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Original

Adhesion of human periodontal ligament cells by three-dimensional culture to the sterilized root surface of extracted human teeth

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Abstract: Residual periodontal ligament (PDL) and cement mass on the roots of extracted teeth are factors that considerably affect tooth transplantation. Therefore, when normal extracted teeth are used for autologous transplantation, it is necessary to regenerate the PDL of the root surface. Here we describe a method to examine human PDL cell adhesion on sterilized root surfaces. Sample teeth were extracted during orthodontic treatment. PDL cells were obtained from healthy periodontal tissue explants from teeth extracted for orthodontic reasons. We developed a method for adhering PDL cells to sterile root surfaces using three-dimensional culture for 3 weeks. We evaluated the adhesion of human PDL cells to the sterilized root surfaces biochemically and histologically. The adherent PDL cells presented new projections on the sterile root surfaces. Therefore, PDL cells can adhere to sterile root surfaces.

Keywords: periodontal ligament cells; tissue regeneration; sterilization; scanning electron microscopy.

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Introduction

In recent years, dentists have adopted a variety of treatment strategies, including orthodontic treatments, in response to emerging patient requirements. Autologous tooth transplantation is increasingly considered during orthodontic treatment, because the autotransplantation of teeth enables the recovery of occlusal function by replacing missing teeth (1-2). Unnecessary wisdom teeth and teeth extracted for other orthodontic treatments are mainly used as donor teeth in this technique (3). The procedure also reduces the amount of tooth movement required during orthodontic treatments by shortening the treatment period. Conventionally, missing teeth are replaced using prosthetics such as bridges, dentures, and implants. However, these prosthetics cannot fully replace natural teeth because they do not have a periodontal ligament (PDL).

Autologously transplanted teeth demonstrate biocompatibility and a functionality similar to that of natural teeth. However, they are also associated with root resorption, attachment loss, ankylosis, and internal absorption. Andreasen and Schwartz (4-6) reported that transplanted teeth have a survival rate of 70-100% and a success rate of 50-97%. Transplant prognoses vary depending on factors such as the type of transplanted tooth, age of the patient, presence or absence of PDL, and surgical procedure used, and numerous studies have investigated the factors affecting the prognosis of autologously transplanted teeth.

The residual PDL and cement mass on the roots of extracted teeth are factors that considerably affect tooth

transplantation (7), because autotransplanted teeth can regenerate the alveolar bone of the socket through the osteoinductive ability of PDL cells (8).

Lesions measuring 1 and 4 mm^2 on the root surface developed transient ankylosis that disappeared after 8 weeks, whereas lesions measuring 9 or 16 mm^2 resulted in ankylosis that in most cases persisted after 8 weeks (9). Previously, we reported that when teeth are extracted without consideration of the remaining human periodontal ligament (HPDL), approximately 50% of the periodontal membrane area remained (10). Therefore, it is necessary to regenerate the HPDL of the root surface.

In our previous experiment, extracted teeth were washed with phosphate-buffered saline (PBS) containing a high concentration of penicillin, and placed upright in three-dimensional (3D) medium filled with culture carrier material. However, this resulted in a high risk of infection due to bacterial adhesion to the tooth crown portion in long-term culture. In addition, for the future reuse of teeth extracted as a result of periodontal disease and trauma, it will be necessary to reliably remove bacteria from the crowns of extracted teeth subjected to organ culture. Thus, in this study, contaminated teeth were sterilized by high-pressure steam sterilization, which is performed in general clinical dentistry to reduce the risk of infection. We believe that high-pressure steam sterilization is feasible and cost-effective. HPDL cells isolated from the root surface of other teeth can then be seeded on to the sterilized periodontal tissues.

HPDL cells were cultured on the sterilized root surface using 3D culture. We hypothesized that this would regenerate the periodontal tissue and enable the teeth to be reused. In this study, we investigated the change in periodontal tissues subjected to high-pressure steam sterilization. We examined the adhesion of HPDL cells to the sterilized root surface biochemically and histologically.

Materials and Methods

Sample collection

Based on the provisions of the Ethics Committee of Kanagawa Dental University, Yokosuka, Kanagawa, Japan (#252), eight fresh human first and second premolars, without caries or periodontal disease, were extracted with informed consent from individuals undergoing tooth extractions for orthodontic reasons. Tooth extraction was performed at the Department of Oral Surgery, Kanagawa Dental College. A randomized design was followed, including patients who were >12 and <35 years old. Tooth extraction was performed using only tooth extraction forceps.

Cell culture

After extraction, the first and second premolars were washed with PBS containing penicillin. Cells were isolated by the outgrowth method from extracted HPDL tissues placed in 90-mL Petri dishes and covered with 15-mm coverslips (11).

The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 0.1% penicillin/streptomycin. The cells were grown in 5% CO_2 and 95% air at 37°C until confluent, trypsinized, and then subcultured for four to five passages. The medium was replaced with fresh DMEM every 7 days.

Detection of mineralized nodules

Isolated cells were evaluated to confirm that they had the phenotype of PDL cells. HPDL cells were seeded in 12-well plates at a density of 4×10^4 cells/per coated disk. The cells were cultured in liquid culture medium in DMEM to a confluent state, and then for 1, 4, 7, 10, and 14 days in DMEM and separately in calcification medium (PromoCell GmbH, Heidelberg, Germany).

The culture medium was replaced every 3 days. The cells were fixed with 4% paraformaldehyde at specific time points, and then stained with 1% Alizarin Red for 10 min to detect calcified nodules. The cells were observed at magnifications of 4× and 20× under a phase-contrast microscope.

Alkaline phosphatase (ALP) activity assay

HPDL cells (5×10^3) were cultured in a 96-well plate for 1, 4, 7, 10, and 14 days in DMEM and separately in calcification medium. ALP activity was measured according to a modified Bessey-Lowry method using LabAssay ALP (Wako Pure Chemical Industries, Ltd., Osaka, Japan). One unit of activity was defined as the activity of the enzyme when hydrolyzing 1 nmol of *p*-nitrophenyl phosphate in 1 min (12). The absorbance of each sample was measured at 405 nm using a microplate reader.

Examination of the root surface before and after high-pressure steam sterilization

After extraction, the teeth were washed 2 to 3 times with PBS. The extracted teeth were divided into the non-sterile (A) and sterilized (B) groups. The teeth in group A were non-sterile, and those in group B were sterilized in an autoclave at 121°C for 20 min. The samples were fixed in 2% glutaraldehyde at 4°C and 1% osmium, and then dehydrated in ascending concentrations of ethanol (50, 60, 70, 80, 90, 95, and 100%) for 15 min each. Dehydration in 100% ethanol was performed twice. Next, the

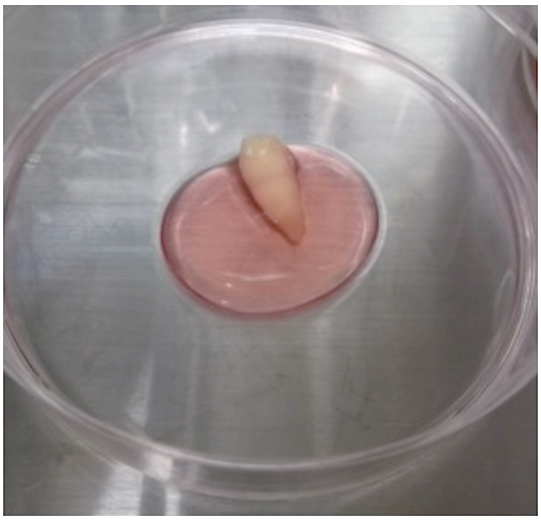

Fig. 1 The method of three-dimensional culture. Human periodontal ligament cells were cultured on the sterilized root surface using three-dimensional culture for 3 weeks in a 90-mL Petri dish.

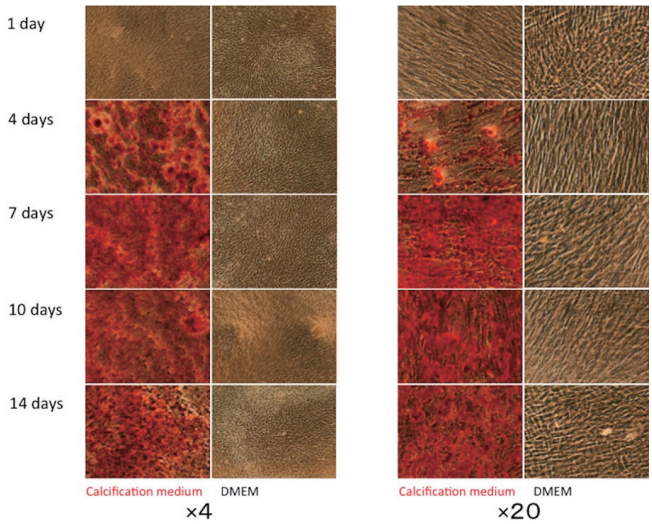

Fig. 2 Detection of mineralized nodules. To visualize the calcification ability of periodontal ligament cells, they were cultured in calcification medium. The cells were observed under a phase-contrast microscope at 4× and 20× magnification. Cells cultured in calcification medium showed staining for calcium deposition from day 4, whereas cells cultured in Dulbecco's modified Eagle's medium showed no such staining at any of the time points.

samples were placed in isoamyl acetate as an intermediate solution, and examined by scanning electron microscopy.

Scanning electron microscopy (SEM)

For SEM analyses, the samples were coated with gold (magnetron sputter) and observed under a microscope at magnifications of 40×, 500×, and 2,000×. Next, each sample was subjected to histologic examinations to determine whether the root surface after sterilization could function as a scaffold.

Examination of periodontal tissue adhesion on the sterile root surface

HPDL cells were cultured on the sterilized root surface using 3D culture (Mebiol Gel; Mebiol Inc., Hiratsuka, Japan). HPDL cells (4×10^5 cells/mL) were mixed on ice with 2 mL of Mebiol Gel. This mixture was poured on the sterilized root surface in a 90-mL Petri dish and allowed to gel at 37°C for 60 min (Fig. 1).

The specimens were then cultured in 10 mL of DMEM supplemented with 10% fetal bovine serum and 0.1% penicillin/streptomycin. The cells were grown in 5% CO_2 and 95% air at 37°C for 3 weeks and then observed by SEM (2,000×). The preparation of the SEM samples was as described above. Fresh medium was provided every 7 days. SEM was performed to examine the morphologic characteristics of the HPDL cells cultured on sterile root surfaces.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

To assess the mitochondrial activity of cementoblasts, we performed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the reduction of resazurin to resorufin by living cells. Adhesion and growth of the cells on the sterile root surface were confirmed by evaluating the activity at 7 and 21 days. After culture, the teeth were washed with PBS and moved to a 12-well plate. The MTT assay was performed using an MTT Assay Kit (Promega Corp., Madison, WI, USA). Cells were incubated for 22 h. The absorbance of each sample was measured at 560/590 nm using a microplate reader.

Statistical analysis

The results were expressed as mean values with standard deviations. Significance was determined using the Student's *t*-test and paired *t*-test. $P < 0.01$ was considered significant.

Results

Detection of mineralized nodules

To determine the calcification potential of cells, the results of Alizarin Red staining were compared between cells grown in calcification medium and those grown in DMEM. The cells grown in calcification medium exhibited red staining from day 4, whereas those grown in DMEM did not show red staining at any time point.

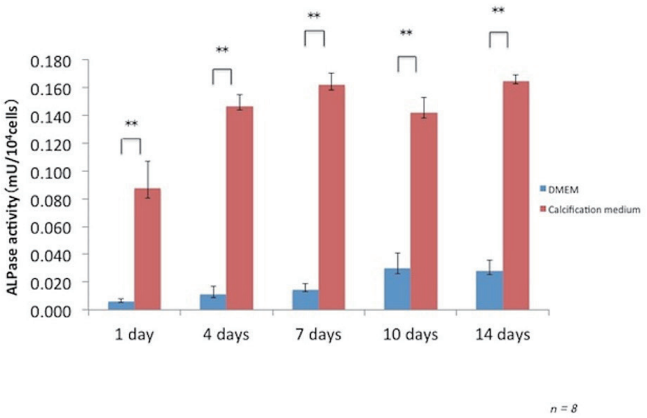

Fig. 3 Alkaline phosphatase activity assay. At all-time points, the ALP activity of cells grown in Dulbecco's modified Eagle's medium was lower than that of cells grown in calcification medium (**$P < 0.01$). Significance was determined using the Student's *t*-test.

Phase-contrast microscopy revealed mineralized nodules stained red in the calcification medium from day 4 (Fig. 2).

Alkaline phosphatase activity

The ALP activity of HPDL cells was evaluated. The ALP activity of cells grown in calcification medium increased with time and peaked on day 7. Subsequently, ALP activity marginally declined. At all-time points, the ALP activity of DMEM-grown cells was lower than that of cells grown in calcification medium (Fig. 3).

Scanning electron microscopy

The root surface was examined before and after high-pressure steam sterilization. Teeth in group A showed fine network structures such as HPDL fibers, nerves, capillaries, red blood cells, and lymphocytes, and spherical and membrane-like structures. HPDL cell-like structures with spindle-shaped projections were also observed. In teeth in group B, the fibrous structures had changed to flat structures under pressure and heat. The number of spherical structures was reduced compared with that evident in group A (Figs. 4-6).

Human periodontal ligament cells adhere to the sterile root surface

The adhesion of HPDL cells on the sterilized root surface was evaluated. The new fibroblasts presented projections on the sterilized root surface as observed at 2,000× magnification (Fig. 7). The cells adherent to the sterile root surface exhibited a dense microstructure and a star-shaped appearance.

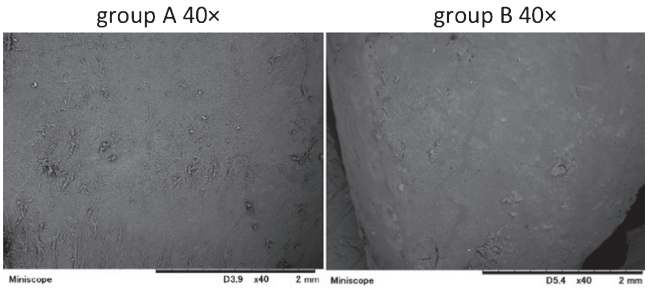

Fig. 4 Scanning electron microscopy observation at 40× magnification.

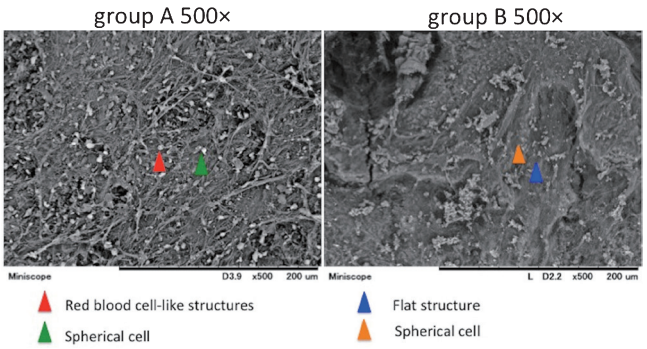

Fig. 5 Scanning electron microscopy observation at 500× magnification.

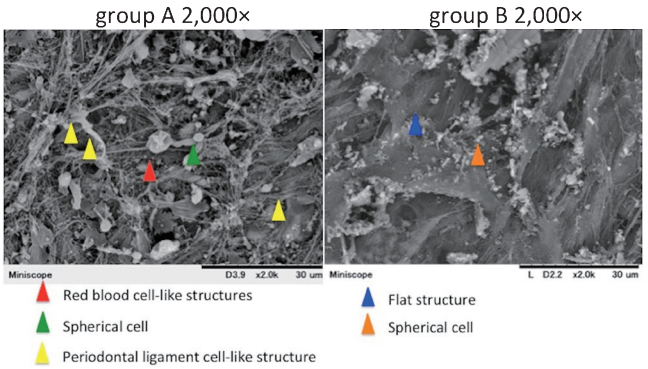

Fig. 6 Scanning electron microscopy observation at 2,000× magnification. In teeth in group B, fine network structures such as periodontal ligament (PDL) fibers, nerves, capillaries, red blood cells, and lymphocytes were observed, in addition to spherical and membrane-like structures. PDL cell-like structures with spindle-shaped projections were also observed. In teeth in group A, fibrous structures had changed to flat structures upon the application of pressure and heat. The number of spherical structures in group A was reduced relative to that evident in group B.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The cell activity as measured by the MTT assay increased from day 7 (Fig. 8). On the 21st day, cell activity of approximately 10 times was recognized in comparison with the day 7.

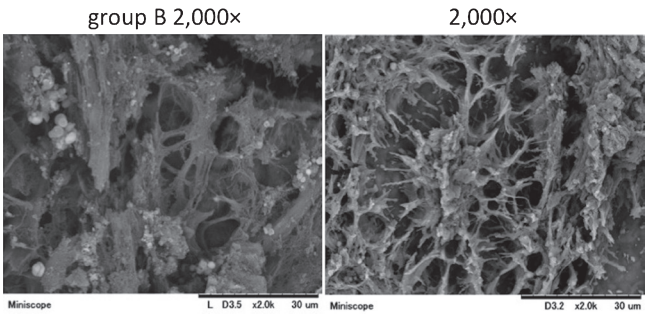

Fig. 7 Human periodontal ligament cells adhere to the sterile root surface. New fibroblasts presented projections on the sterilized root surface, as shown in these images at 2,000× magnification. The fibroblasts had a dense microstructure, and the cells attached to the sterile root surface had a star-shaped appearance. It is possible that these cells communicate with one other through their processes.

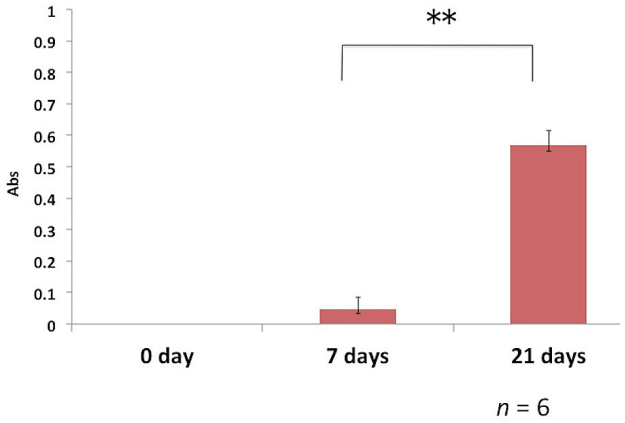

Fig. 8 The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Mitochondrial activity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. Adhesion and growth of the cells on the sterile root surface were confirmed by evaluating the activity at 7 and 21 days. MTT assay increased from day 7 (**$P < 0.01$). Significance was determined using the paired *t*-test. Abs = absorbance

Discussion

The periodontal membrane is important for long-term periodontal regeneration (13). Current tissue-engineering strategies to recover lost tissue typically involve a combination of autologous cells and growth factors with various biomaterials for guided tissue regeneration (GTR) and guided bone regeneration (GBR), or human PDL cell sheets (14-18). However, the outcomes of GTR, GBR, and the use of cell sheets depend on the state of the recipient. In addition, it is difficult to prevent infection because the operation is performed in the oral cavity. Therefore, in this study, we evaluated the adhesion of HPDL cells to sterilized root surfaces because regeneration of the periodontal membrane on a sterile root surface *in vitro* prior to tooth transplantation may minimize negative effects in the host tissue.

Autoclaved bone is used as a bone supplement material in conservative therapy to correct bone defects in patients with bone tumors (19). Autoclaving bone at 130°C for 8 min enables anatomically accurate and immediate filling of the defect. In developing nations, re-implantation of extracorporeally devitalized tumor-bearing bone segments is an appealing option because it allows simple, cost-effective, and anatomically correct filling of defects. In addition, cellular components that cause rejection can be removed by sterilizing biologic tissues (20), because complement components and bacteria are inactivated by sterilization at 121°C for 20 min. Thus, using sterilized teeth is similar to using cadaveric teeth, which presumably have no biologic activity. However, the greatest advantage of using sterile teeth is that the patient's own teeth are used, which ensures a custom fit.

However, one disadvantage is that, similarly to bone, the PDL and cementum become weak upon high-pressure steam sterilization. In addition, cellular components of the PDL are removed by sterilization. Therefore, regeneration of the PDL using autologous cells may represent a viable strategy to improve outcomes. In this study, the HPDL, which normally has a network-like structure, was denatured and flattened by high-pressure steam sterilization and remained on the tooth root surface. We investigated the potential of these autoclaved HPDL tissues to function as scaffolds for HPDL regeneration. Scaffolds possess properties required for cell adhesion; that is, the ability to promote matrix production and control differentiation, biocompatibility, strength, and porosity (21). In this study, SEM images of the sterile teeth showed that the scaffold required for the adhesion of new HPDL cells was present throughout the tooth root. Furthermore, when the HPDL cells were cultured for 3 weeks on the root surface, the adherent fibroblasts exhibited elongated projections. It is possible that the cells communicated with other through their process. The HPDL cells did not peel away from the root surface upon drying and repeated washing during sample preparation for SEM. Cell proliferation was evaluated using the MTT assay, in which living cells cultured on the root surfaces were detected. The sterilized root surface was confirmed free from viable cells. Therefore, the cells adhered and grew on the sterilized root surface after culture.

Notably, the PDL fibroblasts used in this study are known to harbor stem cells (22-26). Indeed, stem cells derived from the periodontal membrane have been found to possess higher osteogenic potential than stem

cells derived from adipose tissues (27). Several studies have investigated the development of scaffolds using biomaterials for periodontal tissue regeneration (28-31). However, none of these scaffolds were able to reproduce the complex structure and function of the PDL. In contrast, the sterilized root surface was able to function as a scaffold during tooth transplantation. Thus, we established a method for periodontal tissue regeneration, and future research must explore the *in-vitro* and *in-vivo* biologic responses to such scaffolds to establish their potential for clinical use.

In conclusion, our results show that HPDL cells can adhere to sterile root surfaces. Further research is needed to establish the clinical applications of our findings.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest for this study or publication.

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