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Maxillofacial bone regeneration with osteogenic matrix cell sheets: An experimental study in rats

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ABSTRACT

Objective: Regeneration of maxillofacial bone defects, characterized by relatively small but complicated shapes, poses a significant clinical challenge. Osteogenic matrix cell sheets (OMCSs) have osteogenic ability and good shaping properties and may be ideal graft materials. Here, we assessed whether implantation of OMCSs could be used to repair maxillofacial bone defects.
Design: We adopted a rat mandibular symphysis model. The rat mandible is formed by a paired bone and the central portion consisting of fibrous tissue. There is no bone tissue at the site; accordingly, this site was interpreted as a physiological bone gap and was used for evaluation. Rat bone marrow cells were cultured in medium containing dexamethasone and ascorbic acid phosphate to create OMCSs. The OMCSs were implanted into the rat mandibular symphysis without a scaffold. Microcomputed tomography and histological analyses were conducted after 2, 4, and 8 weeks.
Results: Two weeks after implantation, microcomputed tomography images and histological sections showed some sparse granular calcification tissue within the bone gap at the mandibular symphysis. At 4 weeks, the calcification tissue spread, and the gap of the mandibles were continued. At 8 weeks, this continuous new bone tissue was matured. The experimental group showed abundant new bone tissue in the group with OMCS implantation, but not in the group with sham implantation.
Conclusions: Our present results indicated that use of OMCSs may be an optimal approach towards achieving maxillofacial regeneration.

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1. Introduction

Maxillary alveolar cleft, facial trauma, bone resection due to cancer, periodontal disease, and bone atrophy after tooth extraction may result in non-healing maxillofacial bone defects. Autologous bone grafts are considered the gold standard for repairing such bone defects (Behnia et al., 2009; Liu, Tan, Luo, Hu, & Yue, 2014; Xie et al., 2007; Yoshioka et al., 2012). However, donor site morbidity is an important consideration. Maxillofacial bone defects are often smaller than those commonly encountered in orthopedic surgery, but have more complicated morphology (d'Aquino et al., 2009). Thus, the ability of the graft material to assume a complex shape is essential for maxillofacial bone regeneration.

Recently, researchers have been working to develop cell-based bone repair methods as a substitute for autologous bone grafts (Kawate et al., 2006; Morishita et al., 2006). We previously developed a cell transplantation method based on cell sheet technology with bone marrow-derived stromal cells (BMSCs), which were cultured in the presence of dexamethasone (Dex) and ascorbic acid phosphate (Akahane et al., 2008). These cells were lifted as cell sheets, termed osteogenic matrix cell sheets (OMCSs),

Abbreviations: BMSC, bone marrow-derived stromal cell; OMCS, osteogenic matrix cell sheet; Dex, dexamethasone; H&E, hematoxylin and eosin; OPN, osteopontin; OCN, osteocalcin; micro-CT, microcomputed tomography; TCP, tricalcium phosphate.

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with no special materials, such as thermosensitive polymers. OMCSs can be transplanted without a scaffold, resulting in bone formation (Inagaki et al., 2013; Nakamura et al., 2010). OMCSs are sufficiently malleable that they may represent optimal graft materials for maxillofacial bone regeneration. However, transplantation of OMCSs at the site of maxillofacial bone defects has not yet been attempted.

Recently, the rat mandibular symphysis, i.e., the central portion of the rat mandible, which consists of fibrous connective tissue and thus can be interpreted as a physiological bone gap, has been used to assess bone graft materials, particularly for the purpose of maxillofacial bone regeneration (Yagyuu, Kirita, Hattori, Tadokoro, & Ohgushi, 2015). Therefore, in this study, we adopted a rat mandibular symphysis model and examined whether implantation of OMCSs could fill the gap with new bone tissue, leading to bone union.

2. Materials and methods

2.1. Animals

All animal studies were approved by the animal care and use committee of Nara Medical University before beginning the experiments. Fischer 344 (F344) rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Seven-week-old male rats were used as donors for marrow cell preparation, and 15-week-old rats were used as recipients.

2.2. Cell culture and cell sheet preparation

OMCSs were used in this study and were prepared as previously reported (Akahane et al., 2008; Inagaki et al., 2013; Nakamura et al., 2010). In brief, rat bone marrow plugs were flushed out and resuspended in basic culture medium, i.e., minimum essential medium (Nacalai Tesque Inc., Kyoto, Japan) containing 15% fetal bovine serum (Gibco, Invitrogen, CA, USA) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Nacalai Tesque Inc.). Cells were cultured in T-75 flasks in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. After reaching confluence, the primary cultured cells were harvested using trypsin/ethylenediaminetetraacetic acid (Gibco, Invitrogen). To generate the OMCSs, the harvested cells were seeded at a cell density of 1×10^4 cells/cm^2 in 6-cm culture dishes with basic culture medium, 10 nM Dex (Sigma-Aldrich, MO, USA), and 0.28 mM ascorbic acid phosphate (Wako Pure Chemical Industrials, Kyoto, Japan) and then subcultured. After reaching confluence, the cells were rinsed twice with phosphate-buffered saline (Gibco, Invitrogen) and then formed into a sheet using a scraper (Fig. 1A).

2.3. In vitro evaluation of OMCSs

Samples of the OMCSs were fixed in 10% formaldehyde neutral buffer solution for 1 week and embedded in paraffin. Each specimen was cut into 5-μm sections, and the sections were stained with hematoxylin and eosin (H&E). Immunohistochemical staining for type I collagen, osteopontin (OPN), and osteocalcin (OCN) was performed on 5-μm sections mounted on glass slides. To enhance antigen retrieval, all sections were treated with 3% hydrogen peroxidase for 10 min to block endogenous peroxidase activity and subsequently blocked for 10 min at 37 °C with 1% bovine serum albumin, followed by overnight incubation at 4 °C with specific primary antibodies, including anti-type I collagen (LB1102; LSL, Inc., Japan; 1:500 dilution), anti-OPN (01-0091; ARP, Inc., USA; 1:100 dilution), and anti-OCN (M186; TaKaRa Bio, Inc., Japan; 1:100 dilution). The slides were then rinsed and incubated for 30 min at 37 °C with biotinylated secondary antibodies. The slides were then washed with Tris-buffered saline, and peroxidase-streptavidin was added for 30 min at 37 °C. Finally, the slides were rinsed well with Tris-buffered saline and developed with 3,3′-diaminobenzidine.

2.4. Implantation protocol

Prior to implantation, we folded the OMCSs into a lump, 2-mm in diameter (Fig. 1B). Shortly thereafter, we implanted the OMCSs into the mandibular symphysis of recipient 15-week-old syngeneic rats, as previously reported (Yagyuu et al., 2015). Briefly, we anesthetized each rat with pentobarbital and shaved the incision site (Fig. 1C). An incision was created in the skin at the inferior margin of the mandible. After exposure of the periosteum of the left and right mandibles, the periosteum was incised and separated. The fibrous tissue between the left and right mandibles was then curetted, creating space for the implant (Fig. 1D). Finally, we implanted a lump of OMCSs into the space (Fig. 1E) and closed the periosteum and skin layers separately. We performed this procedure in 30 rats (experimental group); an additional 10 rats underwent surgery without implantation (control group). Ten animals were sacrificed at each time point (2, 4, and 8 weeks postoperatively) in the experimental group, and 10 animals were sacrificed at 8 weeks postoperatively in the control group. The mandibles were compared using micro-computed tomography (micro-CT) and histological analyses to evaluate the ability of OMCSs to fill the bone at the mandibular symphysis.

2.5. Micro-CT analyses

The harvested rat mandibles were analyzed using a micro-CT (Toscaner–32300 μ-FPD; Toshiba IT and Control Systems Corp., Tokyo, Japan). Each mandible was scanned at intervals of 10 μm at 70 kV and 200 μA. Three-dimensional images were constructed using VG Studio software (Volume Graphics, Heidelberg, Germany). The images were evaluated semiquantitatively using a radiological union scale (Table 1) (Yagyuu et al., 2015). Furthermore, we evaluated the new bone volume. We measured an area of calcification in the mandibular symphysis as a high-density area, defined as a density equal to or greater than 220 CT units, within the region of interest (ROI). To set the ROI, we first established the axial plane perpendicular to the occlusal plane of the molar teeth and on the distal side, 2 mm farther than the plane including the lowest point of the chin, i.e., the menton. In this plane (transverse plane), we defined an ROI as a square area 1.0 mm in height and 1.0 mm in width positioned in the mandibular symphysis. The calcification area (mm^2) was measured using ImageJ software (v. 1.49; NIH, USA).

2.6. Histological analysis

After micro-CT analysis, mandibles from each group of rats were fixed in 10% formaldehyde neutral buffer solution, decalcified (K-CX; Falma Inc., Tokyo, Japan), embedded in paraffin, and stained with H&E and toluidine blue solution. The histological sections were evaluated using a histological union scale (Table 1) (Yagyuu et al., 2015). Next, we performed histomorphometric analysis. We established an ROI as a square area 1.0 mm in height and 1.0 mm in width positioned between the left and right mandibles in the histological slide of the transverse plane, and the new bone formation area (mm^2) was measured using ImageJ software.

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