Osteoanagenesis after transplantation of bone marrow-derived mesenchymal stem cells using polyvinylidene chloride film as a scaffold

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The aim of this study was to develop a new cell transplantation technique for osteoanagenesis at bone defect sites. Polyvinylidene chloride (PVDC) film was evaluated because of its good biocompatibility and flexibility. We used this film as both a cell scaffold and a barrier membrane. Initially, the cell compatibility of the PVDC film for fibroblast-like cells and osteoblast-like cells was confirmed. Subsequently, bone marrow cells were obtained from rats and cultured on PVDC films in two kinds of medium. The PVDC films with bone marrow-derived mesenchymal stem cells (MSCs) were then applied to critical-sized bone defects in the calvarial bone of rats. After the transplantation, the surgical sites were dissected out and evaluated by soft X-ray radiography, micro-CT analysis and histological examinations. The bone marrow-derived MSC-transplanted rats showed greater bone regeneration than the control rats. Therefore, PVDC film is considered to be useful as a scaffold for bone regeneration.

Keywords: PVDC, Bone regeneration, Mesenchymal stem cells

INTRODUCTION

Bone defects of the maxillofacial region can be surgically treated by augmentation procedures. In such cases, the use of autologous bone is still regarded as the gold standard1-2). However, these grafts are limited in quantity and involve invasion of healthy tissue during harvesting. Recent advances in the field of regenerative medicine have led to the development of cell-based therapies based on tissue engineering for bone regeneration3-7). The progenitor cells are referred to as mesenchymal stem cells (MSCs) and have the capability of differentiating into mesenchymal phenotypes, including osteogenic and chondrogenic cell types8,9). Bone marrow-derived MSCs are an attractive cell source for bone regeneration because of their ability for self-renewal with a high proliferative capacity and osteogenic differentiation potential10-14). Importantly, their in vitro differentiation can be achieved by culturing the cells on various materials including hydroxyapatite, β-TCP and polylactic acid (PLA)15-18). Although these materials have good biocompatibility, they usually do not have osteoinductive potential.

In general, the culture of MSCs on the surface of three-dimensional structures of these materials is difficult and requires additional cell manipulation. Consequently, when these materials are used, it is necessary to separate the cells from tissue culture plates for combination with the different materials. During this process, the cells can suffer damage by enzymatic and mechanical means19). Furthermore, during and after combination of the cells with certain carriers and scaffolds, they are left out of the incubator environment until transplantation into the bone defect site. To reduce such damage, it is preferable to avoid cell manipulation processes as much as possible. In addition, these materials do not have sufficient formability after transplantation at the bone defect site. Hence, we evaluated polyvinylidene chloride (PVDC) film as a cell scaffold to develop a new cell transplantation technique because of its good flexibility and biocompatibility. In a previous study according to ISO10993-620. PVDC film was shown to have sufficiently superior biocompatibility for use as a graft material. PVDC film has adhesion properties through a bonding agent and high barrier properties against O2, H2O and organic solvents as its central features. It has been clarified that PVDC film shows decreased cell toxicity of the bonding agents used for the formation and fixation of the PVDC film.

In the present study, cell proliferation tests of fibroblast-like cells (L929) and osteoblast-like cells (MC3T3-E1) were carried on PVDC films out to confirm the cell compatibility. Subsequently, rat bone marrow cells were purified and cultured to collect MSCs. After the MSCs were allowed to proliferate on PVDC films, the cells and PVDC film scaffolds were transplanted into critical-sized defects in the calvarial bone of rats, and the osteoanagenesis abilities were compared between differentiated and non-differentiated MSCs. Bone tissue regeneration was observed by soft X-ray, micro-CT and histological examinations. The non-differentiated MSC-transplanted rats showed better bone repair more than the control rats and differentiated MSC-transplanted rats.

MATERIALS AND METHODS

Cell proliferation tests
PVDC films (thickness 20 µm) were sterilized by autoclaving (121°C, 20 minutes), formed into a round shape and adjusted in size to match the inner diameter of the wells of 24-well microplates. The films were placed on glass plates and pressed with a sterilized cotton applicator. The glass plates were then sterilized by

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MSC culture and differentiation

The methods used were essentially the same as previously reported methods10,13. In the present study, the in vitro differentiation and calcification of an osteogenic cell line were also evaluated21. Briefly, bone marrow cells were obtained from the femora of 4-week-old male Sprague-Dawley rats. Two kinds of culture media were prepared, growth medium and differentiation medium. The growth medium was adjusted alphalit-Eagle’s Minimal Essential Medium containing 15% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin). The differentiation medium was the growth medium supplemented with 100 µM ascorbic acid (Sigma-Aldrich Japan KK, Tokyo, Japan), 50 µg/mL β-glycerophosphate (Sigma-Aldrich Japan KK, Tokyo, Japan), and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin; Sigma, St. Louis, MO, USA). MC3T3-E1 cells were cultured in Alpha-Eagle’s Minimal Essential Medium (Invitrogen Japan KK) containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin). The cells were suspended at 1×10^6 cells/mL in each medium and seeded at 400 µL/well. All culture media were changed every 2 days. The culture period for L929 cells was 8 days and the cell proliferation was measured every day. The culture period for MC3T3-E1 cells was 14 days and measurements of the cell proliferation were carried out after 2, 4, 6, 8, 10, 12 and 14 days. The cell proliferation at each time point was measured by the WST assay using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan; OD at 450 nm). The shapes of L929 cells were observed by phase-contrast microscopy (Model AME-12101; AR Brown Co., Tokyo, Japan).

Sterilized PVDC films were formed to a diameter of 7 mm for culture with the bone marrow-derived MSCs. Briefly, 10 films were placed in a 10-cm tissue culture plate (Becton Dickinson Labware) for subculture of cells. After approximately 1 week of culture, the cells reached confluency and were released from the substratum using 0.25% trypsin-EDTA. The cells were adjusted to 1×10^7 cells/10-cm tissue culture plate for subculture. The subculture was carried out with either the growth medium or the differentiation medium. The cells were rinsed with PBS and the media were renewed every 2 days. The cell proliferation was checked by phase-contrast microscopy.

This study was approved by the Animal Experimentation Committee of Aichi Gakuin University School of Dentistry (AGUD 038).

Alkaline phosphatase (ALP) activity

To confirm that the MSCs differentiated into osteoblastic cells in the differentiation medium, the extracellular ALP activities were measured. Briefly, the MSCs were suspended in the growth medium or differentiation medium, and the cell density was adjusted to 1×10^6 cells/mL. The MSC suspensions were then seeded onto 24-well microplates or PVDC films. Each group was cultured in the growth medium or differentiation medium for 2 weeks. After the cell culture, the ALP activities of the four groups were measured using LabAssay™ ALP (Wako Pure Chemical Industries Ltd., Osaka, Japan). Briefly, the working assay solution was added to the culture medium, and incubated for 15 minutes. After the incubation, the stop solution was added and the absorbance at 405 nm was measured using a microplate reader. This assay is based on the Bessey-Lowry method20. The ALP activity was evaluated by the amount of p-nitrophenol produced and converted to activity units using a calibration curve created with the standard solution. For comparison of the ALP activities, the units were standardized by the sample area.

Surgical procedure

A total of 30 male Sprague-Dawley rats (weighing 130–150 g) were used for transplantation. The rats were anesthetized with a 50 mg/kg intraperitoneal injection of pentobarbital sodium (Somnopentyl®; Schering-Plough Animal Health Co., Kenilworth, NJ, USA). After disinfection of the surgical site, the head skin was dissected for a length of about 10 mm. The calvarial periosteum was separated to expose the skull bone. After the flap was raised, a critical-sized bone defect (5 mm in diameter) was created at the center of the skull using a bone trephine bur with an external diameter of 5 mm (Implatex Co. Ltd., Tokyo, Japan) under running water. Fig. 1 shows a schema of the surgical sites in the five groups. In the three control groups, the defects were treated differently: DEFECT group, no treatment; PVDC group, defect covered with PVDC film using G-BOND (GC Co., Tokyo, Japan) for formation and fixation to the skull bone; GORE group, defect covered with a GORE-TEX® GTR membrane (Japan Gore-Tex Co.,...
Tokyo, Japan). In the two experimental groups, the defects were covered by MSCs cultured on PVDC films, in a similar manner to the control groups. These cells on PVDC films had been cultured in the growth medium (MSC group) or the differentiation medium (D-MSC group). The PVDC, MSC and D-MSC groups were light-cured for 10 seconds after the inner PVDC films were covered by additional PVDC films secured with G-BOND. Finally, the flap was repositioned and sutured. Of the six rats in each group, three rats were respectively euthanized at 6 and 9 weeks after the surgery for analysis (n=3).

**Soft X-ray and micro-CT observations**

To observe the general bone defect sites and compare the bone formation areas, soft X-ray and micro-CT observations were carried out. The samples were placed on soft X-ray films, and exposed to soft X-rays from the top. The soft X-ray observations were performed under the following conditions: exposure time, 40 seconds; tube voltage, 30 kVp; tube current, 4 mA. The films were developed using standard procedures, and the images...
were transferred to a personal computer by a photo scanner (GT-X900; Seiko Epson Co., Tokyo, Japan).

During the fixation process, the samples were also observed by micro-CT (R_mCT; Rigaku, Tokyo, Japan). The micro-CT observations were performed under the following conditions: exposure time, 2 minutes; tube voltage, 90 kV; tube current, 88 µA; magnification, 10-fold.

**Histological examination**

To observe the tissue structures and thickness of the new bone, histological examinations were carried out. Samples were fixed with 10% phosphoric acid in neutral-buffered formalin for 2 weeks. Subsequently, the samples were processed for histological examination according to standard procedures. Briefly, the samples were decalcified, embedded in paraffin, sectioned at 4-µm thickness and subjected to hematoxylin-eosin (HE) staining. All the stained sections were observed by light microscopy (BZ-9000; Keyence Co., Osaka, Japan).

**Statistical analysis**

All data were expressed as the mean±SD. The data were subjected to statistical analysis using a two-tailed Student’s t-test. Probability values for the significance of differences between values were calculated, and values of \( p<0.05 \) considered significant.

**RESULTS**

**Comparison of cell proliferation**

L929 cells cultured on the microplate reached confluency after 4 days of culture (Fig. 2 upper). Although the proliferation of L929 cells on the PVDC film was delayed compared with the control group, the cell proliferation did not differ significantly after 7 days of culture. After 8 days of culture, the cells reached confluency on the PVDC film. Compared with the PVDC film, the cell proliferation on the PVC film was slower and the cells did not reach confluency.

MC3T3-E1 cells cultured on the microplate reached confluency after 12 days of culture (Fig. 2 lower). Although the proliferation of MC3T3-E1 cells on the PVDC film was delayed, the cell proliferation was as high as that of confluent cells after 14 days. Similar to L929 cells, the cell proliferation on the PVC film was slower and the cells did not reach confluency.

**Cell morphology**

The typical cell morphologies are shown in Fig. 3. In the control and PVDC groups, the cells were polygonal and spindle-shaped. In the PVC group, the cells were round and spindle-shaped. As the cells grew, the spindle-shaped cells became rounder on the PVC film. After 4 days of culture, the cells reached confluency in the control group. In the PVDC and PVC groups, cell proliferation was also recognized in the microscopic observations.

**MSC culture**

A typical phase-contrast microscope image of cells after 7 days of culture in the differentiation medium is shown in Fig. 4. The bone marrow cells grew on both the cell culture plate and the PVDC film. However, the cell proliferation on the PVDC film was delayed compared with the cell proliferation on the culture plate. These proliferation properties were similar to the findings for MC3T3-E1 cells.

**Cell differentiation and ALP activity**

The groups in the differentiation medium showed high ALP activity compared with the groups in the growth medium (Fig. 5). In addition, there were no significant differences between the microplate group and the PVDC group. These results confirm that the bone marrow cells reached confluency after 14 days of culture in the growth medium, and that the differentiation medium
Fig. 3  Typical shapes of L929 cells.

Fig. 4  MSCs proliferating on a PVDC film and the underlying tissue culture plate. The arrow indicates the borderline between the PVDC film and the tissue culture plate.

Fig. 5  Comparison of the ALP activities in cells cultured in the two kinds of medium. The data are shown as means±SD. *p<0.05.
successfully induced the cell ability to form calcified substances.

**Soft X-ray and micro-CT observations**

Typical soft X-ray and micro-CT images are shown in Fig. 6. The soft X-ray images revealed less bone repair in the control groups at 6 weeks after implantation. In the MSC and D-MSC groups, considerably more bone repair was observed compared with the control groups. The difference in cell culture between the MSC and D-MSC groups did not affect the bone formation, and the levels of bone regeneration were almost the same. The micro-CT images revealed that bone formation in the experimental groups occurred uniformly from the surface of the bone defect to the inner site. At 9 weeks after implantation, the DEFECT group did not show any bone formation by soft X-ray images compared with the images at 6 weeks after implantation. In the PVDC and GORE groups, little bone tissue was formed compared with the images at 6 weeks after implantation. The experimental groups showed good bone formation, and the bone defects were almost completely repaired. In the experimental groups, the micro-CT images showed a hard tissue layer on the bone defect in accordance with the PVDC film area at 9 weeks after implantation.

**Histological examination**

Typical HE-stained histological sections at 6 and 9 weeks after implantation are shown in Figs. 7 and 8, respectively. At 6 weeks after implantation, the control groups did not show any bone formation, while the experimental groups showed partial bone formation in the bone defects. The new bone formation was both continuous and discontinuous with the calvarial bone. There was no difference in the amount of new bone formation between the MSC and D-MSC groups. The new bone was thin and the tissue structure was incomplete.

At 9 weeks after implantation, bone formation was slightly increased in the PVDC and GORE groups compared with 6 weeks after implantation. In the GORE group, new bone was formed according to the GTR membrane. Although both of the experimental groups showed more new bone formation, the thickness and structure of the new bone were better in the MSC group than in the D-MSC group. The MSC group showed new bone formation across the bone defect site. In the D-MSC group, the continuity of the bone regeneration was incomplete.

None of the samples showed any marked inflammatory reactions caused by the bonding agent or other materials.

**DISCUSSION**

It has been reported that there is a population of undifferentiated cells in the bone marrow, referred to as MSCs, that can differentiate into adipogenic, chondrogenic, myogenic and osteogenic cells8,9). Bone marrow MSCs have been successfully engrafted within critical-sized defects in the long bones of dogs and athymic rats. In general, such implanted cells must escape from a scaffold and induce tissue formation *in vivo*13,17). Other sources of MSCs include the periosteum, synovial membrane, muscle, fat, vascular pericytes, periodontal ligament, deciduous teeth and dermis. Tuli *et al*.23) recently demonstrated that MSCs can also be isolated from explant cultures of collagenase-treated trabecular bone fragments. It has also been reported that MSCs are effective for metabolic bone diseases and bone tumors24,25). In the fields of periodontology and maxillofacial surgery, MSCs are expected to be used in many cases. For such cases, bioresorbable GTR membranes have been investigated for their cell compatibility and operability16,26). In addition, some bone filling materials have been used as carriers or scaffolds for bone defect sites27-29). However, these materials do not have the ability to create new bone or shield the connective tissue from around the bone defect site. The breakthrough achieved in the present study is the development of tools and techniques that allow easy transplantation of MSCs into bone defect sites while avoiding damage to the cells. PVDC films are frequently
used in medical procedures. For example, PVDC films are used for first aid treatment and in the care of pressure ulcers\(^{30}\). Such usage suggested the possibility of using PVDC film as an implant material. However, there was no evidence regarding the biocompatibility of PVDC films. Therefore, the biocompatibility was initially tested according to ISO 10993-6 and confirmed. Furthermore, implantation tests in subcutaneous tissue revealed that PVDC film did not induce inflammatory reactions. Therefore, it was assumed that PVDC film would be useful as a scaffold. Our new cell transplantation technique using PVDC film does not require any preoperative preparation, which usually involves cell ablation and suspension in a carrier. The PVDC film can be formed according to the bone defect size, and the PVDC film with cultured cells can be transplanted immediately and directly. During the cell culture period, the sterilized PVDC film does not require any special

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**Fig. 7** Typical HE-stained histological images at 6 weeks after transplantation. Magnified images of the bone defect sites are shown on the right for the DEFECT, MSC and D-MSC groups. The arrowheads show the borders between the calvarial bone and the new bone. B: bone; NB: new bone; F: fibrous connective tissue; P: PVDC film; G: GORE-TEX\textsuperscript{®} GTR membrane.
treatment before placing on the tissue culture plate because of its viscosity. After seeding, the cells can be observed by phase-contrast microscopy without fixation or staining because of the transparency and thickness (20 µm) of the film. The PVDC film is not absorbed after transplantation and performs effectively as both a scaffold and a shield membrane. Because of these two functions, it is suggested that the bone marrow cells were able to differentiate and proliferate at the bone defect sites, such that the experimental bone defects were regenerated more effectively than the control bone defects.

**Cell adhesion and cell proliferation on PVDC film**

Cell adhesion is influenced by the surface texture of the substrate. To clarify the differences in cell proliferation, the contact angles of the materials were measured: cell culture plate, 53.0±0.6 degrees; PVDC
film, 73.2±0.4 degrees; PVC film, 85.7±1.7 degrees. Cell culture plates are produced to be suitable for cell culture, so their surface texture is smooth and a moderate hydrophilic property is added. The surface texture of sterilized PVDC film is also smooth and contains a higher hydrophilic property than PVC film. Furthermore, the images obtained by phase-contrast microscopy revealed that almost all of the cells on the PVDC films were similar to the cells on the culture plates. These findings suggest that these elements allowed good cell proliferation on PVDC films. Previous findings for initial attachment of osteoblasts for guided bone regeneration were similar to the observations for the PVDC films. In addition, a surface treatment to increase the hydrophilicity of PVDC film has the possibility of increasing the cell proliferation. Taken together, the present findings confirmed that fibroblast-like cells and osteoblast-like cells were able to grow on PVDC film.

**Bone regeneration ability of transplanted bone marrow cells**

Better bone regeneration was observed in the cell transplantation groups than in the control groups. In a previous study, the osteogenic potential of bone marrow cells was verified according to the differentiation period in vitro. The results revealed that cells with a short differentiation period showed better osteogenic potential than cells with a long differentiation period and produced good bone regeneration in vitro. It has been reported that the differentiation rate is influenced by the length of the differentiation period and that the properties of MSCs are lost as the differentiation progresses. In the present study, the osteogenic potential of bone marrow cells was verified according to the differentiation period in vitro. It has been reported that the differentiation rate is influenced by the length of the differentiation period and that the properties of MSCs are lost as the differentiation progresses. In the present study, the osteogenic potential of bone marrow cells was verified according to the differentiation period in vitro. It has been reported that the differentiation rate is influenced by the length of the differentiation period and that the properties of MSCs are lost as the differentiation progresses. In the present study, the osteogenic potential of bone marrow cells was verified according to the differentiation period in vitro. It has been reported that the differentiation rate is influenced by the length of the differentiation period and that the properties of MSCs are lost as the differentiation progresses.

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