Original article

Transplantation of dedifferentiated fat cells combined with a biodegradable type I collagen-recombinant peptide scaffold for critical-size bone defects in rats

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Abstract: Tissue engineering is a promising approach to supplement existing treatment strategies for craniofacial bone regeneration. In this study, a type I collagen scaffold made from a recombinant peptide (RCP) with an Arg-Gly-Asp motif was developed, and its effect on regeneration in critical-size mandibular bone defects was evaluated. Additionally, the combined effect of the scaffold and lipid-free dedifferentiated fat (DFAT) cells was assessed. Briefly, DFAT cells were separated from mature adipocytes by using a ceiling culture technique based on buoyancy. A 3 cm × 4 cm critical-size bone defect was created in the rat mandible, and regeneration was evaluated by using RCP with DFAT cells. Then, cultured DFAT cells and adipose-derived stem cells (ASCs) were seeded onto RCP scaffolds (DFAT/RCP and ASC/RCP) and implanted into the bone defects. Micro-computed tomography imaging at 8 weeks after implantation showed significantly greater bone regeneration in the DFAT/RCP group than in the ASC/RCP and RCP-alone groups. Similarly, histological analysis showed significantly greater bone width in the DFAT/RCP group than in the ASC/RCP and RCP-alone groups. These findings suggest that DFAT/RCP is effective for bone formation in critical-size bone defects and that DFAT cells are a promising source for bone regeneration.

Keywords: adipose-derived mesenchymal stem cells, bone regeneration, critical-size bone defect, dedifferentiated fat cells, recombinant peptide scaffold, type I collagen scaffold

Introduction

Successful repair of jawbone deficiencies is a major concern in dentistry. Such defects require operative transfer of substantial amounts of bone for reconstruction [1-4]. Autologous trabecular bone is widely regarded as the gold standard for bone augmentation, since it supplies growth factors, osteogenic cells, and mechanical support [5,6]. However, the transfer of autologous bone grafts is often inadequate for large defects [7,8], owing to the limited amount of bone available to be harvested. Therefore, dentists must identify a better approach for repairing large bony defects.

Several bone augmentation substitutes based on hydroxyapatite (HA) or beta-tricalcium phosphate are the primary candidates to replace autologous bone grafts for large defects; however, bone augmentation approaches using optimal materials have not been established [9-11]. During osteogenic development, mesenchymal stem cells (MSCs) produce various extracellular matrix (ECM) proteins, such as collagen; in particular, type I collagen is the main structural protein in bone structure. Collagens are the

most extensively used synthetic polymers in bone regeneration approaches. Collagen scaffold systems have advantages for bone tissue engineering, because artificial collagen scaffolds can support proliferation and ECM deposition of encapsulated MSCs. However, a suitable collagen scaffold for bone tissue engineering has not been developed. This can be explained by the lack of specific cell binding domains on the collagen surface and the high hydrophilicity and low protein adsorption of collagen, which limit cell-matrix interactions and diminish osteogenic cellular responses.

Natural type I collagen enhances cellular attachment, because of its abundant Arg-Gly-Asp (RGD) residues, and induces osteogenic differentiation [12-14]. The RGD motif is generally present in ECM components, such as vitronectin, fibronectin, and thrombospondin, and is recognized and bound by integrin receptors expressed on the cell surface [15-18]. Several studies have focused on the use of cell adhesion motifs containing RGD to enhance cell-matrix interactions [14]. In addition, incorporation of RGD-containing peptides on the biomaterial surface has been shown to reinforce cell adhesion and osteogenic differentiation [19]. Fujifilm Corp. (Tokyo, Japan) developed a novel bioabsorbable recombinant protein (RCP) for medical use. An assessment of RCP based on the alpha-1 sequence of human collagen type I confirmed the presence of 12 RGD motifs in a single molecule. RCP produced by the yeast *Pichia pastoris* differs from conventional animal collagen because there is no risk of infections such as bovine spongiform encephalopathy [20,21]. The effect of using RCP in combination with osteogenic cells for bone tissue engineering has not been evaluated.

Dedifferentiated fat (DFAT) cells are established by asymmetrical division of mature adipocytes from adipose tissue with a ceiling culture technique, which relies on the inherent buoyancy of adipocytes [22-25]. Unlike terminally differentiated adipocytes, DFAT cells have the potential to actively proliferate and have the characteristics of MSCs, i.e., multiple differentiation into osteoblasts [26,27], adipocytes [26,27], chondrocytes [26], skeletal myocytes [28], smooth muscle cell lineages [29,30], cardiomyocytes [31], and endothelial cells [32,33] *in vitro*.

Two types of adipose-derived stem cells (ASCs), identified as MSCs and DFAT cells, can be isolated from subcutaneous adipose tissues or buccal fat pads [22-27]. Several studies reported that DFAT cells and ASCs potentially contribute to regeneration of periodontal tissue in animal models [22,24,27]. However, previous results suggest that osteoblastic differentiation potential is greater for DFAT cells than for ASCs [22], which is consistent with data from other studies [34]. Existing evidence indicates that DFAT cells are a particularly promising cell source for bone and periodontal tissue regeneration; however, clinical use of DFAT cells has not been reported. In the context of a clinical need for a more effective approach, it may be feasible to regenerate critical-size bone defects with DFAT cells and RCP scaffolds. However, the combination of DFAT cells and RCP scaffolds has not been evaluated.

The present authors hypothesized that incorporation of RGD into collagen scaffolds would synergistically enhance osteogenic differentiation of encapsulated DFAT cells and bone regeneration. To test this hypothesis, a

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Fig. 1 (A) The upper section illustrates the approach used to isolate DFAT cells from floating mature adipocytes. Floating cells (mature adipocytes) attach to the upper surface of culture flasks. DFAT cells emerged during asymmetrical division of floating cells for 7 days. The lower section illustrates the approach used to isolate ASCs. After centrifugation, the SVF fraction was separated by sedimentation from mature adipocytes and cultured for preparation of ASCs. (B) Morphology of cultured ASCs (P0). Primary ASCs were more spindle-shaped than primary DFAT cells. (C) Morphology of cultured DFAT cells (P0). When culture flasks were inverted for 7 days, some DFAT cells retained lipid droplets. Scale bars = 100 μ m.

collagen scaffold functionalized with the RGD peptide was used to evaluate bone formation, as well as proliferation and osteogenic differentiation of encapsulated DFAT cells. In addition, application of rat DFAT cells and rat ASCs to large bone defects was compared.

Materials and Methods

All animal studies were reviewed and approved by the Animal Research and Care Committee at the Nihon University School of Dentistry (AP17D020).

Preparation of dedifferentiated fat cells

Eight-week-old male F344 rats ($n = 5$, body weight 190 ± 10 g) were purchased from CLEA Japan, Inc. (Tokyo, Japan) for isolation of DFAT cells. Briefly, approximately 1 g of inguinal subcutaneous adipose tissue obtained from 10-week-old rats was processed to isolate adipocytes. Isolated adipocytes were placed in culture flasks (BD Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) filled with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS; 13B103, Sigma-Aldrich) as growth media. Mature adipocytes floated up and adhered to the top inner surface of the culture flasks. After about 1 week, the medium was replaced with growth media, and the flasks were inverted so that the cells were on the bottom of the culture flasks (Fig. 1) [22–25]. The initial DFAT cells, referred to as passage 0 (P0), were passaged at 80% confluence.

Preparation of adipose-derived stem cells

Cultured ASCs were prepared as described previously [27]. Briefly, the stromal vascular fraction (SVF) was isolated by centrifugation as the pellet fraction from collagenase-digested adipose tissue, after collecting the floating uppermost layer, as described above. These cells are referred to as SVF cells and were cultured in DMEM supplemented with 10% FBS (Fig. 1). ASCs and DFAT cells at the second and third passages obtained from the same rat were used for *in vivo* experiments.

Complex of biodegradable scaffolds and cells

The recombinant peptide (RCP, Cellnest; Fujifilm Corp., Tokyo, Japan) from human type I collagen α chain was prepared as previously described [20,21]. The biodegradable RCP porous block scaffolds were resized to approximately $3 \times 4 \times 0.5$ mm, and sterilized biodegradable RCP block

Fig. 2 Macroscopic view of a surgically created critical-size bone defect (4×3 mm) on the buccal surface of the mandible.

scaffolds were purchased from Fujifilm Corp. To prepare the complex of RCP-block scaffold and cells, 500- μ L aliquots of the cell suspension (2×10^6 cells/mL, 1×10^6 cells/RCP) were seeded onto the tops of RCP, centrifuged at $600 \times g$ for 5 min, and then incubated for 6 h before implantation.

To analyze pore size, RCP-block scaffolds were vaporized with an approximately 30-nm layer of carbon by using a vacuum evaporator (JEE-420T; JEOL, Tokyo, Japan) and then evaluated with a field-emission electron probe microanalyzer (JXA-8530FA, JEOL).

Cell transplantation

Fifteen male F344 rats (age, 10 weeks; weight, 200 ± 10 g) were used for the *in vivo* experiment. Under isoflurane (Narcobit-E; KN-1071, Natsume Seisakusho Co. Ltd., Tokyo, Japan) inhalation, critical-size mandibular defects were created as previously described [35]. Briefly, a skin incision was made along the inferior border of the right mandible, and the masseter muscle and periosteum covering the buccal surface of the mandible were elevated as a flap. After measuring $4 \times 3 \times 1$ mm of mandible, a low-speed inverted corn bur was used to grind the bone tissue posterior to the incisor and contiguous to the inferior border of the mandible (Fig. 2). Appropriately sized RCP-block scaffolds were placed in the defects and covered with a membrane (Bio-Gide, Geistlich Pharma, Wolhusen, Switzerland). The masseter muscles were repositioned and the skin was closed with nonresorbable sutures. To prevent infection, Dolmycin ointment (Zeria Pharmaceutical, Tokyo, Japan) was applied to the sutured area for postoperative care. The following four groups were established: (1) blank scaffolds, regarded as the control group (bone defect without any scaffold and cells) ($n = 5$), (2) RCP-block scaffolds covered with the membrane, but without cells (RCP-alone group) ($n = 4$), (3) ASC-loaded RCP-block scaffolds covered with the membrane (ASC/RCP group) ($n = 4$), and (4) DFAT cell-loaded RCP-block scaffolds covered with the membrane (DFAT/RCP group) ($n = 5$). The left side of the mandible was not manipulated, to maintain oral function.

In vivo x-ray micro-computed tomography imaging and analysis

An X-ray micro-computed tomography (CT) device (Rigaku Corp., Akishima, Japan) was used as previously described [22,24]. The exposure parameters were 17 s, 90 kV, and 100 μ A. The isotropic voxel size was 30 μ m.

Briefly, images were obtained from individual rats immediately after the operation and each week thereafter, until 8 weeks after the operation. The images were reconstructed into 3D images with i-View (J. Morita Co., Kyoto, Japan). Bone volume was measured in regions of interest (ROIs) from voxel images by using the bone volume-measuring software package 3 by 4 Viewer 2012 (Kitasenju Radist Dental Clinic I-View Image Center, Tokyo, Japan). The ROI size was $2.4 \times 1.8 \times 2.1$ mm, which covered the surgically created critical-size bone defects. The increase in bone volume in each rat was then calculated by subtracting bone volume on day 0 from

Fig. 3 SEM images of RCP generated by crosslinking the freeze-dried porous peptide block were obtained. Images at lower ($\times 4$, left panel) and higher ($\times 200$, right panel) magnification are shown. A clear porous structure was observed. Scale bars: 100 μm .

Fig. 4 Micro-CT images were obtained from rats implanted with RCP alone (RCP, second line), ASCs plus RCP (ASC, third line), and DFAT plus RCP (DFAT, fourth line) at 4, 6, and 8 weeks after surgery. RCP-implanted groups showed a gradual increase in the radiopacity of bone defects between 4 and 8 weeks. Except for the control (None, 1st line), new bone formation was present at 4 weeks in all groups (arrowheads) and had progressed at 8 weeks. Radiopacity was greatest in the DFAT/RCP group at 8 weeks.

each of the subsequent values.

Histological analysis

Eight weeks after the operation, all animals were sacrificed, and right mandibles were harvested for histological analysis as previous described. Briefly, the mandibles were fixed in 10% neutral-buffered paraformaldehyde, decalcified in 10% ethylenediaminetetraacetic acid (EDTA), dehydrated through a graded series of ethanol solutions, and then embedded in paraffin. The paraffin sections were stained with hematoxylin and eosin (H&E).

Statistical analysis

Data are expressed as means and standard deviation (SD) for each group. Statistical analyses were performed by using GraphPad Prism 5 software (GraphPad Inc, La Jolla, CA, USA). Normality was analyzed with the Kolmogorov-Smirnov test, and homoscedasticity was confirmed with the Bartlett test. One-way analysis of variance (ANOVA) and the Tukey multiple comparisons test were used for intergroup comparisons. Values of $***P < 0.001$ and $****P < 0.0001$ were considered statistically significant.

Results

Pore size of the RCP-block scaffold

Six pores were randomly selected (Fig. 3). On the basis of measurements of diameters, pore size in the RCP-block scaffold was $100.3 \pm 24.2 \mu\text{m}$ (mean $\pm$ SD) (Fig. 3).

Fig. 5 There were significant differences between the DFAT/RCP and ASC/RCP groups at 6 weeks, between the DFAT/RCP and ASC/RCP groups at 8 weeks, and between the DFAT/RCP and RCP-alone groups at 8 weeks ($n = 5$, $***P < 0.001$, $****P < 0.0001$). Normality was analyzed with the Kolmogorov-Smirnov test, and homoscedasticity was confirmed with the Bartlett test. One-way analysis of variance (ANOVA) and the Tukey multiple comparisons test were used for intergroup comparisons.

Implantation of DFAT/RCP enhanced bone regeneration

After large bone defect formation at the inferior border of the mandible, the rats were divided into four groups, as described in the Materials and Methods. A slight loss of body weight (-8.6%) was observed in rats in all groups at the time of sacrifice, but there were no statistically significant differences among groups. No severe inflammation or swelling was seen at any evaluated site during the experimental period. Newly formed bone was clearly observed at the defect sites in all groups at 4 weeks after implantation. At this time point, the range of new bone formation was greater in the DFAT/RCP group than in the ASC/RCP group and RCP-alone group (Fig. 4). In addition, the newly formed cortical bone-like tissue was connected to the native cortical bone at the mesial side at 6 weeks, and at both mesial and distal sides at 8 weeks, in the DFAT/RCP group, but the bone defect was still not occupied by new bone-like tissue in the control, RCP-alone, or ASC/RCP groups at 8 weeks.

The amount of newly formed bone was measured by using micro-CT images. As shown in Figure 5, the amount of newly formed bone was substantial in the DFAT/RCP group (hatched bars) and was significantly greater than in the ASC/RCP group at 6 weeks ($***P < 0.001$) and significantly greater than in the RCP-alone and ASC/RCP groups at 8 weeks ($****P < 0.0001$).

Histological examination

At 8 weeks after implantation, H&E staining and histological analyses were performed. At low magnification, the whole bone defect area was filled with newly formed bone in the DFAT/RCP group (Fig. 6A). Although new bone was generated in the ASC/RCP (Fig. 6B), RCP-alone (Fig. 6C),

Fig. 6 Hematoxylin-eosin (H&E)-stained specimens of samples at 8 weeks (A-D: scale bars: 300 μ m). (A) RCP scaffold seeded with DFAT cells, (B) RCP scaffold seeded with ASCs, (C) RCP-scaffold alone, and (D) no implantation (control). Formation of new bone was limited in the control group. The area enclosed by the dotted line shows the bone defect area. The remnant of the implanted RCP-scaffold was not obvious in the bone defect sites. New bone formation was significantly greater in the DFAT/RCP group than in the ASC/RCP and RCP-alone groups. c, condylar process; m, mandibular bone; i, incisor

and control (Fig. 6D) groups, significantly less bone was regenerated than in the DFAT/RCP group. At high magnification, dense lamellar bone was readily observed in all samples. All samples had osteoblasts lining the bone matrix and numerous osteocytes incorporated in the lacunae of the newly generated bone structure (Fig. 7). In addition, vascularization in newly formed tissues was observed in all samples (Fig. 7).

Discussion

This study used DFAT cells seeded on RCP-block scaffolds to evaluate a prospective therapeutic approach for regeneration of large bone defects. The standard approach for repair of large bone defects caused by tumors or trauma is autogenous bone grafting [36]. However, this approach requires surgical harvesting of bone, and the amount of bone available for harvesting is limited [37,38]. Therefore, several bone-graft substitutes, such as HA and calcium phosphate, have been used [39]. However, a standard method using bone substitutes has not been developed for large bone defects.

Why was an RCP-block scaffold used as a scaffold to repair large bone defects in this study? Successful bone regeneration requires a suitable 3D scaffold that maintains space for cell growth and differentiation. The appropriate scaffold for bone regeneration should be bioabsorbable, so that it can eventually be replaced by regenerated bone tissue [40]. In addition, a 3D bioabsorbable scaffold is necessary in order to deliver MSCs or osteogenic cells to bone defects and to provide initial support for the growth of transplanted cells [41]. The new bioabsorbable biomaterial RCP, developed by Fujifilm Corp., is based on the α -1 sequence of human collagen type I and has 12 RGD (Arg-Gly-Asp) motifs in a single molecule and a repeat of the RGD peptide. Numerous studies have used cell adhesion motifs containing RGD motifs to increase cell-matrix interactions [14]. In addition, incorporation of RGD-containing peptides on biomaterial surfaces increases cell adhesion and osteogenic differentiation [19] because RGD-peptides facilitate interaction of cellular integrin receptors with bone matrix proteins [42]. For example, coating HA surfaces with an RGD-containing peptide increased attachment and differentiation of osteoblasts [43,44]. In sum, existing evidence suggests that the RCP-block scaffold with RGD peptide may be useful for treatment of large bone defects.

Several bone matrix proteins, such as natural collagen and osteopontin, contain the RGD sequence. In particular, natural type I collagen enhances cellular attachment, because of its abundant RGD residues, and induces osteogenic differentiation [12-14]. However, use of natural type I collagen as a scaffold is limited by its potential immune and pathogenic effects. The RCP-block scaffolds produced by recombinant peptides in this study carry no risk of rejection or infection and can be fabricated into custom-designed

Fig. 7 Hematoxylin-eosin stained specimens of samples at 8 weeks. High magnification views of part of the area surrounded by the dotted line in Figure 6 (A-D: scale bars: 50 μ m). (A) RCP scaffold seeded with DFAT cells, (B) RCP scaffold seeded with ASCs, (C) RCP-scaffold alone, and (D) no implantation (control group). In all groups, osteoblasts (arrows) lined the existing bone, which contained osteocytes (arrowheads).

shapes and sizes. To the best of the authors' knowledge, no studies have evaluated the bone regeneration potential of RCP-block scaffolds.

When an RCP-block scaffold alone was implanted into a critical-size bone defect in this study, the defect space was mainly occupied by connective tissue with some bone at 8 weeks after implantation. This result suggests that the RCP-block scaffold helps maintain space for tissue regeneration in critical-size bone defects and supports the use of the RCP-block as a bone substitute to repair defects. However, the bone defect was completely occupied by newly formed connective and bone tissues. Accordingly, additional external interventions, such as the use of osteogenic cells, may be necessary in order to compensate for inadequate bone regeneration with the RCP-block scaffold alone for large bone defects.

Why were DFAT cells combined with the RCP-block scaffold for repairing critical-size bone defects in this study? Several recent studies using a ceiling culture technique showed that adipocytes could be converted into mesenchymal stem cell-like cells, known as DFAT cells [26]. Additionally, ASCs have been widely investigated as potential MSCs in regenerative medicine. In previous studies, DFAT cells exhibited relevant MSC characteristics at frequencies higher than those of ASCs produced from a given amount of the same adipose tissue [22,45]. In addition, compared with ASCs, DFAT cells were a more homogeneous cell population [26]. Furthermore, the capacity for osteoblastic differentiation is greater for DFAT cells than for ASCs [22,34]. The present authors' previous *in vivo* study showed that implantation of DFAT cells with 3D scaffolds was a more effective approach for enhancing periodontal tissue regeneration, as compared with using ASCs in a fenestration defect, and in three-wall intrabony periodontal defect models [22,24]. The results suggest that the DFAT/RCP complex strongly induces bone regeneration. The present study compared the *in vivo* effects of the DFAT/RCP complex and ASC/RCP in critical-size bone defects.

To determine the regeneration potential in critical-size bone defects, reconstituted micro-CT images were prepared until week 8, when the artificially created defect area was nearly filled with newly generated bone in the DFAT/RCP group. The quantity of new bone was calculated at 6 and 8 weeks. Although bone volume at 6 weeks did not significantly differ between the RCP-alone and DFAT/RCP groups, it was significantly higher in the DFAT/RCP group than in the RCP-alone group at week 8.

By week 8, the created defect sites were easily identifiable histologically in the RCP-alone and ASC/RCP groups because the remaining bone defect was clearly visible. In the DFAT/RCP group, bone regeneration was promoted by increasing bone volume, as evidenced by micro-CT analyses. As predicted, implantation of the RCP scaffold carrying DFAT cells enhanced bone regeneration, thus demonstrating potential for healing

critical-size bone defects [14]. However, the precise mechanism by which implanted cells enhance bone regeneration was not investigated in this study. In addition, the present study was limited by the very short duration of the analysis, which was not sufficient to predict differences over time, and the limited number of rats in each study group. To confirm the present conclusions, future studies should use a larger number of animals.

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Conflict of interest

The authors declare that they have no competing interests.

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