Cell culture in vivo by means of diffusion chamber system

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In a diffusion chamber (DC) system, cells are cultured in vivo — hence making it possible to minimize infection and foreign material contamination. In view of this merit, we devised a technique to combine a DC system and a scaffold to the end of incubating sufficient host cells for grafting. In the present study, PLGA sponge and rat bone marrow cells were encapsulated inside a DC and then placed inside the abdominal cavities of rats. DCs were removed at two or four weeks after grafting. At four weeks after grafting, fibrous and calcified tissue matching the shape of the PLGA sponge was formed. These results suggested that the PLGA sponge was an effective scaffolding material in inducing three-dimensional tissue formation and that combination with a DC system resulted in a cell mass matching the scaffold shape. In addition, the cells were cultured in vivo — which meant that DC culturing did not require special incubation facilities or technologies after grafting.

Keywords: PLGA, Bone marrow cell, Diffusion chamber

INTRODUCTION

To reconstruct bone defects in the maxillofacial region, numerous regenerative techniques have been developed. Osteoblast-lineage cells or bone marrow-derived host cells are commonly used for this purpose, and such cells are harvested, cultured in vitro, and then inoculated into scaffolds composed of poly-L-lactic acid (PLLA)¹, poly (DL-lactic acid-co-glycolic acid) (PLGA)²-⁴, or collagen gel⁵-⁶. Scaffolds with osteogenic cells are then implanted into the host body to induce new bone formation⁹,¹⁰. However, cell culture prior to implantation requires medium changes and various supplements to maintain cells, and this can cause side effects in vivo¹¹,¹². To minimize unexpected reactions in the host, a diffusion chamber (DC) system was adopted in this experiment.

Spongy porous scaffold was inoculated with harvested bone marrow cells, and the complex was sealed in a DC. This was followed by implantation into the rat peritoneal cavity to induce hard tissue formation. Therefore with this new method, the PLGA sponge with induced hard tissue could be implanted into the desired site to induce new bone formation after successful cultivation of bone marrow cells in the DC¹³-¹⁵. If this novel method is proven to be clinically viable, it will become possible to grow new bone for all types of repairs instead of removing it from existing bones.

MATERIALS AND METHODS

Preparation of spongy scaffold derived from PLGA
PLGA (75:25, MW=66,000–107,000 Da; Sigma-Aldrich Japan KK, Tokyo, Japan) was solubilized in an acetone solvent, and 10% (w/w) NaCl with a particle size of approximately 300–500 µm was added. The slurry was then poured into a glass die (6 cm i.d.). After hardening, the PLGA sheet was immersed in distilled water for 48 hours at 4°C to elute the NaCl. A sheet with a spongy texture was thereby obtained after 48 hours of lyophilization. PLGA sponges were punched out to form disks of 9 mm diameter × 3 mm thickness, corresponding to the inner diameter of the DC (Fig. 1). Specimens were observed under a scanning electron microscope (SEM). All samples were sterilized with ethylene oxide gas before use.

Preparation of cell suspensions
Male rats were used as both cell donors and hosts for intraperitoneal implantation of DCs. Whole marrow cells were obtained from femoral midshafts of 4-week-old SD rats. Marrow samples were then transferred to Alpha Minimum Essential Medium (Invitrogen Japan KK, Tokyo, Japan). A predominantly single-cell suspension was prepared by repeatedly drawing and expelling marrow through an 18-gage needle. Suspended cells were then counted using a fixed-volume hemacytometer, and the concentration was adjusted to 10⁶ cells/100 µl.

Preparation of DCs
DCs (φ14 mm × 2 mm, 130-µl capacity, 0.45-µm pore
size membrane filters) were assembled from commercially available components (Millipore, Billerica, MA, USA). Cells were inoculated into each DC via a small hole penetrating the sidewall of the chamber ring. After which, a small rod made of the same material as the chamber ring was inserted, and the hole was sealed with cyanoacrylate glue.

Three groups were prepared: PLGA alone (Control group); bone marrow cells alone (Cell group); and bone marrow cells with PLGA (Cell+PLGA group).

DCs were implanted into the abdominal cavities of 4-week-old SD rats. Each rat received three implants, one from each of the three groups. At two or four weeks after implantation, the rats were sacrificed and examined as described below.

All animal experiments were performed in accordance with the guidelines for Animal Experimentation of Aichi-Gakuin University (Nagoya, Japan), and discomfort was kept to a minimum.

**X-ray observation**

At 2 weeks or 4 weeks after implantation, the rats were sacrificed and the DCs retrieved from their abdominal cavities. Recovered DCs were fixed with 10% phosphoric acid in neutral formalin liquid buffer maintained at 4°C for 2 weeks. Samples were then placed on soft X-ray films, and the whole body was exposed to a soft X-ray system for 30 seconds. Films were developed using standard procedures, and images were transferred to a personal computer using a photo scanner (GT-X900, Seiko Epson Corp., Tokyo, Japan).

Microcomputed tomography (micro-CT; SV-CT2500, Shimadzu Co. Ltd., Kyoto, Japan) was also performed under the following conditions: SID (source-image intensifier distance), 500 mm; SOD (source-object distance), 110 mm; 60 kV; 40 mA; slice pitch, 0.03 mm; and 512×512 pixels.

**Histological observation**

Fixed samples were also treated for histological examination according to standard procedures. Embedded samples were sectioned at 4 µm of thickness, stained with HE, and observed under a microscope.

**Calcium content determination**

Recovered DCs were washed with distilled water and lyophilized. Calcium was completely eluted from dry DCs by immersion in 1 ml of 4 N hydrochloric acid for 12 hours. Calcium quantification was performed using Calcium C test (Wako Pure Chemical Industries Ltd., Osaka, Japan). Optical absorbance at 570 nm (OD570) was determined using a microplate reader.

**RESULTS**

**SEM observation**

Inner porosity and three-dimensional structure were observed by SEM. Numerous voids were seen in the specimen, and these reflected the shape of eluted NaCl particles. Pore size was comparable to the size of NaCl particles.

![Fig. 1 SEM observation of scaffold. Whole PLGA sponges were punched out to form disks corresponding to the inner diameter of the diffusion chamber.](image1)

![Fig. 2 SEM observation of scaffold: (a) SEM image of NaCl; (b) SEM image of PLGA sponge. PLGA sponge exhibited isotropic voids reflecting the shape of eluted NaCl, and pore size was comparable to the size of NaCl particles.](image2)
particles (300–500 mm) (Fig. 2).

**Macroscopic findings**

In the Control group, the porous structure of PLGA sponges was maintained at 2 weeks and 4 weeks after implantation. At 2 weeks, neither the Cell group nor the Cell+PLGA group showed macroscopic tissue growth. At 4 weeks, tissue nodules were observed. Localized ectopic tissue was observed in the Cell group (B) and Cell+PLGA group (D).

**X-ray observation**

At 2 weeks after implantation, soft X-ray images revealed no radio-opacity in any of the groups. Opaque images were seen on soft X-ray images at 4 weeks in both the Cell group and Cell+PLGA group, while the Control group did not show any changes. Radio-opacity in the Cell group was localized in the DC. However, in Cell+PLGA group, radio-opacity was diffused throughout the DC and in the meshwork around the PLGA sponge (Fig. 4).

Three-dimensional images reconstructed using micro-CT data revealed calcification with good histological prognosis around the scaffold (Fig. 5).

**Histological observation**

At 2 weeks after implantation in the Cell group, cells were scattered in the DC. In contrast, the Cell+PLGA group showed cell migration into the PLGA sponge. However, no calcification was seen in either the Cell group or the Cell+PLGA group.
At 4 weeks, calcified tissues in the DC were observed in the Cell group. The Cell+PLGA group also showed calcified tissues pervading the structure of the PLGA sponge, where C: calcified tissues, F: fibrous tissue, M: filter membrane, and P: PLGA sponge.

**DISCUSSION**

**Cell culture by DC method**

As cells are cultured *in vivo* using a DC, it becomes possible to minimize infection and contamination by foreign materials, thereby creating an ideal environment for cell incubation. To date, DCs have mainly been used to investigate the effects of cell growth factors, such as BMP, on cells and tissues\(^{16-18}\). However, we devised a technique combining a scaffold and a DC, in which sufficient host cells for grafting were incubated *in vivo*. The resulting cell mass assumed and matched the shape of the scaffold, and...
was then grafted to a target organ. Furthermore, the results of light microscopy and micro-CT confirmed that fibrous and calcified tissue matching the scaffold shape was formed. This indicated that bone marrow cells multiplied and differentiated inside the DC utilizing the biological components that passed through the filter membrane to form tissue \(^19\). In other words, the DC allowed cells to multiply and differentiate within a scaffold \(\textit{in vivo}\), hence providing an effective environment for maintaining cell masses matching the scaffold shape.

**PLGA sponge**

PLGA is widely used as a scaffolding material in regenerative medicine. It is highly malleable, and degradation and strength can be controlled based on polymerization and crosslinks. As a result, these properties enable PLGA sponges to be formed into various shapes.

In the present study, PLGA sponges were prepared by the particle-leaching technique\(^20\). Using NaCl particles dissolved in water as a porous material, pore size was adjusted to 300–500 mm, which is suitable for osteoblast proliferation (continuous porous sponge)\(^21\). The average porosity of scaffolds was 86±5%. Acetone was used as a water-soluble solvent for PLGA, and when eluting NaCl in water, residual acetone was removed by volatilization and elution.

Light microscopy revealed fibrous and calcified tissue matching the PLGA shape in the Cell+PLGA group. SEM revealed that the porosity of PLGA sponge was maintained in a continuous network, thus allowing cells to invade the deep areas of the sponge. Furthermore, soft X-ray imaging at 4 weeks after implantation showed radio-opaque areas matching the three-dimensional shape of the PLGA sponge.

However, soft X-ray imaging did not show radiopacity in the Control group. In addition, the calcium contents of the Cell and Cell+PLGA groups were higher than that of the Control group. These findings suggested that the calcified tissue formed by the present technique was induced by the cells in the DC, not as a result of mineral deposition on the PLGA sponge from the tissue fluid.

Furthermore, when comparing the calcium contents of the Cell and Cell+PLGA groups, calcium content in the Cell+PLGA group was significantly higher. As light microscopy showed marked calcified tissue growth in the Cell+PLGA group, it would seem that calcified tissue formation was aided by the scaffold.

At 4 weeks after grafting, macroscopic and light microscopic findings showed that the PLGA sponge shape was maintained. This was because sponge deformation was suppressed, as host cells could not enter the DC and there was a sufficient amount of space. Therefore, PLGA sponge was an effective scaffold material to induce three-dimensional calcified tissue formation inside a DC.

Bone marrow cells originate from mesenchymal stem cells and differentiate into fibroblasts, as well as osteoblasts, chondroblasts, and myoblasts\(^22,23\). However, inside the DCs, no osseous tissue was seen. Thus, fibroblasts are more likely to multiply in a DC as compared to other precursor cells such as osteoblasts. Studies have been conducted to induce osseous tissue formation from bone marrow cells in a DC using cell growth factors such as BMP\(^24,25\). We suspected that when inducing osseous tissue growth in a DC, addition of growth factors or host-derived PRP (platelet-rich plasma) and BMG (bone matrix gelatin)-containing growth factors are necessary for effective formation\(^26,27\).

**CONCLUSIONS**

Findings in the present study suggested that the PLGA sponge thus prepared served as an effective scaffold for inducing three-dimensional cell growth. Moreover, by combining with a DC, a cell mass matching the shape of the scaffold could be obtained.

The present method can be used to differentiate bone marrow cells using cell growth factors and form artificial tissue inside each patient, whereby the resulting cell mass can be implanted immediately after removal from the DC to treat various maxillofacial defects. In addition, cells are cultured \(\textit{in vivo}\), which means that no special facilities or technologies are required after the DC is placed. Hence, the novel cell culture technique devised in this study is a potential therapeutic option in regenerative medicine.

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REFERENCES


