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Comparison of different tissue-derived stem cell sheets for periodontal regeneration in a canine 1-wall defect model

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ABSTRACT

Cytotherapeutic approaches have been investigated to overcome the limitations of existing procedures for periodontal regeneration. In this study, cell sheet transplantation was performed using three kinds of mesenchymal tissue (periodontal ligament, alveolar periosteum, and bone marrow)-derived cells to compare the differences between cell sources in a canine severe defect model (one-wall intrabony defect). Periodontal ligament cells (PDLCs), iliac bone marrow mesenchymal stromal cells (BMMSCs), and alveolar periosteal cells (APCs) were obtained from each dog; a total of four dogs were used. Three-layered cell sheets of each cell source supported with woven polyglycolic acid were autologously transplanted to the denuded root surface. One-wall intrabony defects were filled with a mixture of β-tricalcium phosphate (β-TCP) and collagen. Eight weeks after the transplantation, periodontal regeneration was significantly observed with both newly formed cementum and well-oriented PDL fibers more in the PDLC group than in the other groups. In addition, nerve filament was observed in the regenerated PDL tissue only in the PDLC group. The amount of alveolar bone regeneration was highest in the PDLC group, although it did not reach statistical significance among the groups. These results indicate that PDLC sheets combined with β-TCP/collagen scaffold serve as a promising tool for periodontal regeneration.

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1. Introduction

Periodontitis is an inflammatory disease that causes pathological alterations in the tooth-supporting tissues and leads to tooth loss. In addition, the associations between periodontitis and systemic diseases, such as cardiovascular disease, diabetes mellitus, and a higher risk of preterm low birth-weight babies, have been reported [1–3]. Various regenerative therapies such as guided tissue regeneration and enamel matrix derivative have been routinely utilized in clinical practice. However, the outcomes have been limited and have been associated with poor clinical predictability [4,5]. Therefore, stem cell-based approaches for periodontal regeneration have been conducted recently [6].

Previous studies have demonstrated that cytotherapies using periodontal ligament cells (PDLCs), bone marrow mesenchymal stromal cells (BMMSCs), and alveolar periosteal cells (APCs) can be effective in periodontal regeneration in large animal models [7–12]. In particular, PDL tissue contains multi-potent stem cell populations and contributes to the regeneration of periodontal ligament, cementum, and alveolar bone [7,8,13]. BMMSCs have been reported to differentiate into periodontal ligament [10,14], and the transplantation of BMMSCs has supported periodontal regeneration [9,10,14]. APCs have been reported to improve periodontal regeneration as well [11,12]. However, no study has compared the periodontal regenerative properties of these different cell sources.

"Cell sheet tissue engineering" is a unique technique in tissue regeneration, with cell sheets harvested from temperature-responsive culture dishes [15]. Recent studies have shown that this technique is effective for the treatment of corneal dysfunction [16], myocardial infarction [17], and oesophageal ulcerations [18]. We have developed cell sheet engineering for periodontal regeneration and shown that PDLC sheets combined with β-tricalcium

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phosphate (β-TCP) induced true periodontal tissue regeneration in a dog model [8].

Therefore, the aim of this study was to compare the potential of different cell sheets obtained from three kinds of mesenchymal tissues (e.g., PDL tissue, iliac bone marrow, and alveolar periosteum) in a canine severe periodontal defect model (one-wall intrabony defect).

2. Materials and methods

2.1. Animals

Four healthy beagle dogs (10 kg, male) were used in this study. The animals exhibited intact dentition with healthy periodontium. All experimental protocols were approved by the animal welfare committee of Tokyo Women's Medical University.

2.2. Cell culture

Dogs were intramuscularly injected with 0.1 mg/kg medetomidine (Domitor, Zenoaq Nippon Zenyaku Kogyo, Fukushima, Japan) and 15 mg/kg ketamine (ketalal, Daiichi Sankyo Propharma, Tokyo, Japan) for anesthetic premedication and then subjected to an intravenous injection of 2.5 mg/kg propofol (Diprivan, AstraZeneca, Osaka, Japan). An endotracheal tube was inserted, and anesthesia was maintained with sevoflurane (Sevofrane, Abbott Japan, Tokyo, Japan). Supra- and subgingival deposits of the premolars and molars were removed with an ultrasonic scaler. Local anesthesia was performed with 2% lidocaine hydrochloride containing epinephrine at a concentration of 1:80000 (Xylocaine Cartridge for Dental Use, Dentsuply-Sankin, Tokyo, Japan). Eight weeks before transplantation, the second and fourth mandibular premolars were extracted, and periosteum (5 × 5 mm) was obtained from the mandibular bone of each dog. Bone marrow aspirates of 2 mL were taken from the iliac crest of each dog and placed into a 50 mL tube containing 20 mL of Dulbecco's Phosphate Buffered Saline (D-PBS) (Invitrogen, Carlsbad, CA) and heparin (100 U/mL) (Mochida Pharmaceutical, Tokyo, Japan).

Extracted teeth and periosteum were rinsed 5 times with α-Minimum Essential Medium (α-MEM) (Invitrogen) containing 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen) for 3 min each. PDL tissues that were gently separated from the surface of the mid-third portion of the extracted root and periosteum were dispersed with α-MEM containing 0.8 PZ-U/mL collagenase type I (Serva Electrophoresis, Heidelberg, Germany) and 1200 PU/mL dispase (Sanko Junyaku, Tokyo, Japan) for 45 min at 37 °C. Single cell suspensions were obtained by passing cells through a 70 μm strainer (Falcon, BD Labware, Franklin Lakes, NJ) and spread on a T25 Primaria culture flask (Falcon) (Passage 0). Single cell-derived colonies were cultured in a complete medium [α-MEM supplemented with 100 U/mL penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS, Moregate Biotech, Queensland Australia)] at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells were subcultured using trypsin and ethylene diamine tetraacetic acid (EDTA) (0.05% Trypsin with 0.53 mM EDTA-4Na) (Invitrogen).

One milliliter of bone marrow aspirates was seeded on a 100 mm culture dish and maintained in 20 mL Dulbecco's modified Eagle's/F12 medium (DMEM/F12) supplemented with 10% FBS, 50 μg/mL gentamicin (Schering-Plough, Osaka, Japan), and 0.25 μg/mL amphotericin B (Bristol-Myers Squibb, New York, NY). Three days after seeding, the floating cells were removed and the medium was replaced by fresh medium. Passages were performed when the cells approached confluence.

2.3. Cell proliferation assay

Cells at passage 4–6 (PDLCs; $n = 3$, BMMSCs; $n = 4$, and APCs; $n = 4$) were plated in 96-well culture plates (1000 cells/well) and cultured in complete medium for 24 h. Cell proliferation was measured using the CellTiter 96 Aqueous Assay kit (Promega, Madison, WI). After 1 h of incubation with CellTiter 96 Aqueous solution, absorbance at 490 nm was determined by a microplate reader (Molecular Devices, Sunnyvale, CA) with SoftMax Pro software.

2.4. Preparation of cell sheets

The preparation of cell sheets was performed as previously described [8]. Briefly, canine PDLCs, BMMSCs, and APCs at passage 3 were seeded on temperature-responsive culture dishes (35 mm in diameter, UpCell®, Cell Seed, Tokyo, Japan) at a cell density of 3–5 × 10^4 cells/dish and cultured in an osteoinductive medium supplement with 50 μg/ml ascorbic acid (Wako, Tokyo, Japan), 10 mM β-glycerophosphate (Sigma–Aldrich, St Louis, MO), and 10 nM dexamethasone (DEXART; Fuji Pharma, Toyama) for 5 days. For the cell sheet harvest, the temperature was reduced to room temperature before the culture medium was aspirated and a wet sheet of woven polyglycolic acid (PGA) (Neoveil®, PGA Felt-Sheet Type, 0.15 mm in thickness: Gunze, Tokyo, Japan) was placed on the culture dish as a reinforced carrier. Cell sheets attached with PGA were harvested by peeling them from the dishes with forceps and then put on the next culture dish. This procedure was repeated two more times until three-layered cell sheets were fabricated.

2.5. Transplantation

All surgical procedures were performed under general anesthesia. After an 8 week period of healing and cell cultures, one-wall intrabony defects (5 × 5 mm in depth, mesio-distal width) were created surgically on the mesial and distal sides of mandibular third premolars and the mesial of mandibular first molars bilaterally. The PDLC sheets transplantation group (PDLC group), the BMMSC sheets transplantation group (BMMSC group), the APC sheets transplantation group (APC group), and the control group were each assigned, in rotation, to one defect of a dog. The remaining defects were used for another study. Root cementum was removed with curettes, followed by conditioning with 19% EDTA (Sigma–Aldrich) for 2 min. Following washing with saline, the three-layered cell sheets supported by PGA sheets were trimmed to the size of the root defects and applied to the exposed root surfaces in the experimental group, while only PGA carriers were applied in the control group (Fig. 2A). Intrabony defects were filled with porous β-TCP (Osferion®, G1, Olympus Terumo Biomaterials, Tokyo, Japan) that was mixed with 3% type I collagen (1:1 ratio by weight, β-TCP/collagen) (Koken, Tokyo, Japan) (Fig. 2B). Finally, gingival flaps were repositioned and sutured. Post-surgical management involved antibiotics (Azithromycin, 250 mg, Pfizer, Tokyo, Japan) daily for 3 days, a soft diet, and topical application of 2% solution of chlorhexidine (Hibitane® concentrate, Sumitomo, Osaka, Japan) twice a week for 8 weeks. The sutures were removed 2 weeks after surgery.

2.6. Histological observation and histometric analyses

Eight weeks after transplantation, all animals were euthanized by an overdose injection of potassium chloride. Surgical sites were dissected and then fixed in 10% neutral buffered formalin (Wako). The specimens were decalcified in Plank-Rychro solution (Wako) for approximately 3 months, routinely processed into 6 μm thick paraffin-embedded sections, stained with hematoxylin and eosin (H&E) or Azan, and observed under a microscope (Eclipse E800, Nikon, Tokyo, Japan). The samples were also observed by a polarizing light microscope under the same illumination conditions (Eclipse TE2000-U, Nikon). Sections were selected from the most central areas of the defects, identified by the sizes of the root canal. Histometric and morphometric analyses were performed with a software package (Photoshop, Adobe, San Jose, CA). The mean value of each histometric parameter was calculated for each site. The following parameters were measured by two calibrated examiners who were blinded to the experimental conditions: (i) Newly formed cementum thickness (μm): the length of cementum regenerated in the direction vertical to denuded root surface. The length were measured every 500 μm on the newly formed cementum, and the mean was calculated [19]; (ii) Periodontal score: each specimen was scored as outlined by Wikesjo et al. [20]; (iii) Bone regeneration ratio (%): the distance between the bottom of bone defect and coronal extension of new alveolar bone by defect height; and (iv) apical extension of the junctional epithelium (mm): the distance between the apical extension of junctional epithelium and cement-enamel junction.

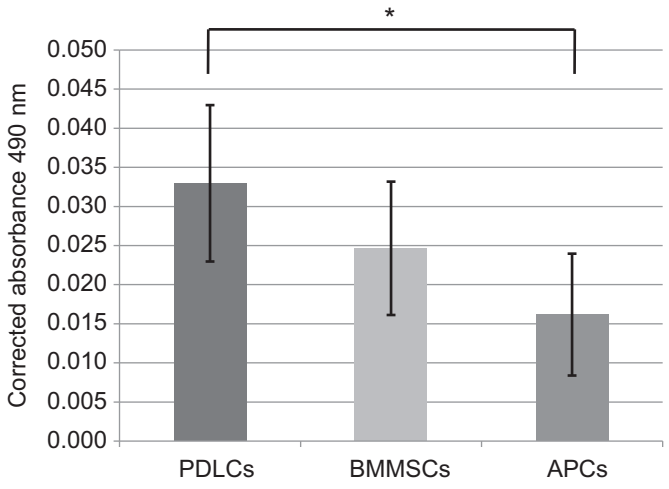

Fig. 1. Cell proliferation assay of PDLCs ($n = 3$), BMMSCs ($n = 4$), APCs ($n = 4$). *Statistically significant difference ($p < 0.05$).

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