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Cementum–periodontal ligament complex regeneration using the cell sheet technique

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Background and Objective: In the present study we evaluated if a multilayered human periodontal ligament cell sheet could reconstruct the physiological architecture of a periodontal ligament–cementum complex.

Material and methods: Human periodontal ligament cells were isolated and then cultured in dishes coated with a temperature-responsive polymer to allow cell detachment as a cell sheet. In the control group, human periodontal ligament cells were cultured in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum and 1% antibiotics. In the experimental group, human periodontal ligament cells were cultured in Dulbecco's modified Eagle's minimal essential medium and osteodifferentiation medium containing dexamethasone, ascorbic acid and β-glycerophosphate. After 3 wk, scanning electron microscopy was carried out, in addition to staining for alkaline phosphatase activity and for calcium (using the Von Kossa stain). Then human periodontal ligament cell sheets were multilayered and placed onto dentin blocks. The constructs were transplanted subcutaneously into the back of immunodeficient rats. At 1 and 6 wk after transplantation, the animals were killed. Demineralized tissue sections were stained using hematoxylin and eosin, and Azan, and then analyzed.

Results: After 3 wk of culture in osteodifferentiation medium, human periodontal ligament cells produced mineral-like nodules and also showed positive staining for alkaline phosphatase, calcium (Von Kossa) and mRNA expression of type I collagen. By contrast, in the control group only weak alkaline phosphatase staining was observed, the Von Kossa stain was negative and there was no mRNA expression of type I collagen. Six weeks after transplantation with human periodontal ligament cells cultured in osteodifferentiation medium, most of the dentin surfaces showed a newly immature cementum-like tissue formation and periodontal ligament with perpendicular orientation inserted into the newly deposited cementum-like tissue.

Conclusion: This study suggests that the multilayered temperature-responsive culture system can be used as a novel strategy for periodontal regeneration. The human periodontal ligament cell sheet technique may be applicable for regeneration of the clinical periodontal ligament–cementum complex.

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Periodontitis is one of the most prevalent infectious diseases and is characterized by the destruction of tissues, such as alveolar bone, cementum and the periodontal ligament, that surround and support the teeth. The goal of periodontal therapy is the complete regeneration of periodontal tissues, particularly where gross periodontal destruction has compromised the support of the tooth. This requires the attachment of new connective tissue to the previously denuded root surface with the formation of new cementum, new bone and functionally oriented collagen fibers emanating from the root (1). However, the reconstruction of periodontal tissue after conventional treatment often results in the formation of a long junctional epithelium that prevents the attachment of connective tissue to the root surface because of the rapid migration and proliferation of gingival epithelial cells. Therefore, the concept of guided tissue regeneration was established to promote the regeneration process (2, 3). This method is based on the assumption that only periodontal ligament cells have the potential to induce regeneration of the attachment apparatus of teeth.

The periodontal ligament is the soft connective tissue interposed between the roots of the tooth and the inner wall of the alveolar socket. Because the fibroblasts of the ligament originate, in part, from the ectomesenchyme in the investing layer of the dental papilla (1, 4), it has been suggested that these cells may have specialized properties (5, 6). Current evidence supports the concept that periodontal regeneration is promoted through the activation of cells in the remaining healthy portion of the periodontal ligament as well as in the perivascular region (5, 7). Several researchers have evaluated the biological properties of periodontal ligament cells *in vitro*, suggesting that these cells can have osteoblast-like properties, including collagen synthesis, response to bone-inductive factors and expression of bone-associated markers such as alkaline phosphatase (8, 9). Furthermore, periodontal ligament cells have the capacity to form mineralized nodules *in vitro*, under culture conditions appropriate for osteodifferentiation (10, 11). These findings have led researchers to hypothesize that progenitor cells of cementoblasts and osteoblasts exist within periodontal ligament tissues and that a portion of periodontal ligament cells have stem cell properties that may be responsible for stimulating the differentiation of the periodontium in cementum, bone and periodontal ligament *in vivo* (12, 13).

Tissue engineering has emerged as a new and ambitious approach that combines knowledge from material chemistry with cell biology and medicine. These strategies used biodegradable polymers to make scaffolds into which cells were inserted to produce tissue in the presence of growth factors (14). The first generation of tissue engineering can be used for constructing cartilage (15, 16) or bone (17). The second generation of tissue engineering requires a new method of tissue reconstruction not based on scaffolds. Cell sheet engineering without scaffolds is a new, alternative approach to tissue engineering. Cell sheet construction involves the use of a biomaterial that is a temperature-responsive culture surface containing a temperature-responsive polymer, poly *N*-isopropylacrylamide (18, 19). Poly *N*-isopropylacrylamide is hydrophilic below 32°C and hydrophobic above this temperature. The cell sheet is harvested by lowering the temperature. We have reported that periodontal ligament cells cultured using this cell sheet technique can regenerate periodontal ligament tissues after transplantation in a rat xenogenic model with human human periodontal ligament cells and in a dog autogenic model (20, 21). In the present experiment we aimed to stimulate periodontal regeneration, including cementum and the formation of new periodontal ligaments, as well as Sharpey's fibers attached to the new cementum-like layer, by using osteodifferentiation medium *in vitro* and the multilayer cell sheet technique, thereby providing a practical clinical approach for the utilization of cell sheet engineering.

Materials and methods

Preparation of temperature-responsive culture dishes

Specific procedures for the preparation of temperature-responsive culture dishes have been described elsewhere (22). Briefly, *N*-isopropylacrylamide monomer in 2-propanol solution was spread onto polystyrene culture dishes (Falcon 3001; BD Biosciences, Bedford, MA, USA). Then, the dishes were subjected to electron beam irradiation with an Area Beam Electron Processing System (Falcon 3001, BD Biosciences, Discovery Labware, Franklin Lakes, NJ, USA). The temperature-responsive polymer-grafted (poly *N*-isopropylacrylamide) dishes were rinsed with cold distilled water to remove ungrafted monomer and sterilized with ethylene oxide.

Cells and cell culture

Following an approved institutional review board protocol from Tokyo Women's Medical University, human periodontal ligament cells were isolated from a second premolar extracted for orthodontic reasons. After extraction, periodontal tissue was scraped from the middle third of the root with a scalpel blade. The harvested tissue was placed into culture dishes containing = Dulbecco's modified Eagle's minimal essential medium (low glucose; Wako Pure Chemical Industries, Tokyo, Japan), supplemented with 10% fetal bovine serum (Japan Bioserum Co. Ltd, Hiroshima, Japan) and 100 units/mL of penicillin–streptomycin (Sigma-Aldrich Japan, Tokyo, Japan). Then, these outgrowth cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C for 48 h to allow attachment of the cells to the dishes. The dishes were washed to eliminate debris and the medium was changed three times per week, as described previously (23). For all subsequent experiments, cells were used from the third to the fifth passage. To harvest the cell sheet, human periodontal ligament cells were plated on temperature-responsive culture dishes (35 mm in diameter) at a cell density of 1×10^5 and cultured at 37°C. In the

control group, cells were cultured in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum and 100 units/mL of penicillin–streptomycin, while the experimental group was cultured in the control medium supplemented with 50 μg/mL of ascorbic acid 2-phosphate (Sigma-Aldrich Japan), 10 nM dexamethasone (Sigma-Aldrich Japan) and 10 mM β-glycerophosphate (Sigma-Aldrich Japan). This supplemented medium functions as an osteodifferentiation medium.

Scanning electron microscopy

For examination using scanning electron microscopy, human periodontal ligament cell sheets were harvested from temperature-responsive culture dishes after 1 and 3 wk of culture, with or without osteodifferentiation medium, by low-temperature treatment at 20°C for 1 h. Afterwards, cell sheets were fixed with 2% glutaraldehyde, post-fixed in 1% OsO_4, dehydrated through a graded ethanol series, lyophilized overnight (ID-2; EIKO Engineering, Tokyo, Japan), coated with OsO_4 and mounted. The mounted sections were examined and photographed with a Hitachi S-4300 Electron Microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

Histochemistry

Alkaline phosphatase activity was examined in human periodontal ligament cells, cultured for 1 and 3 wk with or without osteodifferentiation medium, using an alkaline phosphatase staining kit (Takara Bio Inc., Shiga, Japan) following the manufacturer's suggested protocol. Von Kossa staining was applied to reveal calcium deposition after fixation with 4% paraformaldehyde.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of human periodontal ligament cells

After culture for 1 and 3 wk on temperature-responsive dishes, with or without osteodifferentiation medium, human periodontal ligament cell sheets were recovered by low-temperature treatment at 20°C for 1 h. To isolate fibrillar collagen, which is resistant to pepsin digestion, harvested cell sheets were treated with collagenase (3 mg/mL in 1 mM HCl, pH 3.0) (Sigma-Aldrich Japan) at 4°C overnight. The cell lysate was then pH neutralized to inactivate pepsin and subjected to protein separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 5% polyacrylamide gel. Separated protein bands were stained with Coomassie Brilliant Blue.

Transplantation of multilayered cell sheet–dentin complexes

For the ectopic transplantation, after 3 wk of culture at 37°C, human periodontal ligament cell sheets were harvested using fibrin gel (Tisseel®; Baxter, Vienna, Austria) to support the first cell sheet. First, fibrin gel (approximately 1 mm in thickness) was placed on the upper surface of human periodontal ligament cells cultured on temperature-responsive culture dishes (35 mm diameter) and incubated in a CO_2 incubator at 20°C for 1 h to allow harvesting. Then, all the cultured human periodontal ligament cell sheets were harvested as a construct containing three contiguous cell sheets along with the fibrin gel. The constructs were incubated at 37°C for 30 min to allow stable adhesion between the cell sheets. After incubation at 20°C for 1 h, the constructs comprising fibrin gel and three human periodontal ligament cell sheets were harvested from the dishes (Fig. 1). Human dentin blocks were prepared from teeth without caries or periodontal problems that had been extracted for orthodontic reasons. The dentin blocks were obtained after slicing the roots. Then, from the sections, all the cementum was completely removed with a Faskut® Carbide Cutter (Dentsply-Sankin K.K., Tokyo, Japan) under irrigation with sterile saline solution and contoured until the desirable shape and size were obtained (approximately $20 \times 10 \times 0.5$ mm). Then, the dentin blocks were conditioned with saturated citric acid (pH 1) for 1 min and cleaned in an ultrasonic cleaner. Multilayered cell sheet constructs containing fibrin gel were cut (approximately 25×15 mm), placed onto the dentin blocks in culture dishes and incubated in a small volume of culture medium (1 ml of Dulbecco's modified Eagle's minimum essential medium [low glucose; Wako Pure Chemical Industries, Tokyo, Japan]) at 37°C for 1 h to allow stable adhesion. All animal procedures complied with the guidelines provided by the Institutional Animal Care and Committee (Tokyo Women's Medical University). Twenty-six male, 8-wk-old athymic Fischer 344 rats were purchased from Charles River Japan (Yokohama, Japan). General anesthesia was administered by intraperitoneal injection of sodium pentobarbital sodium (0.1 mL/100 g) for all surgical procedures. Midsagittal incisions were made subcutaneously on the dorsa of the 26 athymic rats. Multilayered cell sheet–dentin constructs were placed at the muscle surface, covered and sutured with a thin polymer membrane (Durapore®; Millipore, Billerica, MA, USA) in order to avoid movement. The incisions were closed with 7-0 nylon sutures. Each athymic rat received a set of three different grafts: a dentin block without cells and two types of multilayered cell sheet–dentin complexes (i.e. one that had, and one that had not, been cultured in the osteodifferentiation medium). Transplanted rats were killed 1 or 6 wk after surgery. At each time point, each group consisted of 13 samples. Excised tissues were fixed in 10% neutral-buffered formalin, decalcified with 10% EDTA and embedded in paraffin. Paraffin sections (5 μm in thickness) were stained with either hematoxylin and eosin or Azan.

Results

Culture of human periodontal ligament cells

Under phase contrast microscopy (data not shown), human periodontal ligament cells cultured in the osteodifferentiation medium supplemented with dexamethasone, ascorbic acid and β-glycerophosphate (experimental group) showed significant accumula-

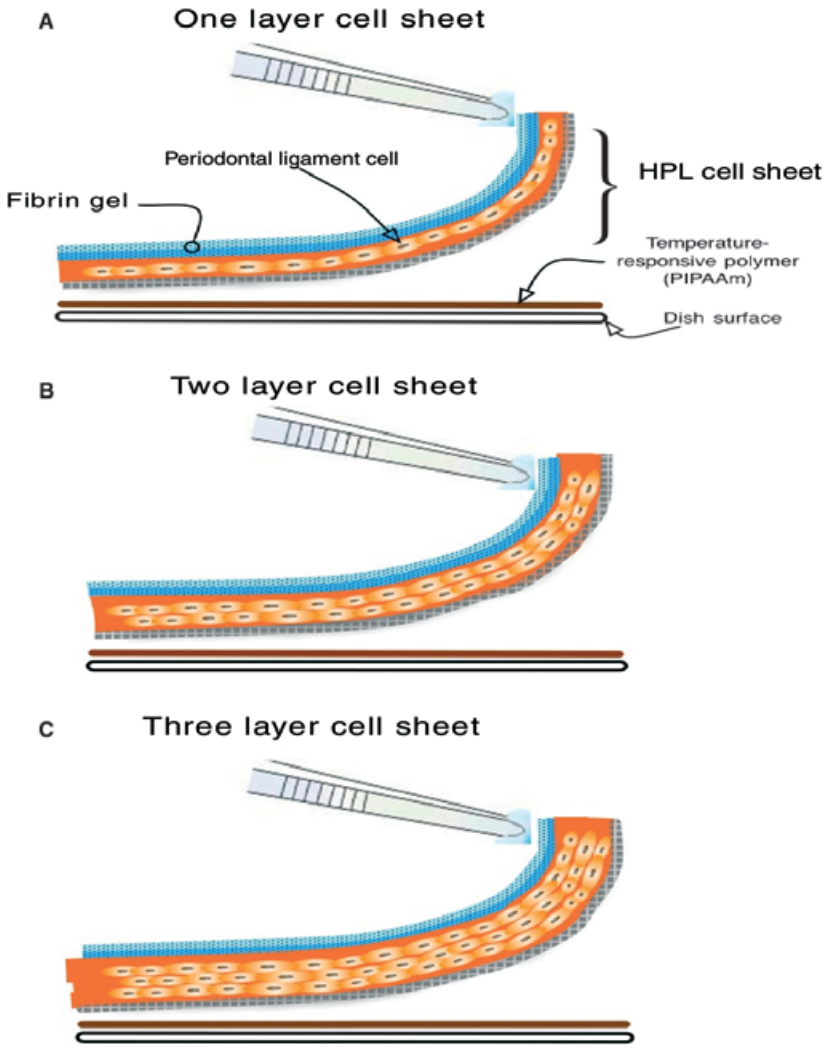

Fig. 1. Construction of a multilayer human periodontal cell sheet. One layer of human periodontal ligament cells (A) was detached from the temperature-responsive culture dish using fibrin gel to support the first cell sheet and incubated at 20°C for 1 h to allow harvesting. Two layers of human periodontal ligament cells (B) were attached after incubation at 37°C for 30 min, incubated at 20°C for 1 h to allow harvesting and transferred to the third dish to obtain the three-layer human periodontal ligament cell sheet construct (C). HPL, human periodontal ligament; PIPAAm, poly *N*-isopropylacrylamide.

tion of abundant extracellular matrix during 3 wk of culture; by contrast, such accumulation was not observed in human periodontal ligament cells cultured without supplements (the control group). Correspondingly, human periodontal ligament cells showed markedly increased alkaline phosphatase activity and calcium deposition after culture in the osteodifferentiation medium, showing a positive result for Von Kossa staining (Fig. 2). The results of the scanning electron microscopy analyses (Fig. 3) suggest that mineral-like nodules were produced by human periodontal ligament cells cultured in the presence of the supplements within 1 wk and that these nodules on the cell sheet surface increased in number during the 3 wk of culture. These nodules were often observed to be associated with high-density extracellular matrix and collagen fiber networks at the cell sheet surface, which is another remarkable characteristic of these cells when cultured in the osteodifferentiation medium. Neither nodules nor abundant extracellular matrix was observed in human periodontal ligament cells cultured without the supplements (Fig. 3). In addition, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of cell lysates showed that a high level of collagenous protein was synthesized and deposited by human periodontal ligament cells cultured in the osteodifferentiation medium during periods of 1 and 3 wk, whereas only a small amount of deposition was observed with cells cultured in the absence of supplements (Fig. 4).

Transplantation of the human periodontal ligament–dentin block construct

Human periodontal ligament cells cultured in the presence or absence of the osteodifferentiation medium were harvested as single contiguous cell sheets from temperature-responsive culture dishes by temperature reduction. Three human periodontal ligament cell sheets were integrated with dentin blocks and these multilayered cell sheet–dentin complexes were subcutaneously transplanted to athymic rats. The postoperative course of all the rats was uneventful throughout the duration of the experiment and no intense inflammatory reactions were observed throughout the healing period. During the first week post-transplantation, cells and/or surrounding soft tissues were detached from the dentin surfaces during histological sectioning in all the groups, implying weak adhesion or integration between dentin surfaces and the cells (data not shown). By contrast, cells and surrounding soft tissues had adhered stably onto the dentin surfaces in eight of thirteen (61.5%) samples of the experimental group (Fig. 6), as well as in two of 13 (14.4%) samples of the control group 6 wk after transplantation (Fig. 5B,D). However, this adhesion was not observed at any time point around dentin blocks without cell sheets (Fig. 5A,C).

Histological observations of the human periodontal ligament–dentin block

To examine whether multilayer human periodontal cell sheets had the potential

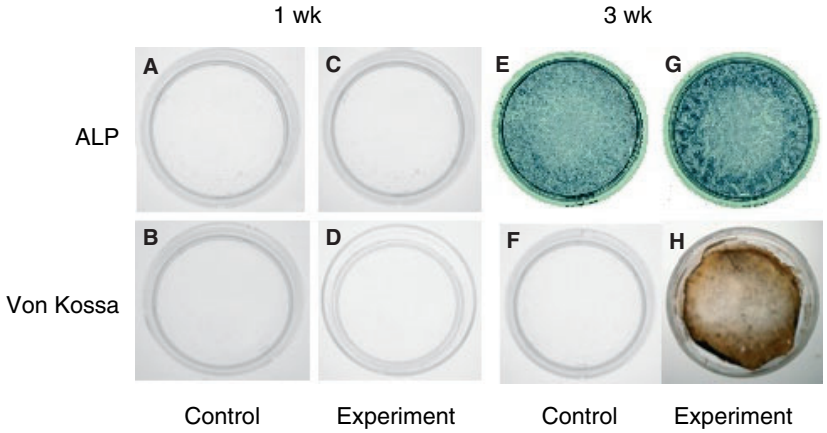

Fig. 2. Staining of human periodontal ligament cell sheets for alkaline phosphatase activity and calcium. Human periodontal ligament cell sheets were cultured for 1 wk in the presence (C, D) or absence (A, B) of osteodifferentiation medium. Human periodontal ligament cell sheets cultured for 3 wk in osteodifferentiation medium stained more strongly for alkaline phosphate activity (G) than human periodontal ligament cell sheets of the control group (E). Human periodontal ligament cell sheets cultured for 3 wk in osteodifferentiation medium and then stained with Von Kossa stain (H) showed that calcium phosphate deposition had occurred during culture, whereas the control group showed no calcium deposition (F) during the same time period. ALP, alkaline phosphatase.

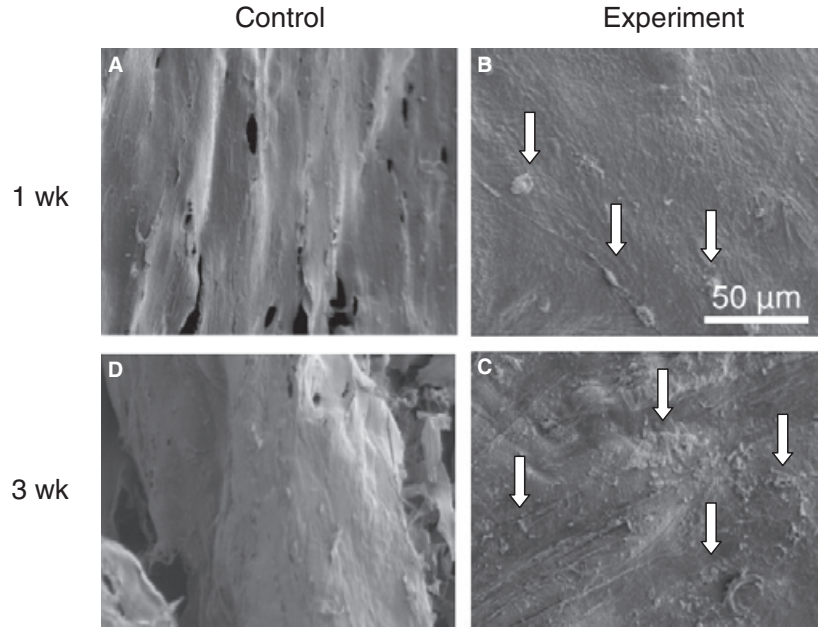

Fig. 3. Scanning electron microscopy of human periodontal ligament (human periodontal ligament) cell sheets. Human periodontal ligament cell sheets were cultured for 1 or 3 wk in the absence or presence of osteodifferentiation medium. Control human periodontal ligament cell sheets (A, D) showed a low level of extra-cellular matrix at 1 and 3 wk incubation periods. After 1 wk of culture in osteodifferentiation medium(B) human periodontal ligament cell sheets showed mineral nodule formation (white arrows). After 3 wk of culture in osteodifferentiation medium (C), human periodontal ligament cell sheets formed mineral nodules (white arrows) associated with a high density of extracellular matrix and collagen fiber networks.

to regenerate the periodontium complex, we transplanted human periodontal ligament–dentin block constructs subcutaneously into athymic rats. A newly immature cementum-like tissue was regenerated on the dentin surfaces in eight of 13 (61.5%) samples of the experimental group 6 wk after transplantation. Histological observations revealed dense extracellular matrix and the adherence of fibers, resembling Sharpey's fibers, to both the newly deposited cementum-like tissue and the dentin surface in samples which showed regeneration of cementum-like tissue at the 6-wk time-point. Higher magnification revealed that collagen fibers were inserted perpendicularly into the newly formed cementum-like tissue, and this orientation closely resembles native periodontal fibers called Sharpey's fibers (Fig. 6). This phenomenon was observed at the peripheral area of the dentin block surface. No sign of continuous dentin resorption was observed on the dentin surface. Transplantation of dentin blocks with cell sheets cultured without supplements resulted in neither cementum formation nor attachment of collagen fibers to the dentin surface (Fig. 5B,D), throughout the observation periods. However, dense extracellular matrix and connective tissue oriented parallel to the dentin surface were found in two of 13 samples 6 wk after transplantation, even in the control group. In the negative control where dentin blocks without cells were transplanted, no tissue regeneration was observed (Fig. 5A,C). Analyses of these data suggest that multilayer human periodontal cell sheets may be useful for therapeutic use.

Discussion

The requirements for periodontal regeneration include the simultaneous regeneration of cementum, the periodontal ligament and alveolar bone. In order to fulfill this requirement, applications of various cells have been considered. Nakahara *et al.* reported that autologous periodontal ligament-derived cells were required for the regeneration of periodontal tissues with collagen sponge scaffold in dogs (9). Mizuno *et al.* attempted to regen-

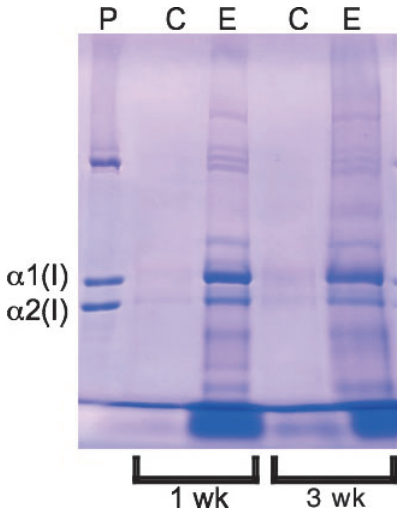

Fig. 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of pepsin extracts from human periodontal ligament cells. Human periodontal ligament cells were cultured for 1 or 3 wk in the absence (C) or presence (E) of osteodifferentiation medium and subjected to pepsin digestion to extract collagen, which is resistant to pepsin. The extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. P, molecular marker of type I collagen.

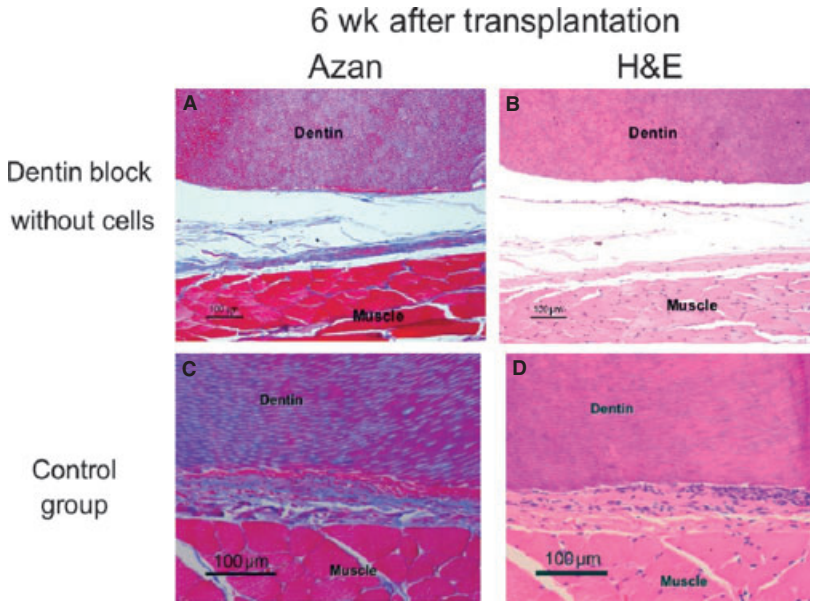

Fig. 5. Histological examination 6 wk after subcutaneous transplantation of tissue-engineered constructs (control group). Athymic rats were subcutaneously transplanted with dentin blocks containing no cells (A, B) in which case tissue adhesion was not observed. The constructs transplanted with multilayer human periodontal ligament cells (C, D), previously cultured in the absence of osteodifferentiation medium (control group), showed stable adhesion of the surrounding tissues, but collagen fibers were not inserted in the dentin block surface. Left columns, Azan staining; right columns, hematoxylin and eosin (H&E) staining.

erate periodontal tissue defects by grafting autologous cultured membrane derived from the periostium (24). Some researchers attempted to apply the mesenchymal stem cells from bone marrow to periodontal defects. Kawaguchi *et al*. and Hasegawa *et al*. reported successful results of autotransplantation of mesenchymal stem cells isolated from bone marrow into furcation defects in dogs (25, 26). Yamada *et al*. reported a novel approach to periodontal regeneration with mesenchymal stem cells and platelet-rich plasma (PRP) (27). The mesenchymal stem cells were isolated from a patient's iliac crest marrow aspirates. However, these procedures are expensive and require large-scale surgical interventions. Recently, stem cells were found in periodontal ligament cells (12, 13).

Seo *et al*. induced mineralization of periodontal ligament stem cells with an osteodifferentiation medium, which was similar to that used in our study. They showed that transplanted periodontal ligament stem cells which were mixed with hydroxyapatite/tricalcium

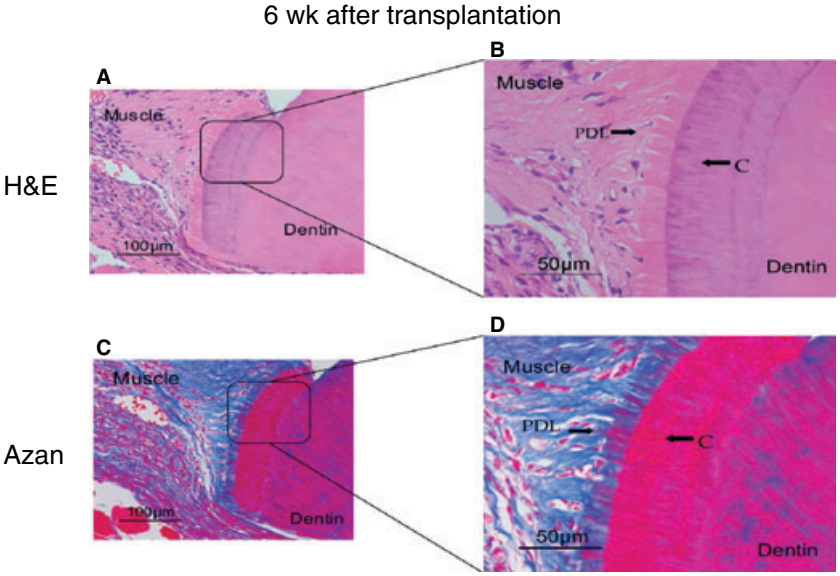

Fig. 6. Histological examination 6 wk after subcutaneous transplantation of tissue-engineered constructs (experimental group) Athymic rats were transplanted with dentin blocks containing multilayer human periodontal ligament cell sheets cultured for 3 wk in the presence of osteodifferentiation medium. A newly formed cementum-like tissue was regenerated at the dentin surface. Collagen fibers adhered to this newly formed cementum-like tissue, resembling Sharpey's fibers (periodontal ligament). C, cementum-like tissue; H&E, hematoxylin and eosin; PDL periodontal ligament.

phosphate ceramic formed a new periodontal-ligament-like structure associated with the newly regenerated cementum (12, 28). However, when these human periodontal ligament stem cells were transplanted into the mandibular molar defects of immunocompromised beige mice, Seo *et al*. reported that very few stem cells attached to the surface of alveolar bone and teeth (12).

On the other hand, Okano *et al*. developed a new method to control cell-surface adhesion by exploiting cell culture temperature and a surface-grafted temperature-responsive polymer, poly *N*-isopropylacylamide. On the grafted surface, culture cells proliferated similarly to those in a conventional culture system at 37°C (22). However, when the temperature was lowered below 32°C, the cells detached spontaneously to float in the medium as a visible cell sheet. This contiguous cell sheet can avoid trypsinization and retain the extracellular matrix, cell-to-cell junctions and differentiated functions. Using this novel cell sheet technique we tried to regenerate periodontal tissue without animal-derived scaffolds or damage to the patient.

An application of this novel cell sheet technique has been performed for periodontal regeneration therapy. Hasegawa *et al*. harvested human periodontal ligament as a contiguous cell sheet and then transplanted it into a periodontal defect model in athymic rats (20). The histological findings with Azan stain showed that the outermost layer of curetted root dentin surfaces were positively stained blue only in the transplanted sides. In the regenerated periodontal ligament tissues, immature fibers were obliquely anchored onto this layer. This orientation highly resembled native periodontal ligament fibers. Akizuki *et al*. applied the periodontal cell sheet to the dog dehiscence model, and periodontal regeneration, including collagen fibers inserted perpendicularly into the newly formed bone and cementum, was reported in more than half of the experiments (21).

For periodontal regeneration, the key issue is to regenerate cementum, including Sharpey's fibers. In this regard, Hasegawa *et al*. and Akizuki *et al*. mentioned that thin new cementum was produced after the transplantation of periodontal ligament cell sheets (20, 21). Periodontal ligament cells (29, 30), as well as human bone marrow cells (31), were found to be capable of producing mineral-like nodules *in vitro* when supplemented with ascorbic acid, glycerophosphate and dexamethasone in the culture medium.

The present study was undertaken to clarify the ability of transplantable human periodontal ligament cell sheets to develop a cementum–periodontal ligament complex in an ectopic implantation site *in vivo*. In order to reinforce the periodontal cell sheet, three layers of periodontal ligament in a cell sheet were prepared. This sheet was rigid and easy to manipulate, indicating usefulness for clinical application. Another modification of the periodontal ligament cell sheet procedure was the inclusion of osteodifferentiation medium in the culture. Nagatomo *et al*. characterized human periodontal ligament cells quantitatively to clarify their stem cell properties, including self-renewal and multipotency (13). They found that about 30% of periodontal ligament cells could differentiate into mineralized tissue after 21 d of culture in the osteodifferentiation medium. Thus, the multilayered periodontal ligament cell sheet cultured in the osteodifferentiation medium was believed to facilitate the production of mineralized tissue, such as cementum, as well as soft tissue-like periodontal ligaments. After 3 wk of culture in the osteodifferentiation medium, we observed that periodontal ligament cell sheets showed significant accumulation of abundant extracellular matrix. In the medium without the osteodifferentiation medium (control group), such accumulation was not observed. Our results demonstrate that numerous periodontal ligaments were inserted into the laminated cementum-like structure showing Sharpey's fibers after transplantation of the periodontal ligament cell sheet covered on dentin block into the dorsum of athymic rats. Such rigid cell sheets were again very easy to transplant on the dentin block surfaces, indicating reliability of the cementum–periodontal complex formation and easy manipulation for clinical use. With this experimental model, we were not successful in formation of a cementum–periodontal ligament complex in all experimental samples. The reason for this may be that blood supply was not sufficient as a result of pressure caused by the membrane we used to avoid the movement of our constructs. This might also explain why the transplanted cells migrated to the peripheral area of the dentin block, resulting in the formation of new cementum-like tissue and the regeneration of Sharpey's fibers. Another possibility is that because the constructs were made up of very heterogeneous populations of cells, some may have had higher proportions of cells capable of aiding the formation of such complexes. It has been reported that root slices stimulate periodontal ligament to form periodontal ligament -like tissue *in vitro* (32), implying that the interaction between periodontal ligament cells and dentin may be a correlated factor in regenerating periodontal ligament tissues. Further studies are required in order to confirm this new regeneration process. We are now studying *in vivo* cementum–periodontal ligament complex experiments with application of the cell sheet, according to King's method, in the rat mandible (33, 34).

In the present work we propose a novel strategy for periodontal regeneration by transplantation of tissue-engineered multilayered human periodontal ligament cell sheets utilizing the osteodifferentiation medium on the tooth surface. This tissue engineered construct would be applicable for periodontal regeneration in clinical settings.

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