness of the procedure is addressed in our previous reports[4,5)]. Furthermore, the authors reported the clinical applications of this procedure for regenerative therapy of periodontal diseases[6)]. To enhance bone regeneration by securing the spaces for the implanted cells to proliferate and differentiate, the authors have developed a 3-D woven fabric composite scaffold.

The 3-D woven fabric composite scaffold is made from biodegradable poly-L-lactic acid (PLLA) fibers and designed to secure internal spaces to retain the seeded MSCs. Graft materials are injected into the spaces between the fibers of the scaffold either before or during surgery. The osteogenic ability of two different procedures —pre-seeding human marrow MSCs on the scaffold and culturing them before surgery and injecting *ex vivo* cultured MSCs into the scaffold during surgery— was evaluated under the periosteum of the cranial bone of rats.

Color figures can be viewed in the online issue, which is available at J-STAGE.
Received Nov 20, 2009: Accepted Jul 2, 2010
doi:10.4012/dmj.2009-123 JOI JST.JSTAGE/dmj/2009-123

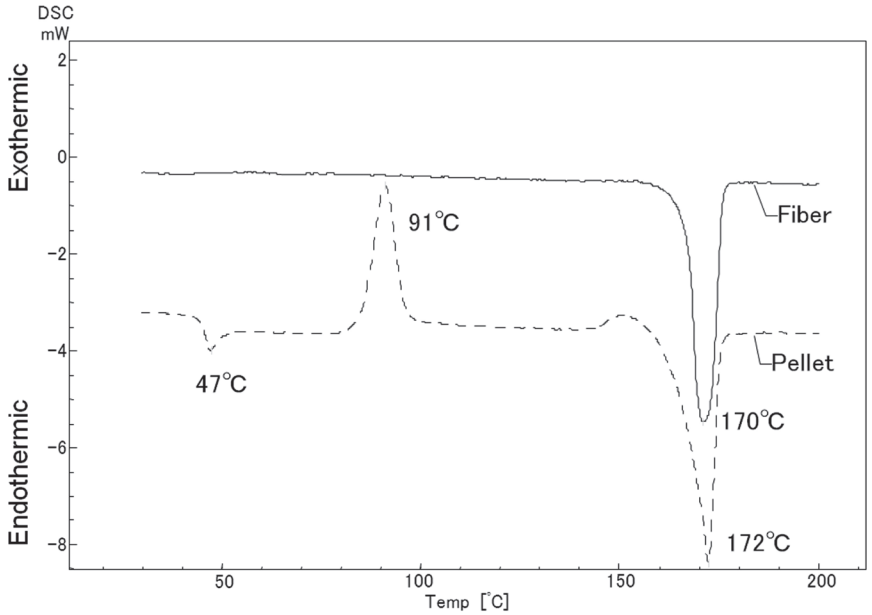

Fig. 1 Differential thermal analysis of PLLA fibers. The raw material pellets had a glass transition temperature of 47°C and a crystallization temperature of 91°C. On the other hand, no glass transition and crystallization temperatures were detected with the PLLA fibers, but the latent heat of melting increased from 54 J/g (pellets) to 68 J/g (monofilaments).

MATERIALS AND METHODS

3-D woven fabric composite scaffold

PLLA[7,8] manufactured at a United States Food and Drug Administration current good manufacturing practice (cGMP) compliant production facility (Birmingham Polymers, Inc., Birmingham, USA) was used in this study. The PLLA had a density of 1.24 g/cm^3, a melting point of 172°C, and an average molecular weight of 160,000 Da. About 300 meters of PLLA monofilament (0.09 mm in diameter) was produced from each lot (2.5 grams). For the production of PLLA fibers, two-step heat-drawing, comprising a primary drawing (draw ratio: 4-fold) and a secondary drawing (draw ratio: 2-fold), was chosen. Figure 1 shows the raw material pellets of the PLLA used in this study and the results of the differential thermal analysis. The PLLA pellets had the glass transition (47°C) and crystallization (91°C) temperatures associated with the presence of amorphous molecules. On the other hand, with the PLLA fibers, no glass transition and crystallization temperatures were detected but the latent heat of melting increased from 54 J/g (pellets) to 68 J/g (monofilaments). These findings indicate that, during the two-step heat-drawing, most of the molecular chains were arranged in the axial direction of the fibers.

The PLLA fibers were woven to form a 3-D scaffold such that the weft fiber (X-Y direction) was drawn under and over the warp fibers (Z direction) that were arranged in a grid pattern perpendicular to an orthogonal weaving base (12×10×10 mm^3; 1.5 mm pitch) (Fig. 2).

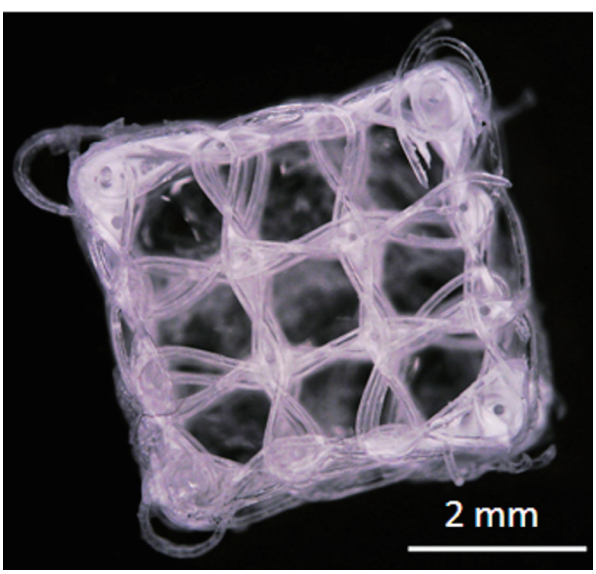

Fig. 2 3-D woven fabric composite scaffold. The PLLA fibers were woven to form a 3-D-structured scaffold.

Animals

The animal experiment (JBS-06-RXGE-380) was approved by the Institutional Animal Care and Use

Committee of the Japan Biological Science Center (JBS Inc., Gifu, Japan), in accordance with the Guide for the Care and Use of Laboratory Animals.

Six-week-old male nude rats (F344/NJcl-rnu/rnu) bred by CLEA Japan, Inc. were purchased. They underwent quarantine and habituation for 9 days, and their general condition and survival were monitored once daily. The body weight of each rat was measured on the day after delivery and at the end of the 9-day period. Nine rats, which showed healthy development during the quarantine and habituation period, were used for the study. The body weight of the rats on the day of surgery was within the range of 138.2 to 153.2 grams.

Cell culture

Human marrow MSCs (Lot No. 4F0312, Cambrex Co., East Rutherford, USA) were used in this study. After being recovered from the frozen state, the cells within three culture passages were used. Dulbecco's modified Eagle medium (Invitrogen Co., Carlsbad, USA) was used as the basal medium, to which fetal bovine serum (FBS, Invitrogen Co., Carlsbad, USA), 200 mM L-glutamine solution (Invitrogen Co., Carlsbad, USA), and antibiotic-antimycotic (100×) liquid (Invitrogen Co., Carlsbad, USA) were added to produce the final concentrations of 10% FBS, 4 mM L-glutamine, and 1% antibiotic-antimycotic.

Cells were seeded at a density of 2,000 cells/cm^2 in a polystyrene cell culture flask with a base area of 75 cm^2 (Falcon, Becton Dickinson Company, Franklin Lakes, USA), and the culture medium was replaced once every 2 to 3 days. Cells were cultured in a carbon dioxide gas incubator (CO_2 gas concentration 5%, temperature 37°C, and relative humidity 100%). Cells were subcultured when a subconfluent cell layer was formed after 5 to 7 days of incubation. Adhered cells were exfoliated with 0.05% trypsin-0.53 mM EDTA solution (Invitrogen Co., Carlsbad, USA). The cells were collected by centrifugation at 500 g for 5 minutes and then seeded in a new culture flask at a density of 2,000 cells/cm^2.

Cell morphology and proliferation on the 3-D woven fabric composite scaffold

The cell suspension (2×10^6 cells/mL) was prepared by adding 1×10^5 cells into 50 μL of growth medium and was injected into a 3-D woven fabric composite scaffold on a rectangular solid form of 5×5×2 mm^3. Then, the scaffold with adhered cells was moved to a 48-well plate and incubated with 0.5 mL of growth medium. After incubation for 24 hours, cells were fixed for 10 minutes in phosphate-buffered saline (PBS) containing 0.5% (w/w) Triton X-100, 4% (w/w) paraformaldehyde, and 5% (w/w) sucrose. The fixed cells were fluorescently stained for 1 hour with PBS containing 6×10^{-8} M phalloidin (Molecular Probes, OR, USA). Cell morphology was determined using an automated fluorescence microscopy system (BZ-9000, Keyence, Osaka, Japan) equipped with a computer-controlled translation stage. After incubation for 24 and 72 hours, the lysates obtained from the cells were homogenized in 10 mM Tris (pH 7.4) and sonicated for 15 minutes on ice. A fluorometric assay was performed to ascertain the total amount of DNA. Briefly, immediately before the analysis, the lysate was adjusted to PicoGreen (Molecular Probes, OR, USA). Fluorescence was then quantified on a SpectraMax M5 (Molecular Devices, CA, USA) at an excitation wavelength of 435 nm and an emission wavelength of 455 nm.

Preparation of platelet-rich plasma

On the day before surgery, 50 mL of venous blood was taken from the median antebrachial vein of each volunteer and placed in a conical tube system for PRP preparation. The volunteers were in good health, and we received prior informed consent from all of them. A citrate phosphate dextrose (CPD) solution (Kawasumi Chemical Co., Japan) was used as an anticoagulant at a ratio of 14 mL of CPD in 100 mL of peripheral blood. The blood was centrifuged at 1,100 g for 4 minutes and the supernatant, except erythrocytes and buffy coats, was removed into an empty conical tube. Then, the blood was subjected to a second centrifugation at 3,000 g for 6 minutes, and the supernatant in an amount equivalent to one-tenth (5 mL) of the volume of the collected blood was retained and stored in a reciprocal shaker. On the day of surgery, platelet pellets were evenly suspended to produce PRP.

Implant tests under the periosteum of the cranial bone of nude rats by 3 methods using PLLA mesh

All 9 nude rats received intraperitoneal administration of thiopental sodium for anesthesia. Hair at the surgical site was removed as necessary. Each rat was immobilized on a table and the surgical site was carefully cleaned by spraying alcohol so that no shaved hairs were left. The surgical site and its surrounding area were further disinfected with Isodine. An incision was made in the skin of the head and the subcutaneous flap was fully elevated. The periosteum of the skull was detached with a sterilized swab to create a pocket in which to place the graft material.

Three groups were prepared for the experiments (Fig. 3). In Group A, the cell suspension (2×10^6 cells/mL) was prepared by adding 1×10^5 cells into 50 μL of growth medium, and then injecting into a 3-D woven fabric composite scaffold on a rectangular solid form of 5×5×2 mm^3. The scaffold was left still in a carbon dioxide gas incubator (5% CO_2, 37°C, and 100% relative humidity) for 3 hours after seeding. Then, the scaffold with adhered cells was moved to a 6-well plate and supplemented with 5 mL of growth medium (differentiation-inducing medium) containing 50 μM ascorbic acid-2-phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 100 mM β-glycerophosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 10^{-8} M dexamethasone (SIGMA-ALDRICH Inc., St. Louis, MO, USA) as their final concentrations to induce the cells to undergo

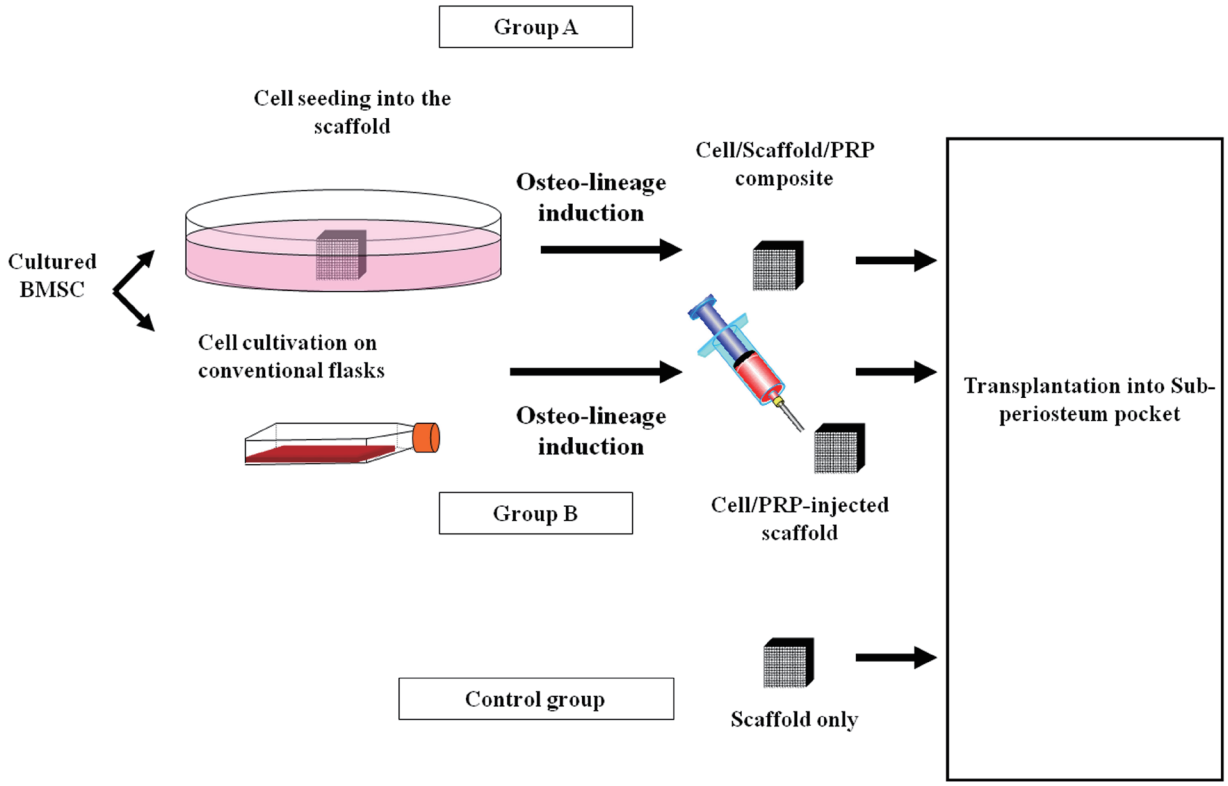

Fig. 3 Experimental concept. In Group A, the cell/scaffold composites, which were prepared by pre-seeding human MSCs onto the scaffold were implanted in combination with coagulated PRP. In Group B, the graft materials were prepared immediately before implantation by injecting a coagulated cell/PRP mixture into the scaffolds. In the control group, the 3-D-woven scaffolds were implanted into subperiosteum pockets created on the calvaria of immune-compromised rats.

differentiation. After 7 days of induced differentiation, the cell/3D scaffold composite produced was inserted into the subperiosteal pocket of 3 rats after being mixed with 1mL of PRP (Fig. 4). The PRP added to the cell/scaffold composite at this stage had been precoagulated by connecting a 2.5 mL disposable syringe containing 1mL of PRP and a 2.5-mL disposable syringe containing about 160 μL of thrombin/$CaCl_2$ solution with a three-way stopcock and exchanging the contents in the two syringes several times.

For Group B, the cells were prepared in a polystyrene cell culture flask with a base area of 75 cm^2, and cultured with 10 mL of growth medium (differentiation-inducing medium) as described above to induce the cells to undergo differentiation. After 7 days of induced differentiation, osteoblast-like cells were exfoliated with 0.05% trypsin-0.53 mM EDTA solution. The human osteoblast-like cells (2×10^6 cells) were collected by centrifugation at 500 g for 5 minutes and were ready for implantation. The cell/PRP mixture was coagulated using thrombin and injected into a 3-D woven fabric composite scaffold for implantation. A 2.5-mL disposable syringe, which contained a 1-mL mixture of cells and PRP, and a 2.5-mL disposable syringe which contained about 160 μL of thrombin/$CaCl_2$ solution were connected with a three-way stopcock to exchange the contents in the two syringes several times to produce a gel-like graft material. This graft material was injected into a $5\times5\times2$ mm^3 3-D woven fabric composite scaffold and inserted into the subperiosteal pocket created in 3 rats.

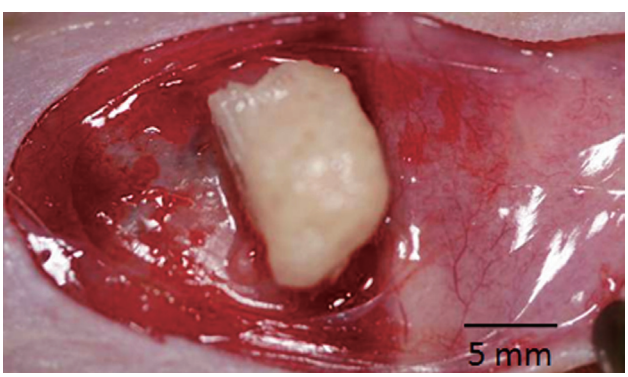

Fig. 4 A 3-D-structured scaffold placed in a subperiosteum pocket. A $5\times5\times2$ mm^3 3-D woven fabric scaffold was implanted immediately after the injection of the gel-like cell/PRP mixture.

For the Control group, the 3-D woven fabric composite scaffold (5×5×2 mm^3) was rinsed in physiologic saline solution 3 times and placed in the subperiosteal pocket of the rats.

At the end of the observation period (8 weeks after grafting), the animals were euthanized by exsanguination under intravenous narcosis using sodium pentobarbital. All animals were visually checked for any abnormality in the organs or tissues, and then the tissues peripheral to the implanted graft (including the cranial bone) were excised and fixed in 10% formalin neutral buffer solution. After fixation, the tissues were decalcified by hydrochloric acid to prepare paraffin blocks. The cranial bone at the grafted site was sliced into coronal sections. A section in which the edge of the graft material was exposed was identified, and from this section, three 3 μm-thick consecutive sections were sliced. These sections were stained with hematoxylin-eosin stain and examined microscopically.

Histometric analysis

Histometric analysis of newly formed bone was carried out using automated fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan). Computerized BZ-H1M (Keyence, Osaka, Japan) was used for histomorphometric measurements to calculate the total area of newly formed bone in each group.

Statistical analysis

The mean and standard deviation of each parameter were obtained for each group. Data on cell proliferation were analyzed using the Student's *t* test.

RESULTS

The morphology and proliferation of the cells are summarized in Figs. 5 and 6. The MSCs on the scaffold spread extensively and showed apparent actin stress fibers in 24 hours (Fig. 5). A slight increase in total DNA level was found in the MSCs grown on the scaffold for 24 to 72 hours (Fig. 6) ($p<0.05$).

In Group A, in which the cell/scaffold composite was implanted immediately after being combined with PRP, the graft materials were coagulated using thrombin/$CaCl_2$ solution so that a sufficient amount of gel-like graft materials could be retained inside the scaffold. Histologic sections of Group A at 8 weeks after grafting are shown in Fig. 7. At this time point, the remaining PLLA fibers were still clearly observed and island-like formations of new bone were found, not on the PLLA fibers to which human osteoblast-like cells were adhered, but in the spaces between the fibers.

In Group B, the mixture of human osteoblast-like cells and PRP was coagulated with thrombin, injected into the 3-D woven fabric composite scaffold, and grafted. Bone regeneration was found inside the scaffold (Fig. 8).

In the control group in which only the scaffold was implanted (Fig. 9), a sign of new bone formation was found in the scaffold. However, new bone formation was observed in only one rat among 3 individuals.

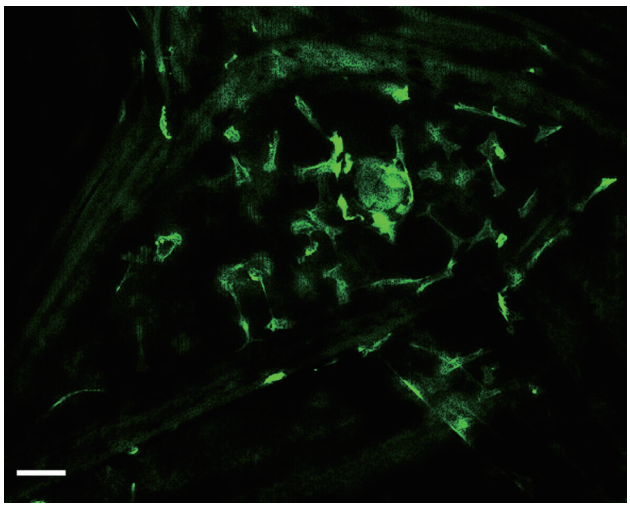

Fig. 5 Morphology of MSCs on a 3-D woven fabric scaffold. MSCs (1×10^5 cells per scaffold) were incubated on a 3-D woven fabric scaffold for 24 hours. Cells were fixed and then stained with phalloidin (bar=100 μm).

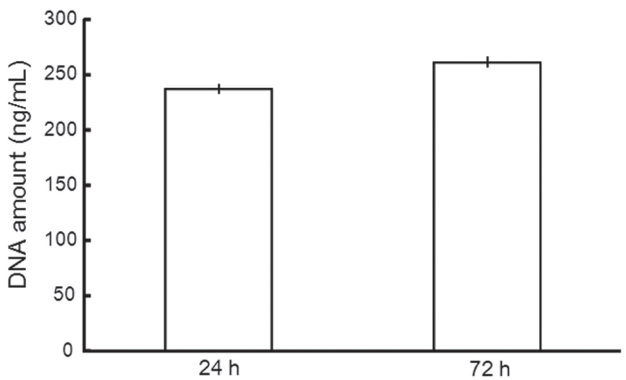

Fig. 6 Total amount of DNA in the MSC/scaffold composite. MSCs (1×10^5 cells per scaffold) were incubated on a 3-D woven fabric scaffold for 24 and 72 hours. Cells proliferation was determined by fluorometric assay.

The results of the histometric quantitative analysis are shown in Fig. 10. The percentage of newly formed bone area in the scaffold was measured in each group. The amount of new bone in the scaffold increased with the presence of the pre-seeded MSCs and PRP. Accordingly, the area of new bone in Group A was greater than that in Group B and the Control group ($p<0.05$).

DISCUSSION

The effectiveness of scaffolds made from bioabsorbable

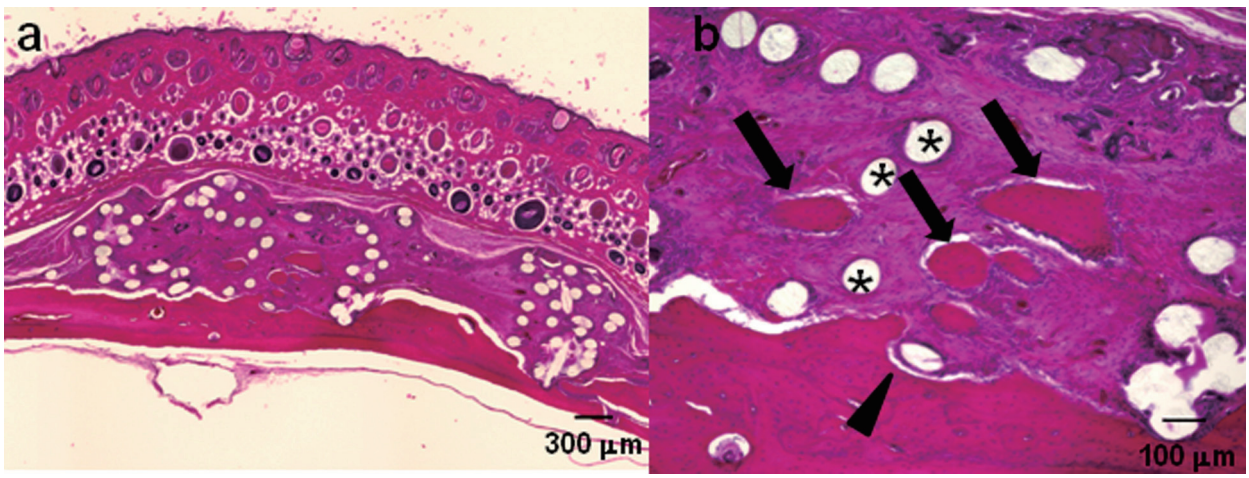

Fig. 7 Histologic observation of Group A. New bone formation was observed after the transplantation of a human osteoblast-like cells/3-D-woven fabric composite scaffold under the periosteum of the skull of a nude rat. (a) A histologic section prepared 8 weeks after transplantation (H&E staining, bar=300 µm). (b) Newly formed bone (area shown by arrows) and resorption of the native bone (shown by triangular marks) at a higher magnification (* Scaffold fiber residues, H&E staining, bar=100 µm).

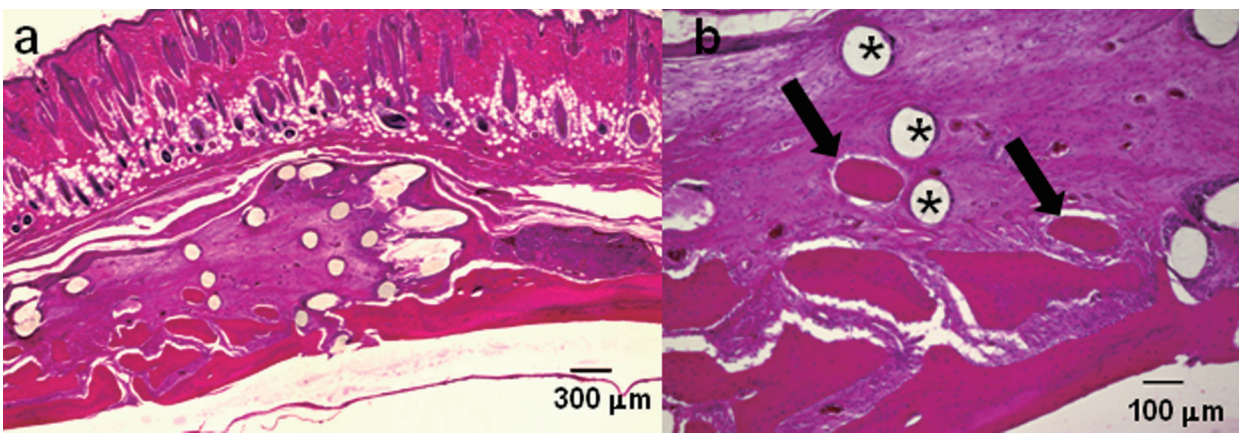

Fig. 8 Histologic observation of Group B. The human osteoblast-like cells/PRP mixture was coagulated using thrombin and then injected into a 3-D woven fabric composite scaffold for transplantation under the periosteum of the skull of a nude rat. (a) A histologic section prepared 8 weeks after transplantation (H&E staining, bar=300 µm). (b) New bone formation (area shown by arrows) is scarcely observed at a higher magnification. Areas marked with * are scaffold fiber residues (H&E staining, bar=100 µm).

materials such as PLLA in bone regeneration has been reported[9]. There are reports that osteogenic ability could be enhanced by improvements in the microstructure of such scaffold[10,11]. Unlike the raw material pellets, neither a glass transition point nor crystallization temperature was detected in the differential thermal analysis of PLLA fibers of the scaffold we tested. The latent heat of melting increased from 54 J/g (pellets) to 68 J/g (monofilaments). These results indicate that, during the two-step heat-drawing, most of the molecular chains were arranged in the axial direction of the fibers. A tensile strength of more than 400 MPa and an elongation after fracture of more than 40% represent some of the features of this new fiber.

Cellular interaction with the scaffold surfaces was clearly observed on fluorescence images. In the presence of MSCs and PLLA fibers, the cells could stretch and were able to adhere to the scaffold. In addition, the biocompatibility of PLLA microfibers and MSCs was tested by cell proliferation assays. The results showed that our woven fiber scaffolds had good compatibility with MSCs, allowing their proliferation. On the other hand, several different methods are available for seeding cells on a scaffold. A frequently used method[10] is to wet the scaffold surface with a

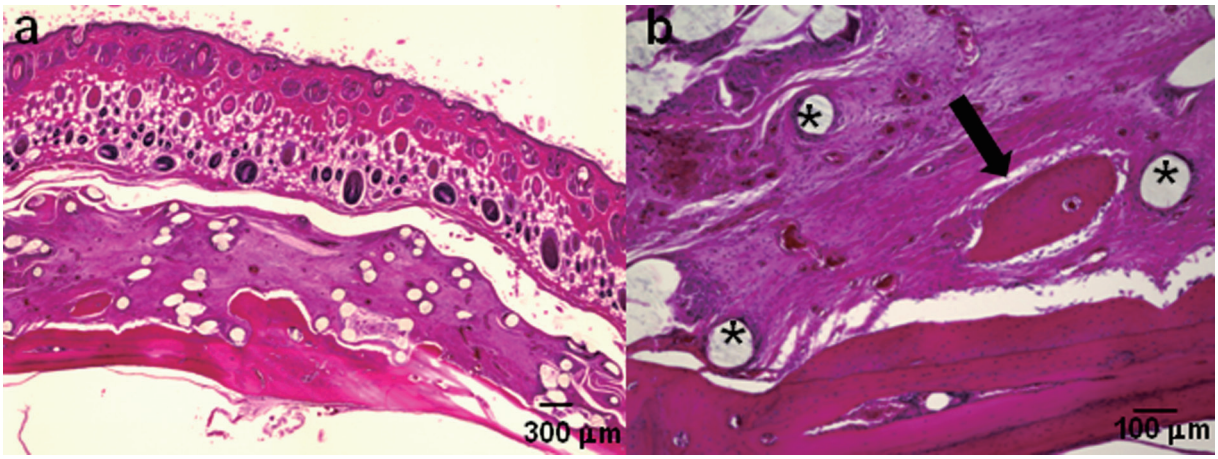

Fig. 9 Histologic observation of Control group. A 3-D woven fabric scaffold without cells was placed under the periosteum of the skull of a nude rat. (a) A histologic section prepared 8 weeks after transplantation (H&E staining, bar=300 μm). (b) New bone formation (area shown by arrows) is scarcely observed at a higher magnification (*Scaffold fiber residues, H&E staining, bar=100 μm).

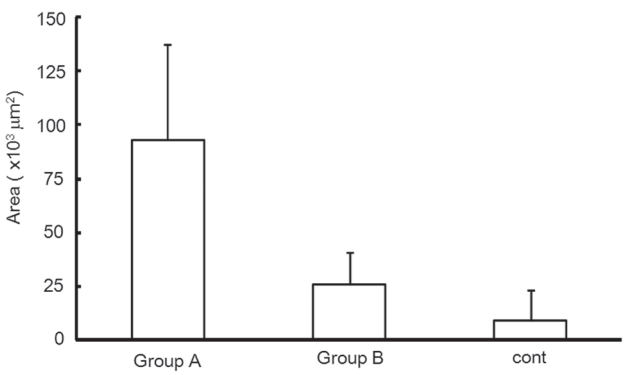

Fig. 10 Histomorphometric analysis of new bone areas in a 3-D woven fabric scaffold 8 weeks after grafting. The area of newly formed bone in each group was calculated.

solvent (*e.g.*, ethanol) with high affinity for the material of the scaffold for ease of introduction of the cell suspension into the porous structure, and then to replace the culture medium in the scaffold. Another method[12] is to immerse the scaffold into the cell suspension and then remove the air trapped in the porous structure by vacuum so that the cell suspension is easily introduced into the porous structure. In the case of the 3-D woven fabric composite scaffold, regularly arranged pores form a lattice-like porous structure, and therefore, cells are seeded just by immersing the scaffold into a comparatively highly concentrated cell suspension. As indicated by Bruder *et al.*[13], an increase in cell adhesion is expected by pre-coating the scaffold surface with adhesive molecules such as fibronectin. Further studies are necessary to develop new methods for improved cell seeding.

Yoshikawa *et al.*[14] assessed the effectiveness of osteogenic differentiation induction using hydroxyapatite as the scaffold in a model of heterotopic bone formation. In this model, the marrow-derived mesenchymal cells of rats were implanted subcutaneously into rats of the same species. Therefore, it is assumed that the bone matrix formed in an *in vitro* environment by the culture of cell/scaffold composite with osteo-lineage inducing factors can enhance the osteogenic ability of the implanted graft. In fact, we have confirmed the *in vitro* deposition of bone matrix when osteo-lineage induction of human marrow-derived mesenchymal cells was used on polylactic fibers of a scaffold[15]. Here, we implanted the composite of the scaffold and the cells which had undergone previous osteo-lineage induction into Group A.

It has been reported that the amount of new bone formed in a xenograft model in which human mesenchymal cells were implanted into animals was less than that observed in the xenograft model for implanting mesenchymal cells derived from a different species of animals[16]. According to the standard procedure, we assessed sympatric osteogenic ability in immunocompromised animals, in which bone formation was considered to occur easily when compared with that in an allopatric environment. Reports of new bone formation in immunocompromised animals include the study by Martin *et al.*[17] on bone formation from osteogenic precursors from human bone marrow and a study by Kuznetsov *et al.*[18] on the formation of bone marrow from human marrow stromal cells. Both studies demonstrated effective bone formation.

In this study, we used human MSCs derived from iliac bone marrow since the iliac bone is frequently used as the donor site for harvesting MSCs. In clinical application, however, the optimal donor site should be

chosen to achieve a minimally invasive procedure.

The shape and amount of graft materials and the site of implantation may affect the biocompatibility of polylactic acid. Therefore, it is necessary to thoroughly examine the biocompatibility of the scaffold made from polylactic acid according to the planned implantation site. As shown in Fig. 7, bone resorption was found in an area where the PLLA fibers of the scaffold were in contact with the cranial bone. Because of skin tension generated by suture, the PLLA fibers may have been located too close to the bone and may have induced bone resorption. Inflammatory response induced by polylactic acid is an important problem and has a negative effect on bone formation, and long-term careful follow-up is necessary when using a bioabsorbable scaffold made from polylactic acid.

The periosteum is known to play an important role in hard-tissue regeneration. Tieliewuhan *et al.*[19] grafted collagen sponge incorporating carbonate apatite in rats so that the graft could remain in contact with the surface of the periosteum, and they observed new bone developed from the surface of the periosteum. In contrast, Estrada *et al.*[20] compared the repair of bone on the bone and periosteum sides during bone expansion and reported that the bone repair associated with angiogenesis was observed on the bone side. The present results showed less bone formation on the periosteum side compared with island-like formations of new bone in the region adjacent to the cranial bone; however, it remains unclear whether the new bone was formed by bone conduction or from the implanted cells. Further detailed investigation will be needed.

Taken together, this study demonstrated the osteogenic ability of the material comprising a 3-D woven fabric scaffold made from biodegradable resin, bone morphogenic cells, and PRP. Further improvements in the design of the 3-D woven fabric composite scaffold, such as optimization of the fiber intervals and porosity, are expected to enhance the osteogenic ability of the material. Theoretically, the 3-D woven fabric composite scaffold can be manufactured in any size, shape, and strength to meet the needs of each individual application. This new material is considered to be beneficial for patients who require bone augmentation for dental implant treatments. We will conduct further animal experiments to simulate clinical applications.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to Mr. Toshiyuki Imoto (CEO) of Imoto Machinery Co., Ltd. (Kyoto, Japan) who provided us with the materials for the scaffolds in this study. The authors also thank ArBlast Co., Ltd., Kobe, Japan for assistance in this study. This study was conducted in collaboration with JST Innovation Plaza Kyoto and in part by a Grant-in-Aid for Encouragement of Scientists (C) No. 20592317 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This study was also supported by MEXT. HAITEKU (2007) and an Oral Implant Research Grant (10-04).

REFERENCES

1) Nyman S, Lindhe J, Karring T, Rylander H. New attachment following surgical treatment of human periodontal disease. J Clin Periodontol 1982; 9: 290-296.
2) Hammarstrom L. Enamel matrix, cementum development and regeneration. J Clin Periodontol 1997; 24: 658-668.
3) Langer R, Vacanti JP. Tissue engineering. Science 1993; 260: 920-926.
4) Yamada Y, Ueda M, Naiki T, Nagasaka T. Tissue-engineered injectable bone regeneration for osseointegrated dental implants. Clin Oral Implants Res 2004; 15: 589-597.
5) Ueda M, Yamada Y, Kagami H, Hibi H. Injectable bone applied for ridge augmentation and dental implant placement: Human progress study. Implant Dent 2008; 17: 82-90.
6) Yamada Y, Ueda M, Hibi H, Baba S. A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: A clinical case report. Int J Periodontics Restorative Dent 2006; 26: 363-369.
7) Matsusue Y, Yamamuro T, Oka M, Shikinami Y, Hyon SH, Ikada Y. *In vitro* and *in vivo* studies on bioabsorbable ultra-high-strength poly(L-lactide) rods. J Biomed Mater Res 1992; 26: 1553-1567.
8) Nakamura T, Shimizu Y, Okumura N, Matsui T, Hyon SH, Shimamoto T. Tumorigenicity of poly-L-lactide (PLLA) plates compared with medical-grade polyethylene. J Biomed Mater Res 1994; 28: 17-25.
9) Turhani D, Watzinger E, Weisenbock M, Yerit K, Cvikl B, Thurnher D, Ewers R. Three-dimensional composites manufactured with human mesenchymal cambial layer precursor cells as an alternative for sinus floor augmentation: An *in vitro* study. Clin Oral Implants Res 2005; 16: 417-424.
10) Ishaug-Riley SL, Crane GM, Gurlek A, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG. Ectopic bone formation by marrow stromal osteoblast transplantation using poly(DL-lactic-co-glycolic acid) foams implanted into the rat mesentery. J Biomed Mater Res 1997; 36: 1-8.
11) Redlich A, Perka C, Schultz O, Spitzer R, Haupl T, Burmester GR, Sittinger M. Bone engineering on the basis of periosteal cells cultured in polymer fleeces. J Mater Sci: Mater Med 1999; 10: 767-772.
12) Saito N, Murakami N, Takahashi J, Horiuchi H, Ota H, Kato H, Okada T, Nozaki K, Takaoka K. Synthetic biodegradable polymers as drug delivery systems for bone morphogenetic proteins. Adv Drug Deliv Rev 2005; 57: 1037-1048.
13) Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res 1998; 16: 155-162.
14) Yoshikawa T, Ohgushi H, Tamai S. Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. J Biomed Mater Res 1996; 32: 481-492.
15) Baba S, Inoue T, Tanaka N, Hiwa C, Adachi T, Hojo M, Morikuni H, Nishiura A, Matsumoto N, Kawazoe T. *In-vitro* assessment of fabric structural composite scaffold applicable to tissue regeneration around implants. J Osaka Dent Univ 2008; 42: 107-112.
16) Siddappa R, Martens A, Doorn J, Leusink A, Olivo C, Licht R, Van Rijn L, Gaspar C, Fodde R, Janssen F, Van Blitterswijk C, De Boer J. cAMP/PKA pathway activation in human mesenchymal stem cells *in vitro* results in robust

bone formation *in vivo*. Proc Natl Acad Sci U S A 2008; 105: 7281-7286.
17) Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R. Fibroblast growth factor-2 supports *ex vivo* expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology 1997; 138: 4456-4462.
18) Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, Robey PG. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation *in vivo*. J Bone Miner Res 1997; 12: 1335-1347.
19) Tieliewuhan Y, Hirata I, Sasaki A, Minagi H, Okazaki M. Osteoblast proliferation behavior and bone formation on and in CO_3 apatite-collagen sponges with a porous hydroxyapatite frame. Dent Mater J 2004; 23: 258-264.
20) Estrada JIC, Saulacic N, Vazquez L, Lombardi T, Ramirez JUC, Bernard JP. Periosteal distraction osteogenesis: Preliminary experimental evaluation in rabbits and dogs. Br J Oral Maxillofac Surg 2007; 45: 402-405.