Tissue Engineering

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In vivo periodontium formation around titanium implants using periodontal ligament cell sheet

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Abstract

Osseointegrated implants have been recognized as being very reliable and having long term predictability. However, host-defense mechanisms against infection have been known to be impaired around a dental implant because of the lack of a periodontal ligament (PDL). The purpose of our experimental design was to produce cementum and PDL on the implant surface adopting cell sheet technology.

To this aim we used PDL-derived cells, which contain multi-potential stem cells, as the cell source and we cultured them on an implant material constituted of commercially pure titanium treated with acid etching, blasting, and a calcium phosphate (CaP) coating to improve cell attachment. Implants with adhered human PDL cell sheets were transplanted into bone defects in athymic rat femurs as a xenogeneic model. Implants with adhered canine PDL-derived cell sheets were transplanted into canine mandibular bone as an autologous model.

We confirmed that PDL-derived cells cultured with osteoinductive medium had the ability to induce cementum formation. The attachment of PDL cells onto the titanium surface with 3 surface treatments was accelerated, compared with that onto the smooth titanium surface, at 40 minutes after starting incubation. Results in the rat model showed that cementum-like and PDL-like tissue was partly observed on the titanium surface with 3 surface treatments in combination with adherent PDL-derived cell sheets. On the other hand, osseointegration was observed on almost all areas of the smooth titanium surface that had PDL-derived cell sheets but did not have the 3 surface treatments.

In the canine model, histological observation indicated that formation of cementum-like and PDL-like tissue was induced on the titanium surface with surface treatments and that the PDL-like tissue was perpendicularly oriented between the titanium surface with cementum-like tissue and the bone.

Results demonstrate that a periodontal-like structure was formed around a titanium implant, which is similar to the environment existing around a natural tooth. The clinical application of dental implants combined with a cell sheet technique may be feasible as an alternative implant therapy. Furthermore, application of this methodology may play an innovative role in the periodontal, prosthetic, and orthodontic fields in dentistry.

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Introduction

Since an osseointegrated implant was introduced by Branemark et al. approximately 50 years ago (1), root form titanium implants have been used worldwide. Dental implant therapy helps provide good masticatory function and dental aesthetics for patients. Regarding implant stability, the concept of osseointegration has been recognized as being fundamental for predictable long-term clinical success of dental implant therapy.

However, peri-implant lesions have been reported with a high prevalence after 5 to 10 years in use without systematic supportive treatment in mixed dentition (2-4).

One of the reasons for peri-implantitis is related to the nature of the environment surrounding osseointegrated implants. The host-defense mechanism against infection is impaired around an implant because of the lack of a periodontal ligament (PDL) which contains vascular networks (5). Therefore, the infection around the implant easily spreads to the surrounding bone. Furthermore, elimination of causative factors, including bacteria and their toxic products, from the implant surface, is difficult to accomplish with daily homecare.

In 2010, Giannobile indicated that the presence of a PDL allows for a more dynamic role beyond that seen with a functionally ankylosed osseointegrated implant (6). A dental implant accompanied by a periodontal structure similar to that associated with a natural tooth would ameliorate such conditions.

PDL-derived cells possess mesenchymal stem cell-like properties and are regarded as useful sources for reconstruction of periodontal tissues (7, 8).

Basic and clinical research, using PDL-derived cells in conjunction with “cell sheet engineering” for periodontal regeneration, has been conducted. The results demonstrated periodontal regeneration with inserting PDL fibers and newly-formed cementum in periodontal defects (9-11).

The cell sheet technique uses a cell culture dish with an intelligent cell culture surface that responds to temperature changes allowing cells to detach (12). The application of this technology enables harvesting cells by a reduction of culture temperature without using

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any enzymes such as trypsin. Cell-cell interactions, cell surface proteins, and extracellular matrix proteins are preserved within the sheet and allow the cell sheets to adhere to living tissues without suturing (13). Using this technique, cell sheets were applied to regenerative therapy in various fields (14-16). We expect that cell sheet technology will be effectively applied in dental implant therapy.

Implant surface treatment techniques have been developed to improve the peri-implant environment. For improved stabilization of cells on implants, the implant surface needs to be rough to facilitate cell attachment and induce tissue formation on the implant surface. Surface treatment methods, of blasting and acid etching, are traditional methods to create such a porous surface on materials (17). A CaP coating is also reported to improve cell attachment (19). For these reasons, in our protocol, the titanium surface was morphologically changed by blasting, acid etching, and calcium phosphate (CaP) coating. A biological surface, coated with cells or tissue, is now envisioned as the next generation of modified implant surface (18).

In this study, we investigated whether periodontal structures can form using cultured PDL cell sheets on titanium, with morphologically changed surfaces, in a xenogeneic athymic rat femur model and an autologous canine mandible model.

Materials and Methods

Animals

Athymic rats (F344/NJcl-rnu/rnu, 5 males, 5 weeks old, Japan Laboratory Animals, Inc., Tokyo, Japan) were used for the experiment on the transplantation of titanium with cell sheets into a bone marrow cavity. Beagle dogs (2 males, 2 females, 8 months old, mean weight of almost 10 kg, Institute for Animal Reproduction, Ibaraki, Japan) were used for the experiment on cell attachment to titanium surfaces and transplantation of titanium, with or without cell sheets, into a mandibular bone defect. The animals exhibited intact teeth with a healthy periodontium.

All experimental protocols were approved by the animal welfare committee of Tokyo Women's Medical University (Agreement number AE17-5).

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Human samples

This study was conducted according to the principles of the Declaration of Helsinki. The institutional review board of the Tokyo Women's Medical University approved the collection of human third molars, which were to be treated as clinical waste and used for the procurement of cells for this experiment. The collected human teeth were extracted for various reasons other than periodontal disease (tooth impaction, caries, and occlusal problems, etc.), after obtaining informed consent by the patients, at the clinic of Tokyo Women's Medical University

Isolation, culture and preparation of human PDL-derived cells and cell sheet

Human PDL-derived cells were harvested from the collected human third molars. The method to obtain PDL-derived cells is as follows (20): extracted teeth were rinsed 5 times, for 3 min each, with alpha-Minimum Essential Medium (α-MEM; Life-technologies, Carlsbad, CA, USA) containing 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO). PDL tissues were gently removed from the surface of the mid-third of the extracted root by scalpel. The tissue was digested with collagenase (NB6; 0.8 PZ-U/mL, SERVA Electrophoresis, Heidelberg, Germany) / dispase (1200 PU/mL, Sanko Junyaku, Tokyo) with vigorous shaking at 37°C for 45 min. The cells were cultured at 37°C with basic medium which consists of minimum essential medium alpha (Thermo Fisher Scientific, MA), 10% fetal bovine serum (Japan Bio Serum, Japan) and 1% penicillin streptomycin (Sigma-Aldrich Japan, Japan). Medium change for cell passage using 0.25% Trypsin-1mM EDTA (Thermo Fisher Scientific) was conducted every 3-4 days. At third passage, cells were cryopreserved with cell freezing medium (CELLBANKER® 1, Zenoaq, Fukushima, Japan) at -150°C until use.

The cryopreserved PDL-derived cells were thawed two weeks prior to transplantation and were cultured with basic medium to reach an 80% confluence. For the production of the cell sheets, the cultured human PDL-derived cells were spread on 35 mm temperature-responsive culture dishes, which were coated with Poly (N-isopropylacrylamide) on the surface (UpCell®, CellSeed, Tokyo, Japan), at a density of 4×10^4 cells/dish. The culture medium was changed, after cultivation with basic medium for 2 days, to an osteoinductive

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medium, in which basic medium is supplemented with 82 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate (Wako Junyaku, Tokyo, Japan), 10 nM dexamethasone (DEXART; Fuji pharma, Toyama, Japan), and 10 mM β-glycerophosphate (Sigma-Aldrich, MO). The osteoinductive medium was changed twice a week and the cells were incubated for 10 days. This temperature-responsive surface allows cells to proliferate in the same manner as being cultured on commercial dish surfaces at 37°C. The cells detach from the surface spontaneously when the temperature is reduced below 32°C to obtain a "cell sheet".

Isolation, culture and preparation of canine PDL-derived cells and cell sheet

Canine PDL-derived cells were harvested from extracted mandibular premolars under general anesthesia. Prior to the tooth extraction, the dogs were intramuscularly injected with 0.08 mg/kg medetomidine (Domitor; Nippon Zenyaku Kogyo, Fukushima, Japan) and 0.3 mg/kg midazolam (Dormicum; Astellas Pharma Inc, Tokyo, Japan) for anesthetic premedication and then subjected to an intravenous injection of 1-2 mg/kg propofol (Diprivan; AstraZeneca, Osaka, Japan). An endotracheal tube was inserted and anesthesia was maintained with 2-4% sevoflurane (Pfizer Japan Inc., Tokyo, Japan). Local anesthesia was injected using 2% lidocaine hydrochloride containing epinephrine at a concentration of 1:80000 lidocaine (ORA Injection Dental Cartridge; Showa Yakuhin Kako Co,Ltd, Tokyo, Japan) to reduce intra-operative bleeding. The method of harvesting cells, culturing, and cryopreservation followed the same protocol as performed with human PDL-derived cells as described above. Approximately two weeks before a transplantation, the frozen canine PDL cells were thawed and cultured in a basic medium. After the subculture, the cells were plated at $4x10^4$ cells / UpCell® (35 mm dish). The medium was changed to an osteoinductive medium which was refreshed every 3 to 4 days. Subsequently, the canine PDL cell sheet was obtained after 5-7 days of additional culture (10).

Gene expression of cementum markers in cultured human PDL-derived cells

Human PDL-derived cells (passage 6) were cultured, using a six-well plate, at a density of $3x10^4$cells/well for 2 days in basic medium, then the medium was shifted to an osteoinductive medium for an additional 14 or 21 days. Total RNA was isolated using the QIA shredder and RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the

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manufacturer's instructions. For the TaqMan gene expression assay, cDNA was synthesized from 500 ng of total RNA using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time PCR using StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) was performed in triplicate using a cementum protein 1 probe (CEMP1; Hs04185363_s1), a bone sialoprotein probe (BSP; Hs00173720_m1), and a b-actin probe (43263215E) (Thermo Fisher Scientific). Messenger RNA expression levels relative to β-actin were determined and fold changes were calculated using the values obtained by the ΔCT method (21).

Titanium specimens

Commercially pure titanium (grade 2) was used in this study. For the in vitro experiment, titanium foil, 10 μm thick, was cut into a circular shape 5 mm in diameter (Titanium Foil / TF). For transplantation into the bone marrow cavity of the rat femur, a titanium rod 1 mm in diameter and 2-3 mm in length (Titanium Rod / TR) was used. For transplantation into an implant preparation site of the canine mandible, a tapered cylindrical shape of titanium with a diameter of 3.5 mm at the upper end and 3 mm at the lower end, and a length of 8 mm, (Implant / TI) was used. The design of the Titanium Implant used in the canine experiment is similar to narrow diameter implants in clinical use, but there is difference between the two types of implants. The currently used implants have screw design, but the TI has no screw design.

To improve cell attachment, titanium specimens in the experimental groups were treated by blasting, acid etching, and CaP coating. The surface treatment procedures were performed sequentially as follows: 1) Blasting: zirconia powder with a particle size of 75 to 106 μm (TZ-B90: Tosoh Corporation) was blasted onto the titanium surface. 2) Acid etching: the titanium specimen was immersed at room temperature for 4 hours in a 1:1 mixed solution of undiluted reagents hydrogen peroxide (Kanto Chemical Co.) and sulfuric acid (Wako Pure Chemical Industries, Ltd.). The titanium was then washed with ultrapure water numerous times. 3) CaP coating: the titanium specimen was placed in a Hanks' solution without glucose and kept immersed at 37℃ for 168 hours. The titanium was then washed with 70% ethanol and subjected to autoclave sterilization before use.

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All the titanium specimens were washed with phosphate-buffered saline (PBS: Thermo Fisher Scientifics, USA) or saline (Otsuka Pharmaceutical Factory, Inc., Japan) for 5 min twice before use.

Cell attachment to Titanium Foil (TF) surface

After trypsinization of canine PDL-derived cells (passage 5), the cells were collected in 1.5 mL tubes (Eppendorf, Hamburg, Germany) at a density of $4x10^5$ cells/ 500 µL medium, and then a TF was soaked in the tube. TF without surface treatments was used as the control. The tubes with cells and TF were incubated at 37℃ for 20, 40, 60, and 120 min. After incubation, the number of cells remaining in the tube was counted and the number of attached cells on the TF was calculated by subtracting the number of cells remaining in the tube from the total cell number ($4x10^5$ cells).

PDL cell sheets adhered to Titanium Rod (TR) and Implant (TI)

The cell sheets were obtained by the reduction of culture temperature from 37°C to below 32°C (at room temperature of 20~30°C). The three-layered cell sheets were adapted to the titanium surface. The titanium specimen with cell sheets was incubated with basic medium at 37°C for more than 1 hour prior to transplantation and washed with PBS just before use.

Transplantation of Titanium Rod (TR) with cell sheets into bone marrow cavity of rat femur; specimen collection for histological analysis

Under anesthesia of 2-3% isoflurane, a small access incision was made in the rat's leg using a scalpel. The knee joint was exposed by moving the rectus femoris muscle to the outside of the knee joint using a dental excavator. A bone defect 2-3 mm in diameter was made with a dental bur on the intercondylar fossa of the rat femur. After washing the defect with saline, the TR (with or without surface treatments) including adherent cell sheets was inserted into the bone marrow cavity via the bone defect. The displaced muscle was repositioned onto the knee joint and the skin incision was sutured. Six weeks after transplantation, the rat was sacrificed using 3-4% isoflurane after anesthesia with 2-3% isoflurane. The inserted TR and covering tissue were removed together with the femur as a

specimen for histological analysis. The experimental procedure in the rat model is illustrated in Fig. 1A.

Transplantation of Titanium Implant (TI) with cell sheets into canine mandibular bone; specimen collection for histological analysis

Six months after the extraction of the donor teeth, for use in cell sheet preparation, the extraction wound sockets were completely repaired. A bone defect was prepared, under anesthesia, with a diameter of 4.5 mm, and depth of 10 mm. The defect's dimensions were slightly larger than the dimensions of the TI. The TI with adherent cultured cell sheet was inserted into the canine mandibular bone defect. After implant placement, titanium mesh (Bone Plate®, Jeil Medical Corporation, Korea) secured by titanium screws was placed to cover and stabilize the TI. A periosteal releasing incision was performed to allow all materials to be passively covered by the gingival flaps. An interrupted GORE TEX® suture (Gore Medical, USA) was performed in order to achieve flap stability and primary closure. From preliminary studies, involving transplantation of a TI with canine PDL cell sheets into canine mandibular bone, we found that by covering the transplanted materials with a titanium mesh we were able to avoid exfoliation of the implants (data not shown). In the control group, the TI with surface treatment was inserted without cell sheets. Eleven weeks after the transplantation, the canine was sacrificed using potassium chloride (KCl; Terumo, Tokyo, Japan) under anesthetic condition in the same manner as previously performed at the time of tooth extraction. The inserted TI and covering tissue were removed together with the mandibular bone as a block specimen for histological analysis. The number of TI transplanted with cell sheet was 6. The number of TI without cell sheet, as control, was 4. The experimental procedure in the canine mandibular bone model is illustrated in Fig.1B.

Tissue preparation and histological observation and measurement

The histological preparation was conducted at a commercial laboratory (Kureha Special Laboratory, Tokyo, Japan, URL for this method is http://www.kureha-bunseki.co.jp/english/bone/). Briefly, the specimen was dehydrated with 70% ethanol and embedded in methylmethacrylate (MMA) resin for non-decalcified ground section, a

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method commonly used for preparing sections of hard tissue and tissue containing metallic materials. The sections were stained using the Villanueva-Goldner method. The staining can distinguish calcified tissues stained in green and other tissues (eg. osteoid and connective tissue etc.) stained in red. A light microscope (Eclipse E800, Nikon, Japan) was used for morphological observation of the stained sections. Imaging software (NIS-Elements D, Nikon) was used for image capture and histological measurement. The section areas examined by light microscope were also re-examined by phase contrast light microscope (ECLIPSE Ti, Nikon) in order to observe the newly formed fibrous tissue morphologically.

Observation using transmission electron microscopy (TEM)

For TEM observation, the harvested specimens were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (PB) for 2 h. The samples were washed with 0.1M PB, post-fixed in 1% OsO4 buffered with 0.1M PB for 2 h, dehydrated in a graded ethanol series, and embedded in Epon 812. Semi-thin sections were cut at 1µm and stained with toluidine blue. Ultrathin sections, 90 nm, were collected on copper grids, double-stained with uranyl acetate and lead citrate, and then observed using TEM (H-7100, Hitachi, Tokyo, Japan).

Statistical analysis

All the data are expressed as the mean ± standard deviation. Statistical analysis of histological measurements and of PCR data were conducted using Student's t-test. The data from the experiment of cell attachment to the titanium surface was analyzed by a one-way repeated analysis of variance followed by Fisher's protected least significant difference post hoc test. A p-value < 0.05 was considered statistically significant.

Results

Expression of cementum gene markers in cultured human PDL-derived cells

To confirm the ability of cementogenesis induction in the obtained human PDL-derived cells, the gene expression of CEMP1 and BSP was observed at day 14 and 21 of culture with osteoinductive medium. The gene expression of CEMP1 in PDL-derived cells observed at day 14 was low. However, at day 21, the CEMP1 expression level was increased up to

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approximately ten-fold compared with the expression at day 14 (Fig. 2A). The expression level of BSP was also up-regulated at day 21, in a similar manner to that of CEMP1 (Fig. 2B). The results revealed that culturing human PDL derived cells in an osteoinductive medium could up-regulate cementogenesis-related genes.

Attachment of canine PDL-derived cells to Titanium Foil with or without surface treatment

To investigate an appropriate titanium surface for cell attachment, canine PDL-derived cells were incubated with TF with or without surface treatments. The number of attached cells on both titanium surfaces was increased time-dependently, and more than half the spreading cells were attached by 120 min on both types of surface (Fig. 3). A significant acceleration of cell attachment on the treated titanium surface was observed at 40 min compared with the control (untreated) titanium surface ($p < 0.05$) (Fig. 3).

Transplantation of a Titanium Rod with human PDL-derived cell sheets in the bone marrow cavity of rat femur

To confirm the cell attachment and formation of periodontal-like tissues on titanium from human PDL-derived cells in vivo, we transplanted the TR with human PDL-derived cell sheets into the bone marrow cavity of athymic rat femur for 6 weeks. In the experimental group using the TR with surface treatments and adherent PDL-derived cell sheets, the transplanted PDL cell sheets remained on the TR surface, were red-stained, and were observed between the TR and newly formed bone on approximately half of the TR surface (Fig. 4A(a)). Cementum-like hard tissue (green arrow) and PDL-like fibrous tissue (red arrow) were observed in the red-stained area on the TR surface (Fig. 4A(b)). In the control group using the TR without surface treatments and with adherent PDL-derived cell sheets, the transplanted PDL cell sheets detached from the TR surface and osseointegration occurred almost completely around the TR surface (Fig. 4A(c)). Bone marrow cells filled in the red stained area between the TR and new bone, but periodontal-like tissue was seldom observed in the control group (Fig. 4A(d)).

The length of attached cell sheet and newly formed PDL-like tissue on the TR surface was measured for a statistical comparison of the effect of titanium surface treatments. There is a trend towards an increased cell attachment onto the TR with surface treatments as

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compared to the control without surface treatments (Fig. 4B(a)). Furthermore, the formation of PDL-like tissue observed on the TR surface with treatments tended to increase as compared with the control surface (Fig. 4B(b)), though these differences were not statistically significant.

Observation of newly formed tissue on titanium using transmission electron microscopy (TEM)

For further understanding of regenerated tissue on the treated titanium surface, observation using TEM was conducted. The observation area with regenerated tissue was confirmed using light microscopy (Fig. 5A). The results showed that a calcified layer, containing cementoblast-like cells and rich in hydroxyapatite, existed adjacent to the treated titanium surface, and PDL-like tissue was observed on the calcified layer (Fig. 5B).

Transplantation of a Titanium Implant with canine PDL-derived cell sheets

3 implants with surface treatments and adherent PDL cell sheets (experimental group), and 2 implants with surface treatments but without cell sheets (control group), were successfully stabilized inside the bone defects. In the experimental group, 2 of the 3 implants successfully retained cell sheets around the TI surfaces. From the histological observation at 11 weeks after transplantation, red-stained soft tissue formed between the titanium and mandibular bone (Fig. 6A). In the area close to the mandibular bone, PDL-like tissue (P) was oriented perpendicularly to the titanium surface as well as to the cementoid-like tissue (CO), and a blood vessel (BV) was observed (Fig. 6A orange box, 6B, and 6E). In the control group, osseointegration predominated on the titanium surface (Fig. 6C, D).

Discussion

The presence of a PDL helps protect against infection and helps protect against bone absorption associated with mechanical stress, such as seen with occlusal loading and orthodontic tooth movement. These benefits contribute to maintenance of alveolar bone height (22).

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PDL-derived cells are capable of periodontal regeneration including cementum induction. CEMP-1 was identified as one of the cementum marker genes found to be expressed in cementoblasts, PDL cells, and cells located around vascular networks (23, 24). In this study, CEMP-1 expression was confirmed for PDL cells cultured with osteoinductive medium for 21 days. This evidence suggests that the obtained PDL cells have the capacity for cementogenesis induction. In our protocol, the cell sheet was transplanted with a TI after 14 days of culture. Our results suggest that the cell sheet possesses the ability to induce cementum formation after transplantation. Some research indicates that CEMP-1 expression level in human PDL cells is affected by culture conditions (25, 26). Thus, further investigations will need to be conducted to find the most suitable culture conditions in order to accelerate cementum and PDL formation on a TI.

Researchers anticipate developing periodontal tissues on titanium surfaces by taking advantage of the multiple attributes of PDL cells. The possibility of new periodontal tissue formation around implants has been investigated since 1990 when initial observations suggested the possibility of achieving anchorage of a dental implant with a PDL (27). Subsequently, many studies were conducted on the combination of dental implants and periodontal tissue (28-30), and on cell culture methods around implants utilizing bio-engineering (31, 32) as well as tissue engineering (22, 33, 34). Various procedures have been investigated with an ultimate goal of obtaining an intimate and stable interface between an implant and PDL tissues.

We focused on “cell sheet engineering” in this study because a suitable attachment of cells on the titanium surface is needed for a more biologically stable implant surface. This technology has already produced tissues from cells without any scaffolds (35, 36) and the efficacy of PDL cell sheets cultured with osteoinductive medium, for periodontal regeneration on dentin surfaces, has already been reported (10, 13).

Furthermore, it was reported recently that a transplanted PDL cell sheet with a CaP-coated polycaprolactone scaffold, in a rat periodontal defect model, promoted new periodontal attachment formation (37). That finding is in keeping with our in vivo results that PDL cell sheets induced PDL-like tissue on titanium with surface treatments including CaP coating.

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It was already reported that the CaP coating had the ability to accelerate calcification from osteoblasts using scaffolds (38, 39). Furthermore, as shown in Fig. 3 at 40 minutes after starting incubation, and by our trial experiments of surface treatments (data not shown), it is suggested that PDL cell attachment on the titanium surface was improved by CaP coating. This improvement may help induce formation of cementum-like tissue on the titanium surface. In our experiments, the structure of the PDL-like fibrous tissue anchored to the cementum-like layer was similar to that of natural periodontal tissue.

In athymic rat experiments with human PDL cells, the differences in the cell sheet covering ratio and the PDL formation ratio between the control and experimental groups were not statistically significant. This discrepancy between statistical analysis and histological observation might be due to variability in the measurement data. Additionally, the location and mobility of the TR in the rat femur might adversely influence stability for the attachment of cell sheets and periodontal-like tissue formation on the titanium surface.

Since the athymic rat model is useful for validity assessment of regeneration from human cells, we chose it to assess periodontal tissue formation induced by human PDL cell sheets. It was also necessary to confirm that newly formed PDL fibers are inserted between the calcified tissue on the titanium and the bone, with a structure similar to that seen with natural periodontal tissue, and the rat femur is suitable for the observation of calcified tissue formation because of its rich blood supply and cancellous bone.

However, since the femur and mandible develop through different ossification processes, in order to help translate our experiment to a future clinical setting, we considered the use of the autologous canine mandibular model to be an appropriate protocol.

With the canine control model, in order to exclude the effect of periodontal-like tissue formation associated with adhered PDL cell sheets, PDL cell sheets were not transplanted along with the implant. With the experimental group, newly formed periodontal-like tissue was observed between the implants and bone. On the other hand, osseointegration, which is the current method used to stabilize implants, occurred in the control group.

With osseointegration, dental implants are directly connected to the alveolar bone as ankylosis without the following benefits and functions of a natural PDL. The main functions

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of the PDL are the absorption of occlusal loading and the maintenance of alveolar bone height. PDL cells form cementum and provide nutritional supply to the cementum, which contribute to the maintenance of alveolar bone height. Clinically, orthodontic tooth movement, tooth supported fixed prosthetics and periodontal regenerative therapy depend on functions of the PDL. In addition, PDL tissues have many vascular capillaries. This blood supply helps induce the local immunological defense mechanism to act against external noxious stimuli such as in bacterial infection. Thus, we hypothesized that an implant with PDL-like tissue will help prevent peri-implantitis.

Recent reports clarify that peri-implantitis is not uncommon following implant treatment and that it is considered a significant and growing problem in dentistry (40, 41). One of the reasons for these complications is related to the nature of present implant systems that depend on osseointegration without the benefits of a PDL-like structure as discussed above. Therefore the formation of periodontal-like tissue in association with a titanium implant has potential for benefits similar to those existing around a natural tooth.

Conclusion

This study demonstrated that periodontal-like tissue formation occurred when using cultured PDL cell sheets on the titanium surface, in combination with acid etching, blasting, and CaP coating, in a xenogeneic rat femur model and in an autologous canine mandible model. The newly formed tissue contained inserting PDL-like fibers and newly-formed cementum-like tissue, which are structures similar to those found in periodontal tissue around a natural tooth. The clinical application of bio-engineering implants with PDL-like tissue is anticipated to present an alternative therapeutic method in dental implant therapy allowing for a more natural and stable tooth-like dentition for a life time.

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Disclosure Statement

The following authors disclosed financial relationships relevant to this publication: T.O. is a founder and director of the board of CellSeed, Inc., which licenses technologies and patents from Tokyo Women’s Medical University;

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Figure Legends

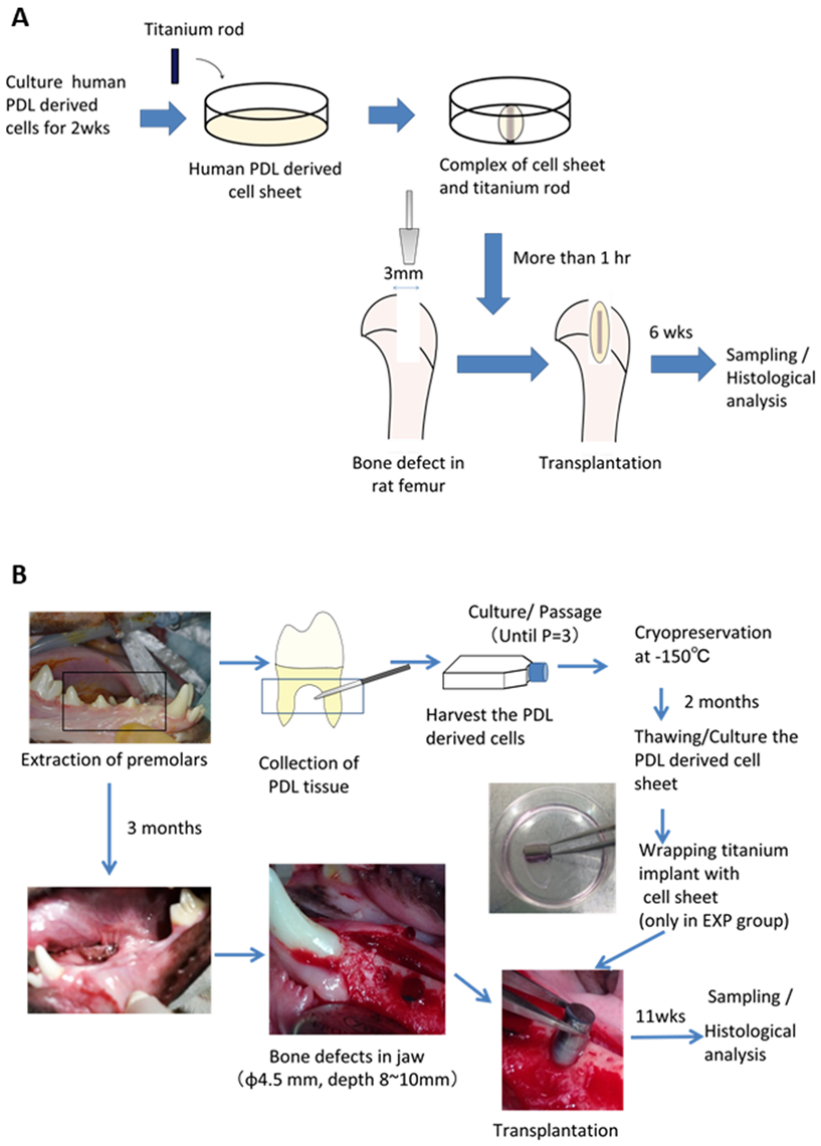

Fig. 1 Illustration of experimental protocol

The experimental procedure of cell culture and transplantation in the rat bone marrow cavity model is shown in Fig.1A.; the experimental procedure of cell culture and transplantation in the canine mandibular bone model is shown in Fig. 1B.

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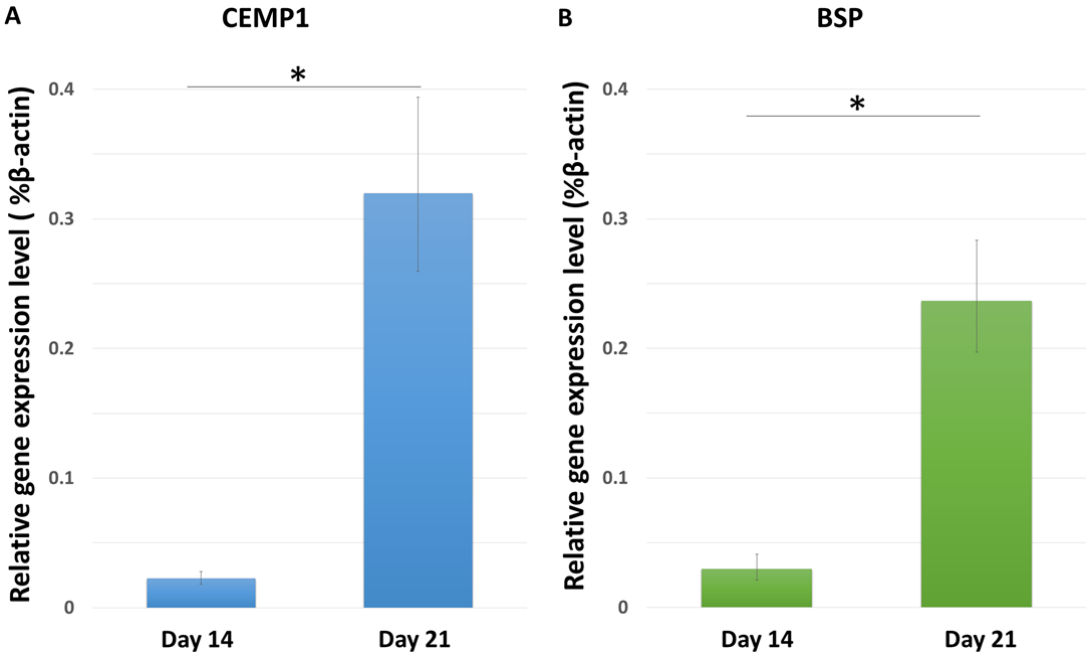

Fig. 2 Expression of cementum markers in human PDL cells

Gene expression of cementum markers, CEMP1 (A) and BSP (B), from human PDL-derived cells cultured with osteoinductive medium for 14 days and 21 days. * denotes a *p*-value < 0.001.

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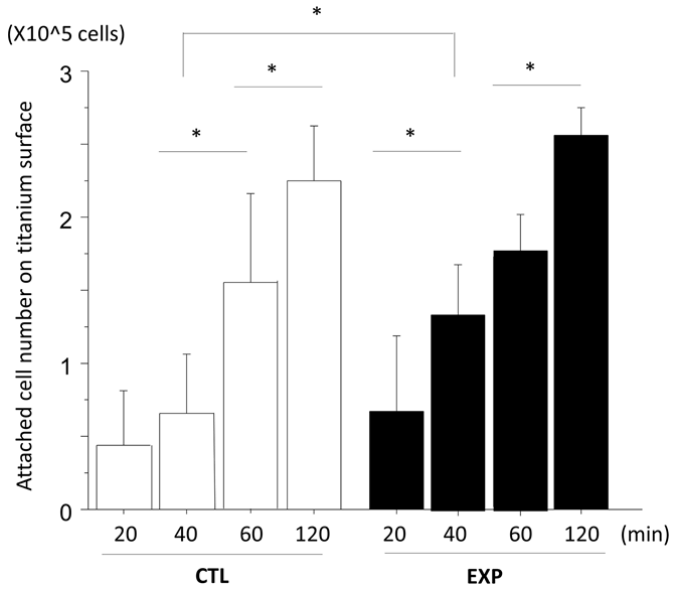

Fig. 3 PDL-derived cells attached more quickly to Titanium Foil with surface treatments

This figure shows the number of cells attached to the TF surface with or without acid etching, blasting, and CaP coating. Time points show the cultivation period of the PDL cells with TF. CTL (white bar) shows the control group using TF without surface treatments; EXP (black bar) shows the experimental group using TF with surface treatments. * denotes a *p*-value < 0.05.

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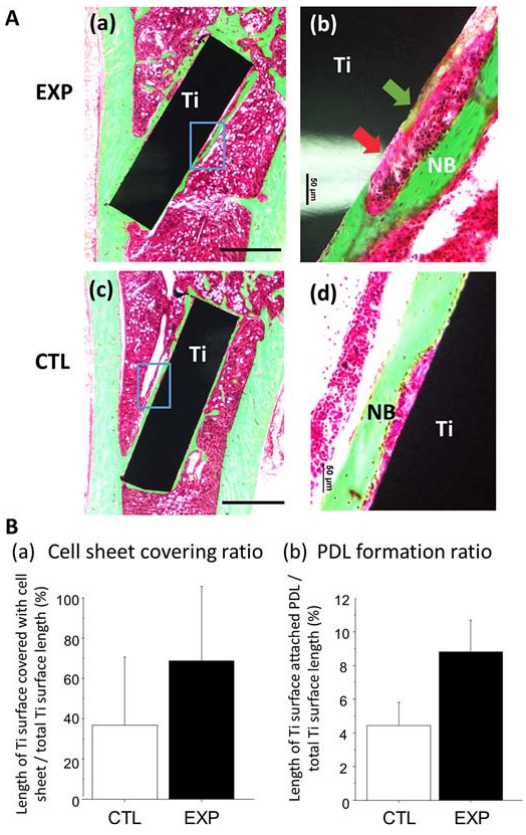

Fig. 4 Transplantation of Titanium Rod and human PDL-derived cell sheets in a rat bone marrow cavity model induced periodontium-like structure formation on the titanium surface

A. The histological results of the transplantation of the complex of TR [with surface treatments (experimental group (a, b), or without surface treatments (control group (c, d)] and human PDL-derived cell sheets for 6 weeks. (b, d) is magnified image of a square area of (a, c). NB, newly formed bone; TR, Titanium Rod; Green arrow, newly formed cementum-like tissue; Red arrow, newly formed PDL-like tissue

B. The comparison of the length of formed tissue observed by histological study. (a) indicates the ratio of the length of the titanium surface covered with PDL cell sheets per total titanium surface length. (b) indicates the ratio of the length of the titanium surface attached PDL per total titanium surface length.

CTL, control group (white bars); EXP, experimental group (black bars). Scale bar indicates 1 mm in (a, c), and 50 μm in (b, d).

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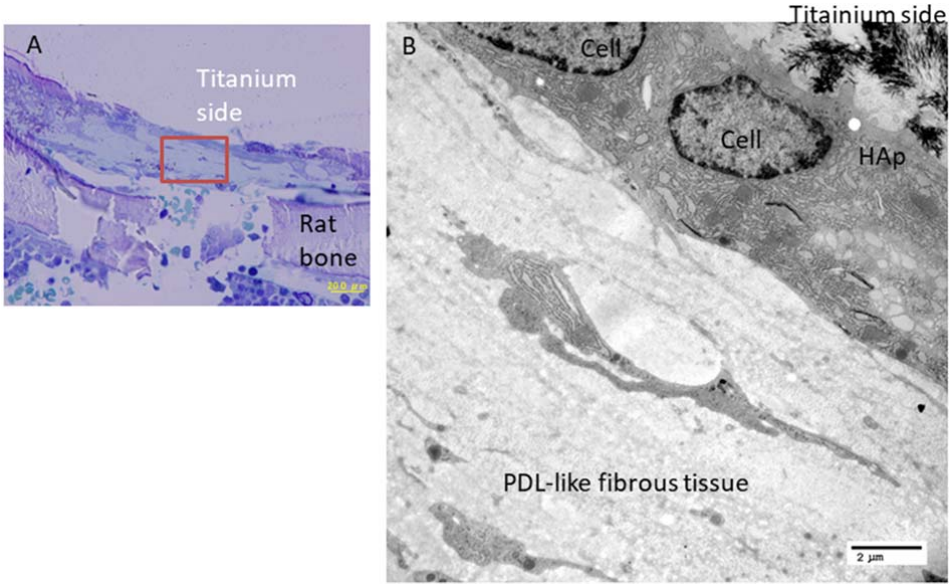

Fig. 5 Observation of newly formed tissue on titanium using TEM

The regenerated tissue was first observed with the light microscope (A). The red square in (A) shows the location area of (B). (B) shows TEM image of regenerated tissue. HAp, crystal of hydroxylapatite; Cell, nucleus of cementoblast-like cell.

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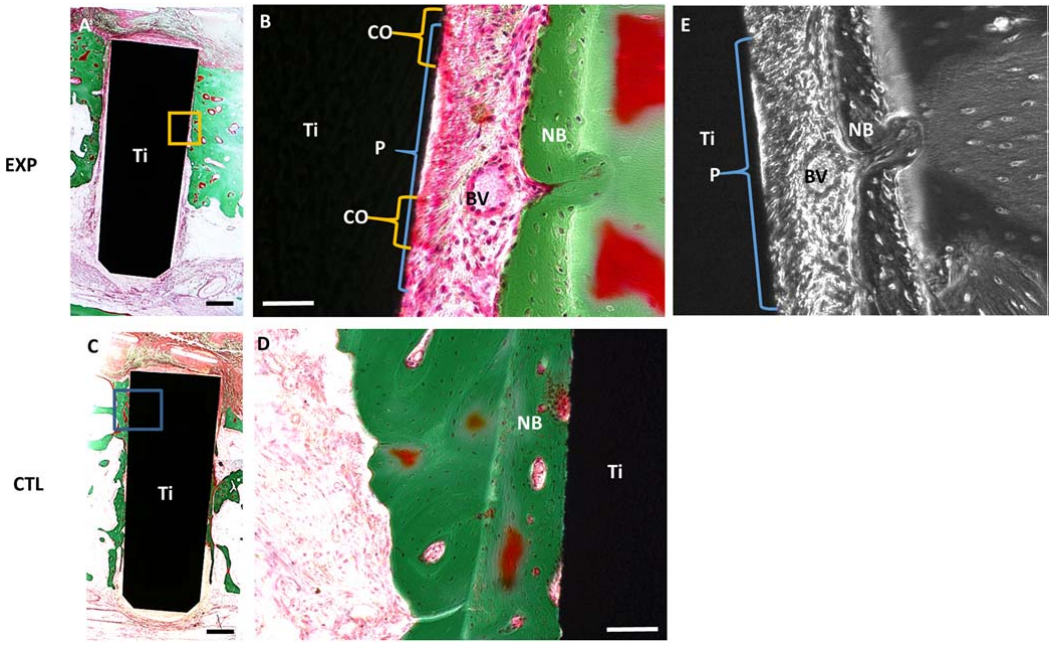

Fig. 6 Transplantation of TI with cell sheet to the canine mandibular bone defects

Histology of transplanted TI and surrounding tissue at 11 weeks after transplantation. The experimental group was transplanted as TI with surface treatments and with adapted canine PDL-derived cell sheets (A, B). The control group was transplanted as TI with surface treatments but without cell sheets (C, D). A, C demonstrate the total image and B, D show the magnified images. E shows the image using phase contrast microscope. NB, alveolar bone; BV, blood vessel; CO, cementoid-like tissue; P, PDL-like tissue; Ti, titanium. Black scale bar indicates 1 mm in (A, C), and white scale bar indicates 50 μm in (B, D).