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Periodontal ligament cell sheet promotes periodontal regeneration in athymic rats

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Abstract

Aim: The primary goal of periodontal treatment is regeneration of the periodontium. Current theories suggest that the periodontal ligament (PDL) cells have the capacity to participate in restoring connective and mineralized tissues, when appropriately triggered. We evaluated whether human PDL cell sheets could reconstruct periodontal tissue.

Material and Methods: To obtain the cell sheet, human PDL cells were cultured on temperature-responsive culture dishes with or without osteogenic differentiation medium. The cell sheets were transplanted on periodontal fenestration defects of immunodeficient rats. Forty rats were divided in two groups: in one group, cell sheets cultured with control medium were transplanted and in the other, cell sheets cultured with osteogenic differentiation medium were transplanted. The defects were analysed histologically and histomorphologically after healing.

Results: Most of the experimental group exhibited a new cementum-like layer and new attachment of collagen fibres to the layer. Histomorphological analyses indicated significant periodontal regeneration. The control group revealed dense extracellular matrix and fibre formation, but an obvious cementum layer was not observed.

Conclusions: Transplanted PDL cell sheets cultured with osteogenic differentiation medium induced periodontal regeneration containing an obvious cementum layer and Sharpey's fibres. Thus, the method could be feasible as a new therapeutic approach for periodontal regeneration.

Key words: cell sheet; cementum; King's method; periodontal ligament; regeneration; temperature responsive; tissue engineering; transplantation

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Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.

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The periodontal ligament (PDL) is a soft connective tissue that surrounds and supports the tooth. It is located between two mineralized tissues; cementum covering the tooth root and the inner wall of the alveolar bone socket. The PDL plays an important role in anchoring the tooth to the alveolar bone and in maintaining the structural integrity. It has also been reported that the PDL has the capacity to induce the regeneration of periodontium. PDL cells consist of a heterogeneous cell population such as fibroblasts, cementoblasts and osteoblasts (Pitaru et al. 1994). Current theories suggest that because of the stem cells in the PDL, the periodontium has regenerative capacity, which, when appropriately triggered, participates in restoring connective tissues and mineralized tissues (Seo et al. 2004, Bartold et al. 2006, Nagatomo et al. 2006).

Periodontal disease results in the destruction of periodontal tissues including cementum, bone, PDL and gingiva, and finally leads to tooth loss. The ultimate goal of periodontal therapy is regeneration of damaged periodontal tissues. Formation of new cementum with the attachment of Sharpey's fibres on the denuded root surfaces is an essential process in the regeneration of periodontal tissues. This process requires organized and selective repopulation of cementum and fibroblast populations that are generated within

the PDL. As a method to induce the regeneration of periodontium with cementum and PDL, we focused on cell sheet engineering.

Cell sheet engineering emerged as a novel alternative approach for tissue engineering involving the use of a temperature-responsive polymer *Poly N-isopropylacrylamide* (PIPAAm) surface without the use of scaffolds (Okano et al. 1993). PIPAAm is hydrophilic below 32°C and hydrophobic above this temperature. This specific nature allows the cells to attach and proliferate at 37°C and a continuous cell sheet can be obtained by lowering the temperature to 20°C with good preservation of the cell surface junction and intact cell–cell junctions and deposited extracellular matrix (ECM) (Hirose et al. 2000, Nishida et al. 2004). We have shown that cell sheet engineering could be applied for regeneration of PDL cells and the cell sheets could induce formation of periodontal tissue containing cementum and PDL in vivo and in vitro (Akizuki et al. 2005, Hasegawa et al. 2005). In a previous paper, we revealed that human PDL cell sheets plated onto a dentin block could induce regeneration of cementum in the back of a rat and the cell sheets cultured with osteogenic differentiation medium could induce the formation of an obvious cementum layer compared with the cell sheets cultured with control medium (Flores et al. 2008)

To verify the regeneration of periodontal tissue induced by the PDL cell sheet in the oral region, we applied periodontal cell sheets in mandibular molar defects in athymic rats according to the method of King et al. (King et al. 1997, King & Hughes 2001) to regenerate cementum and PDL-like tissues.

Material and Methods

Isolation and culture of human PDL cells

Following an approved institutional review board protocol from Tokyo Women's Medical University, human PDL cells were gently removed with a scalpel blade from the middle third of the root of a human second premolar extracted for orthodontic reasons. The harvested tissues were placed into culture dishes with GIBCO™ minimum essential medium (MEM) alpha medium (1X) (Invitrogen, Tokyo, Japan), 10% foetal bovine serum (Japan Bioserum Co. Ltd., Hiroshima, Japan) and 100 units/ml penicillin–streptomycin (Sigma-Aldrich, Tokyo, Japan). Thereafter, these outgrown cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 48 h to allow cell attachment, as described previously (Hasegawa et al. 2005). For subsequent experiments, human PDL cells from the third to fifth passage were used. In order to obtain the human PDL cell sheet, the human PDL cells were plated on 35 mm diameter temperature-responsive culture dishes Up Cell® (CellSeed Inc., Tokyo, Japan) at a density of 1×10^5 and cultured at 37°C. After the human PDL cells had reached confluence, the dishes were divided into two groups; the control group was cultured with GIBCO™ MEM alpha medium (1X) (control medium), while the experimental group was cultured with the control medium supplemented with 50 μg/ml ascorbic acid 2-phosphate (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich) and 10 mM β-glycerophosphate (Sigma-Aldrich) for 3 weeks. This medium was named osteogenic differentiation medium.

Phenotypic markers of human periodontal cell sheets in vitro

Immunostaining for α-smooth muscle actin, osteopontin and bone sialoprotein

Human PDL cells were cultured in 35 mm temperature-responsive culture dishes at 1×10^5 and cultured at 37°C in order to obtain periodontal cell sheets. All the experiments were performed in triplicate. The dishes were cultured with control medium or osteogenic differentiation medium containing 50 μg/ml ascorbic acid 2-phosphate, 10 nM dexamethasone and 10 mM β-glycerophosphate for 3 weeks. To examine the osteoblastic characteristics of the human PDL cell sheets, we stained the dishes immunohistochemically and could facilitate the evaluation of the functional activity of cell proteins. Dishes containing human PDL cell sheets were fixed with equal parts of methanol and acetone for 20 min. at 4°C. The dishes were carefully washed with distilled water until the fixation agents were completely eliminated. Endogenous peroxidase was quenched by incubating with Dako Cytomation Peroxidase Blocking Reagent (Dako, Carpinteria, CA, USA) for 30 min. Non-specific staining was blocked by using Dako Cytomation Protein Block Serum-Free (Dako) for 30 min. The cell sheets were incubated with the primary antibody at 4°C overnight in a moisture chamber. Immunolocalization for α-smooth muscle actin (α-SMA) was performed with a mouse monoclonal anti-α-SMA antibody (Clone 1A4; Sigma-Aldrich Inc., St. Louis, MO, USA; 1:50 dilution), for osteopontin (OPN) with rabbit polyclonal anti-OPN (American Research Products Inc., Belmont, MA, USA) dilution 1:100 and for bone sialoprotein (BSP) with rabbit polyclonal anti-BSP (Chemicon International Inc., Tokyo, Japan) and then incubated with the second antibody Dako Cytomation LSAB®2 System-HRP (Dako) for 30 min. For visualization, dishes were treated with Dako Envision/HRP (DAB) Chromogen System (Dako) according to the manufacturer's specifications. Non-immune rabbit serum and non-immune mouse immunoglobulin G were used as negative controls for rabbit and mouse primary antibodies, respectively.

Transplantation of human PDL cell sheet into root defects

Human PDL cells were cultured in the presence or absence of osteogenic differentiation medium for 3 weeks in temperature-responsive culture dishes (35 mm diameter). After 3 weeks of culture, the human PDL cell sheets were detached by lowering the culture temperature to 20°C for 1 h, and then were harvested as a continuous cell sheet and applied for transplantation.

All animal procedures complied with the guidelines provided by the Institutional Animal Care and Committee (Tokyo Women's Medical University). Forty male, 5-week-old athymic Fischer 344 rats were purchased from the Charles River Japan (Yokohama, Japan). General anesthesia was administered by intra-peritoneal injection of pentobarbital sodium (0.1 ml/100 g) for all the surgical procedures. The incisions were performed according to the King's method (King et al. 1997, King & Hughes 2001) at the base of the right side of the mandible. The superficial fascia was separated to expose the masseter muscle. The masseter muscle and the periosteum were separated from the bone to expose the mandible. The cortical buccal bone overlying the first and the second mandibular molars was removed using a size 3 round-head bur (Dentsply-Sankin K. K., Tokyo, Japan) at slow speed under saline irrigation. We performed the bone removal proce-

dure under a microscope Leica M651 (Leica Microsystems GmbH, Wetzlar, Germany) at 10 × and 16 × magnification. The defect was carefully created at all times in order not to perforate the intra-oral mucosa. The superior margin of the bone defect was located 1 mm inferior to the oral mucosa attachment and extended to either side of the two exposed buccal roots until 4 mm in length. The apical margin was extended 2 mm in width to avoid exposure of the roots apices and to ensure their vitality. The PDL was carefully removed from the buccal roots of the first and second molars, and overlying cementum and dentin were debrided by curettes under saline irrigation. The depth of the defect was determined when the pulp in the root canal was visible through the translucent dentin. The root surface was acid etched with EDTA chelating and filing lubricant gel, File-Eze® (Ultradent Products Inc., South Jordan, UT, USA) for 15 s. The defects were washed with abundant saline solution and were dried with sterile cotton tips. The animals were divided into two groups. The control group (20 rats) received human PDL cell sheet cultured in the absence of osteogenic differentiation medium, and the experimental group (20 rats) received human PDL cell sheet cultured with osteogenic differentiation medium. The human PDL cell sheets were placed on the root defect and covered with a 5 × 2 mm non-absorbable GORE-TEX® membrane (W. L. Gore & Associates Inc., Flagstaff, AZ, USA) in order not to disturb the human PDL cell sheets. The masseter was re-positioned and sutured with Vicryl, PDS®II monofilament 7-0 (Johnson & Johnson, North ride, NSW, Australia), the external incision was sutured with nylon 5-0 (Nescosuture®; Alfresa, Osaka, Japan), and removed 7 days after surgery. The animals were sacrificed 3 or 5 weeks after transplantation. The right side of the mandibles was dissected from the rats, fixed with 4% formalin, decalcified with 10% EDTA in Dulbecco's phosphate-buffered saline for 3 weeks and then embedded in paraffin. Serial horizontal 5 μm paraffin sections were prepared and stained with hematoxylin–eosin and Azan.

Histomorphometric analyses for regenerated periodontal tissues

To evaluate newly formed periodontal tissues statistically, following parameters were measured on the sections of rat mandible 3 weeks after cell sheet transplantation: cementum regeneration rate, newly formed cementum thickness and vertical ligament regeneration rate. Cementum regeneration rate represented the length of newly formed cementum attached to denuded root surface normalized to the length of denuded root surface. Newly formed cementum thickness was the length of cementum regenerated in the direction vertical to denuded root surface. The lengths were measured every 50 μm on the newly formed cementum and the mean was calculated. Vertical ligament regeneration rate showed the length of newly formed PDL-like fibres attached to denuded root surface at an angle of more than 45° divided by the length of denuded root surface.

Statistical analysis

Data were presented as mean values ± standard deviations (SD). Differences between control and experimental groups were analysed using Student's *t*-test. When *p*-values were less than 0.05 (*), the differences were considered to be statistically significant.

Results

Expression of differentiation markers in human PDL cell sheets

To investigate the potential of human PDL cell sheets to undergo osteoblastic–cementoblastic differentiation, secondary cultures of human PDL cell sheets were supplemented with dexamethasone, ascorbic acid and β-glycerophosphate (osteogenic differentiation medium), and analysed immunohistochemically. After 3 weeks of culture with osteogenic differentiation medium, human PDL cell sheets showed positive expression of α-SMA, OPN and BSP, which were used as differentiation markers of connective tissue cell population in the PDL (Fig. 1a–c). The expression of OPN and BSP was not observed in the human PDL cell sheets cultured with control medium; however, the expression of α-SMA was positive (Fig. 1d–f).

Transplantation of human PDL cell sheet into root defects

To investigate whether the human PDL cell sheets had the potential to differentiate into functional cementoblast-like cells and to contribute to periodontal tissue regeneration, human PDL cells were seeded in temperature-responsive culture dish with or without osteogenic differentiation medium, harvested as a single contiguous cell sheet and then transplanted into surgically created defects on the distal root in mandibular molars in athymic rats according to the experimental schedule described in Fig. 2.

Three weeks after transplantation, supplemented human PDL cell sheets started to form newly mineralized layers of cementum-like tissue at the denuded root surface and the attachment of fibres in six out of 10 (60%) examined samples. Newly formed cementum layers were confined to the margins of the denuded root surface, and collagen fibres were attached perpendicularly to the thin acellular cementum-like tissue layer (Fig. 3A). In the control group, only a dense mass of disorganized fibres was observed in all the samples (Fig. 3B). Additionally, 5 weeks after transplantation, new cementum-like tissue had regenerated at the denuded root surface in seven out of 10 (70%) examined samples of the experimental group and repair of alveolar bone was evident (Fig. 3C). Higher magnification revealed a thin band of acellular cementum-like tissue overlying the denuded root surface and the formation of functionally attached fibres resembling Sharpey's fibres connected to this newly formed cementum-like tissue. No sign of continuous dentin resorption was observed at the peripheral area of the surgically created defects. On the other hand, in the control group in which human PDL cell sheets cultured with control medium were transplanted, only dense ECM and parallel fibre formation were observed in eight out of 10 samples, obvious cementum regeneration was not observed during the experimental period (Fig. 3D).

To evaluate regenerated periodontal tissues induced by transplantation of human PDL cell sheets, we conducted histomorphometric analyses (Table 1). The rats, which were transplanted with cell sheet for 3 weeks, were used to verify the early periodontal regeneration. Our results showed that the newly formed cementum-like tissue covered approximately 75% of the denuded root surfaces in the experimental group, whereas only 25% was covered in the control group. The cementum regeneration rate was significantly higher in the experimental group compared with the

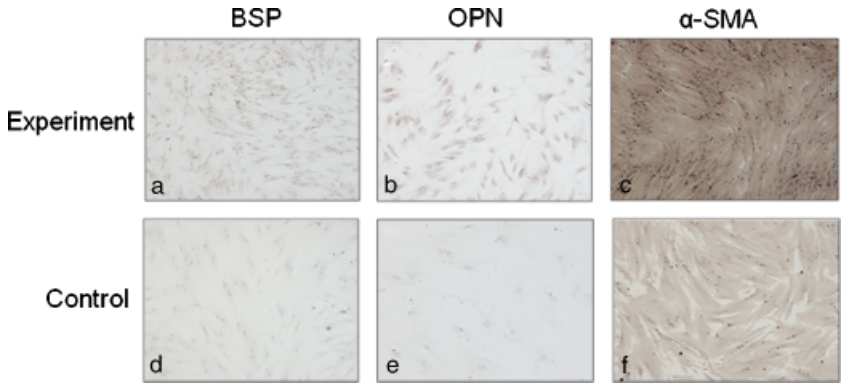

Fig. 1. Immunohistochemestry of phenotypic markers in human periodontal ligament (PDL) cell sheets in vitro. Human PDL cells were cultured with control medium (control) or osteogenic differentiation medium (experiment) and analysed immunohistochemically. Human PDL cell sheets cultured with osteogenic medium showed positive expression of α-smooth muscle actin (α-SMA) (c), osteopontin (OPN) (b) and bone sialoprotein (BSP) (a). The expression of OPN (e) and BSP (d) was not observed in the human PDL cell sheets cultured with control medium, in contrast the expression of α-SMA was positive (f).

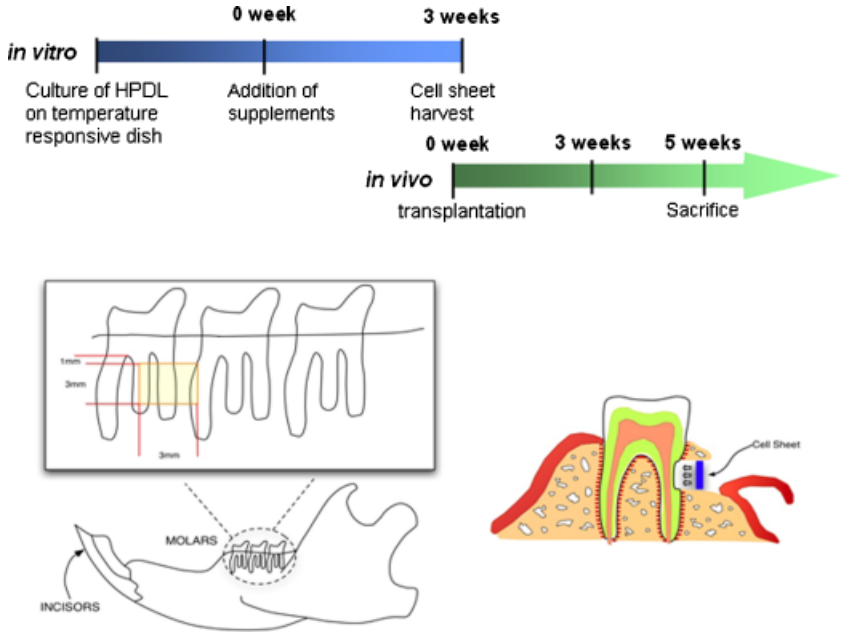

Fig. 2. Time course of cell culture and transplantation. Defect in rat mandible according to King's method. Human periodontal ligament cells were cultured on a temperature-responsive dish. After the culture with supplements for 3 weeks, the cell sheets were harvested and transplanted onto the defect. The defect was made in the rat distobuccal root of the first molar, with 3 mm width, 4 mm length and approximately 1 mm depth according to the King's method. The animals were sacrificed 3 or 5 weeks after transplantation.

control group. The newly formed PDL-like fibres resembling normal PDL covered approximately 65% of the denuded root surfaces and were only observed in the experimental group.

These findings might indicate that the transplanted human PDL cell sheet generates a cementum–periodontal complex when induced with osteogenic differentiation medium.

Discussion

The aim of this study was to evaluate cementum and periodontal regeneration when using periodontal cell sheet engineering without scaffolds. The ideal periodontal therapy entails simultaneous regeneration of cementum, PDL and alveolar bone. Many procedures have been advocated for periodontal regeneration. Isaka et al. (2001) isolated PDL cells and transplanted them in an autologous dog model and found that PDL cells were capable of differentiating into the osteoblast lineage and they were effective for periodontal regeneration. Dogan et al. (2002, 2003) assessed periodontal healing by seeding regenerated periodontal tissue underneath a Teflon membrane in fenestration defects in dogs. The procedure induced effective bone formation; however, connective tissue formation was limited. Other attempts for regeneration were based on the use of fibroblast growth factor in combination with BMP-2, and periosteum cells enhanced the proliferation rate and subsequent bone formation (Agata et al. 2007). Combination of growth factors such as platelet-rich-plasma preparations were also shown to be capable of promoting regeneration of connective tissues (Kawase et al. 2005, Yamada et al. 2006). However, these procedures were mainly focused on bone formation and the clinical outcomes were still insufficient.

Melcher (1970) proposed that PDL cells induced the formation of new PDL (Melcher 1970). Furthermore, it has been reported that PDL cells exhibit properties of stem cells such as self-renewal and multilineage differentiation potential, have high ability to proliferate and differentiate, and play an important role in the regeneration of the periodontal tissues (Trubiani et al. 2005, Chen et al. 2006, Nagatomo et al. 2006). Previous experiments have shown that PDL cells have the potential to express osteoblast-like phenotype, such as the capacity of mineralized nodule formation and the expression of several bone-associated proteins, i.e. OPN, BSP, alkaline phosphatase (ALP) when the PDL cells were cultured in the presence of dexamethasone, β-glycerophosphate and ascorbic acid (Arceo et al. 1991, Nohutcu et al. 1997, Lekic et al. 2001). In the present study, we confirmed that human PDL cell sheets cultured with osteogenic differentiation medium expressed OPN and BSP, and transplanted human PDL cell sheets induced cementum-like tissues in vivo. The findings suggested that human PDL cell sheets contain a heterogeneous stem cell population and have the capacity to differentiate cementoblast-like and PDL tissues. Seo et al. (2005) found that PDL cells were similar to other mesenchymal stem cells, and transplanted PDL cells mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic formed

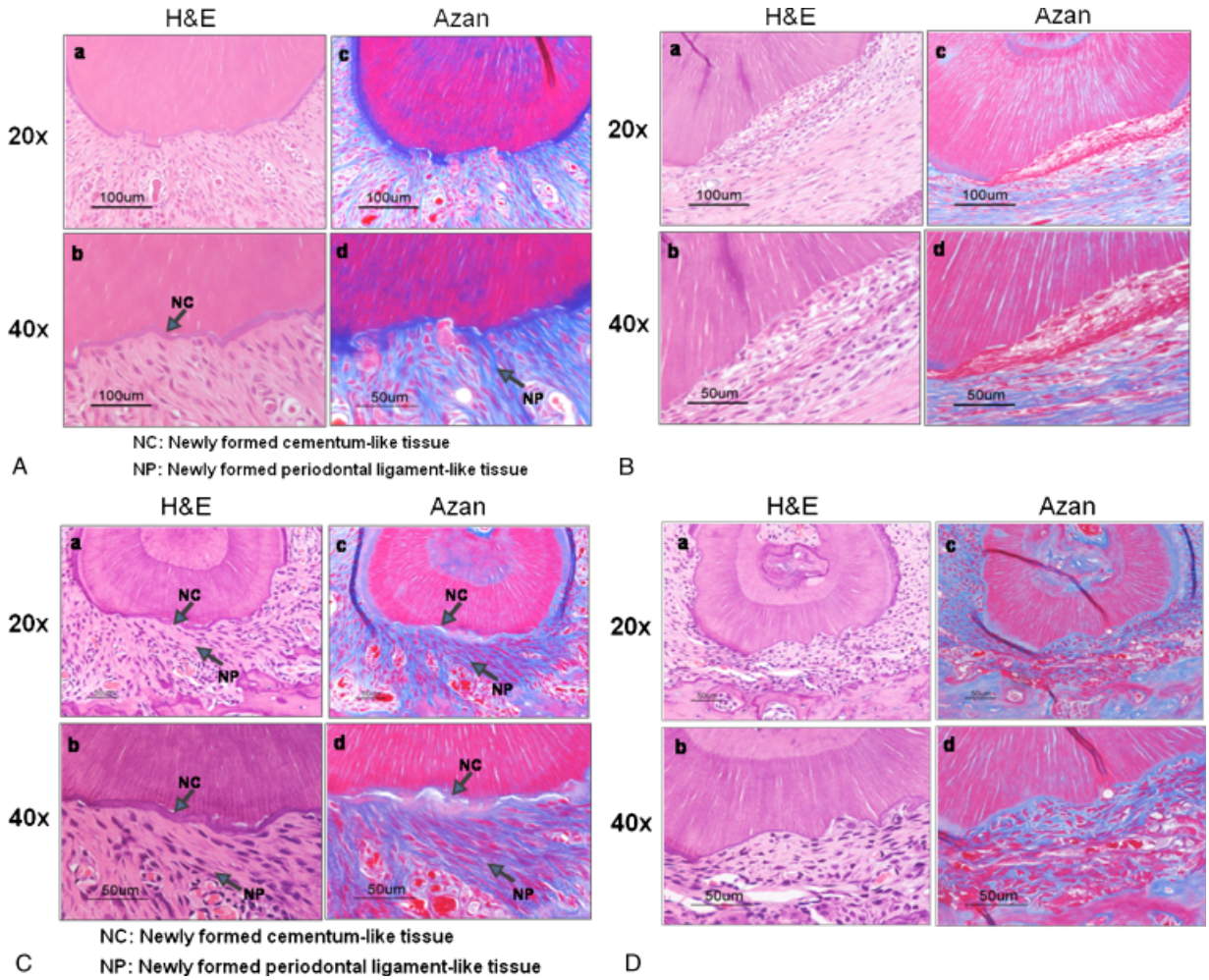

Fig. 3. (A) Three weeks after transplantation with human periodontal ligament (HPDL) cell sheet cultured in osteogenic differentiation medium (experimental site). Three weeks after transplantation with human PDL cell sheet cultured in osteogenic differentiation medium (experimental site), newly formed layers of cementum-like tissue at the denuded root surface. HE staining revealed newly formed cementum layers on the margins of the denuded root surface [Part A (a, b)]. Azan staining showed collagen fibres attached perpendicularly to the thin acellular cementum-like tissue layer [Part A (c, d)]. (B) Three weeks after transplantation with HPDL cell sheet cultured in D-minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) (control site). In the control group, newly formed cementum layer was not observed clearly [(Part B (a, b)] and only a dense mass of disorganized fibres was observed in all of the samples [(Part B (c, d)]. (C) Five weeks after transplantation with HPDL cell sheet cultured in osteogenic differentiation medium (experimental site). The periodontal defects exhibited a newly formed layer resembling cementum-like tissue [Part C (a, b)]. The new attachment of periodontal ligament-like tissue to this layer was observed in HE and Azan staining. The collagen fibres of the periodontal like-tissue were attached perpendicularly into the newly formed cementum-like tissue [Part C (c, d)]. (D) Five weeks after transplantation with HPDL cell sheet cultured in D-MEM supplemented with 10% FBS (control site). The periodontal defects did not exhibit newly formed cementum-like tissue [Part D (a, b)] and the orientation of the collagen fibres adjacent to the root surface was parallel to the root surface compared with the experimental site [Part D (c, d)].

Table 1. Statistical analyses for histomorphometry

	Experiment group		Control group	
	mean	SD	mean	SD
Cementum regeneration rate (%)	74.4*	7.4	26.4*	21.6
Newly formed cementum thickness (μm)	4.24*	1.65	0.28*	0.23
Vertical ligament regeneration rate (%)	64.8	9.2	0	–

SD, standard deviation.
n (number of sites) = 3.
*Statistically significant difference ($p < 0.05$).

cementum-like structure which connected to newly formed collagen fibres that mimicked Sharpey's fibres on ceramics. However, they reported that very few stem cells attached to the surface of alveolar bone and teeth when the human PDL stem cells were transplanted in the mandibular molar defects of immunocompromised beige mice (Seo et al. 2004). Recently, Liu et al. (2008) reported successful PDL stem

cell-mediated treatment for experimental periodontitis in swines. Autologous PDL stem cells, which were obtained from extracted root surface and cultured for 2 weeks, were seeded onto HA/TCP and then transplanted into swine periodontal bone defects. They found that transplanted PDL stem cells contributed to periodontal regeneration in vivo.

On the other hand, the possibility of periodontal regeneration without using scaffolds via cell sheet engineering technique has been studied. Cell sheet engineering is a novel approach to control cell surface adhesion by exploiting cell culture temperature and a surface grafted with a temperature-responsive polymer, PIPAAm (Okano et al. 1993). The cultured cells can attach and proliferate at 37°C on the grafted surface. However, when the temperature is lowered below 32°C, the cells detach spontaneously as a contiguous cell sheet. The cell sheet can avoid trypsinization and retain the ECM, cell-to-cell junctions, and differentiated features.

We have used this novel cell sheet technique in order to regenerate periodontal tissues and have reported that the PDL cell sheet when transplanted to surgically created dehiscence defects in dogs induced new bone, PDL and cementum formation (Akizuki et al. 2005). Hasegawa et al. (2005) applied human periodontal cell sheets in a periodontal defect model of athymic rats, and the histological findings with Azan stain showed that the outermost layer of curetted root dentin surfaces was positively stained blue only on the transplanted sides and the immature PDL fibres anchored obliquely onto this new layer. In our previous study (Flores et al. 2008), human periodontal cell sheets were cultured in the presence of osteogenic differentiation medium, harvested and multilayered onto a dentin block and then transplanted subcutaneously into the back of immunodeficient rats. Most of the dentin surfaces had newly immature cementum-like tissue formation and PDL fibres with perpendicular orientation resembling Sharpey's fibres inserted into the newly deposited cementum-like tissue.

To verify the regenerative potential of human periodontal cell sheet stimulated with osteogenic differentiation medium, we transplanted the cell sheet onto the defect on the rat mandible created by the King's method (King et al. 1997, King & Hughes 2001). The method allowed us to observe the regenerated human cementum and PDL clearly. The PDL induced by the human PDL cell sheets showed thicker layers than PDL of rat and stained strongly with aniline blue. These data suggested that the regenerated PDL cells were derived not from rat, but from human cells. We had shown that the human PDL cell sheets also induced the regeneration of cementum. Akizuki et al. (2005) already had shown the regeneration of periodontal tissue in dog model using the dog PDL cell sheet.

A novel point of this study was that the human PDL cell sheets were cultured with osteogenic differentiation medium containing ascorbic acid, β-glycerophosphate and dexamethasone. We found that the human PDL cell sheets cultured with osteogenic differentiation medium induced an obvious cementum layer containing acellular cementum as well as cellular cementum, and the amount of newly formed cementum was almost triplicate compared with control at 3 weeks after transplantation. The data conformed with the previous study that the human PDL cells cultured with osteogenic differentiation medium could induce mineralization (Nagatomo et al. 2006).

As shown in this experiment, PDL cells cultured with osteogenic differentiation medium could contribute to simultaneous regeneration of cementum and PDL. To obtain obvious cementum from PDL cells, addition of dexamethasone and β-glycerophosphate into the culture medium is needed to induce cell mineralization in vitro. But it was reported that also when the PDL cells were cultured with IL-11 instead of dexamethasone, the ALP activity was enhanced (Leon et al. 2007). This might be a new method for regeneration of an obvious cementum layer.

For clinical application of the cell sheet engineering, we are now conducting a study in a dog model by transplanting the PDL cell sheet cultured with osteogenic differentiation medium. Furthermore, we have gained approval to apply the PDL cell sheet obtained from human wisdom teeth in patients with periodontal disease. For this purpose, we should create safer and sterile PDL cell sheets at a cell-processing centre. These procedures will surely contribute to the development of regenerative medicine in dentistry.

In conclusion, our results suggest that after transplantation of human PDL cell sheet treated with osteogenic differentiation medium onto denuded root surfaces, cementum-like structure including Sharpey's fibres and PDL fibres were seen attached and regenerated in vivo.

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Clinical Relevance

Scientific rationale for the study: To investigate the possibility of periodontal regeneration, a novel tissue engineering approach is important for regeneration of the complicated periodontal tissue structure.
Principal findings: The human PDL cell sheet induced periodontal regeneration consisting of a cementum–PDL combined structure on the root surface.
Practical implications: The PDL cell sheet technique might be a possible therapeutic approach for reconstruction of destroyed periodontal tissues.