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Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs

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Objective: The ultimate goal of periodontal treatment is to regenerate the damaged periodontal support. Although periodontal ligament (PDL) cells are essential for periodontal regeneration, few studies have reported the transplantation of periodontal ligament cells to periodontal defects. We developed a new method to apply periodontal ligament cells as a sheet to the defect. The aim of this study was to investigate the periodontal healing after application of the periodontal ligament cell sheet in beagle dogs.

Methods: Autologous periodontal ligament cells were obtained from extracted premolars of each beagle dog. Periodontal ligament cell sheets were fabricated using a temperature-responsive cell culture dish. Dehiscence defects were surgically created on the buccal surface of the mesial roots of bilateral mandibular first molars of each dog. In the experimental group (five defects), periodontal ligament cell sheet with reinforced hyaluronic acid carrier was applied to the defect. Only the hyaluronic acid carrier was applied to the contralateral side as a control (five defects). Eight weeks after surgery, the animals were sacrificed and decalcified specimens were prepared. Healing of the periodontal defects was evaluated histologically and histometrically.

Results: No clinical signs of inflammation or recession of gingiva were observed in both experimental and control groups. In the experimental group, periodontal tissue healing with bone, periodontal ligament and cementum formation was observed in three out of five defects. In the control group, such periodontal tissue formation was not observed except in one defect. Histometric analysis revealed that the formation of new cementum in the experimental group was significantly higher than that in the control group.

Conclusion: The periodontal ligament cell sheet has a potential to regenerate periodontal tissue and may become a novel regenerative therapy.

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Periodontal regeneration is the ultimate goal of periodontal treatment. Several procedures, such as grafting, root surface conditioning, guided tissue regeneration and application of growth factors, are performed for periodontal regeneration. Among these methods, guided tissue regeneration is currently the most predictable regenerative procedure. Guided tissue regeneration is based on the hypothesis that only cells from the periodontal ligament have the potential to regenerate periodontal tissue (1–4). In guided tissue regeneration, the downgrowth of epithelial cells is prevented by placing membrane over the defect. Thus, it provides space for ingrowth of

cells from periodontal ligament (5). This treatment results in significant new attachment formation on previously exposed roots. However, the lack of adequate remaining periodontal ligament may affect the outcome of this method and it requires a relatively long healing period (6).

Although periodontal ligament cells are essential for periodontal regeneration, few studies have reported the grafting of periodontal ligament cells to periodontal defects (2, 7–9). If tissue engineering is applied, appropriate methods to manipulate adequate number of periodontal ligament cells without any damage are required (10).

A new temperature-responsive cell culture dish, which responds reversibly to temperature changes, was developed by Okano and coworkers (11). It was suggested that non-enzymatic cell harvest from the temperature-responsive cell culture dish surface is non-invasive, gentle and harmless to cells (12–14). Cell sheets fabricated from various types of cells including fibroblasts, endothelial cells, hepatocytes, macrophages and retinal pigmented epithelial cells have been reported (15, 16). Some of these cell sheets have already been applied *in vivo* and showed remarkable results (17–19). In the field of periodontics, we have succeeded in fabricating the human periodontal ligament cell sheets and reported that the sheets preserved the intact cell-to-cell attachment and the extracellular matrices such as type I collagen and fibronectin. It was also indicated that the sheet possesses the potential to regenerate the periodontal ligament in an athymic rat model (20).

The aim of this study was to investigate periodontal healing after application of periodontal ligament cell sheets to surgically prepared periodontal defects in beagle dogs.

Material and methods

Animals

Five healthy female 3-year-old beagle dogs, weighing between 9.8 and 11.2 kg, were used in this study. The protocol design and procedures were approved by the Animal Research Center of Tokyo Medical and Dental University.

Periodontal ligament cell culture

Supra- and subgingival deposits of the premolars and molars were removed with ultrasonic scaler and the teeth were wiped with gauze soaked in povidone-iodine. Under general anesthesia, mandibular premolars in each dog were extracted to obtain the periodontal ligament cells. The extracted teeth were immediately immersed in a sterilized plastic tube filled with culture medium (Dulbecco's modified Eagle's medium: Invitrogen Corp., Carlsbad, CA, USA), supplemented with 15% fetal bovine serum (Invitrogen) and 1% antibiotic-antimycotic solution (ABAM) (Invitrogen) at room temperature. The teeth were washed twice with the medium. To avoid contamination from gingival and apical tissue, only periodontal ligament tissue attached to the middle third of the root was removed carefully with the scalpel. The obtained tissue was suspended in 50 μl of type I atelocollagen neutral solution, cooled in 4°C (Kokencellgen, Koken, Tokyo, Japan). The periodontal ligament tissue in collagen solution was placed in the center of a 35 mm dish (Falcon, Becton Dickinson Labware, NJ, USA) and incubated for 1 h at 37°C in a fully humidified atmosphere of 5% CO_2–95% air. After the collagen solution solidified by incubation at 37°C, periodontal ligament tissue in the solidified collagen gels were overlaid with pre-warmed culture medium and incubated for another 2 weeks. When the periodontal ligament cells were confluent, the culture medium was discarded and rinsed with phosphate-buffered saline. The outgrown periodontal ligament cells inside the gels were released by digesting in 1 ml of 0.1% type 1 collagenase (Invitrogen) in phosphate-buffered saline at 37°C for 1 h. Then, 0.5 ml of 0.25% trypsin-EDTA (Invitrogen) was added and incubated for a further 5 min. The cells were collected by centrifugation at 100 ***g*** for 5 min and then the cells were inoculated in culture medium (passage 1) on a 10-cm culture dish (Falcon, Becton Dickinson Labware) and subcultured.

Periodontal ligament cell sheets

The periodontal ligament cell sheet was prepared using a temperature-responsive cell culture dish. The temperature-responsive cell culture method has been already described elsewhere (11, 20). Here the method is explained in brief. The temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), was covalently attached to solid surface by specific chemical immobilization reaction or electron beam irradiation. This surface shows similar hydrophobicity as a normal cell culture dish when the PIPAAm-coated dish is placed at 32°C or above, as PIPAAm chains are dehydrated on the surface. PIPAAm molecules rapidly hydrate and the surface becomes hydrophilic when the temperature is reduced below 32°C. Monolayers of confluent periodontal ligament cells on the PIPAAm-coated dish at 37°C are promoted to detach by reducing the medium temperature without any enzymatic digestion or divalent cation chelators. Periodontal ligament cells between the fourth and sixth passages were used in this study. A total of 1×10^5 cells were seeded on a temperature-responsive cell culture dish (35 mm diameter) in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and 1% ABAM and incubated for 2 weeks at 37°C in a fully humidified atmosphere of 5% CO_2–95% air. The culture medium was changed every 2 days. The culture surface of the temperature-responsive culture dish was covalently coated by the temperature-responsive polymer, PIPAAm and exhibits the same hydrophobicity as a normal cell culture dish at 37°C. When the cells reached confluence, the medium was replaced with culture medium supplemented with 50 μg/ml of ascorbic acid to produce extracellular matrix, type I collagen for further manipulation of the periodontal ligament cell sheet and incubated for another 2 weeks.

At the time of application of the periodontal ligament cell sheets,

hyaluronic acid sheets (5 × 5 mm, 1 mm thickness, average pore diameter 30 μm: Seikagaku Kogyo, Tokyo, Japan) were placed in the culture dishes as a reinforced carrier (hyaluronic acid carrier) and medium was discarded. Then the culture dishes were placed in a low temperature incubator (20°C) for 30 min. The surface of the temperature-responsive cell culture dish became hydrophilic and periodontal ligament cells were promptly detached from the surface preserving cell-to-cell interaction. The periodontal ligament cell sheet with the hyaluronic acid carrier was detached from the surface of the dish using tweezers and applied to the defects with the bottom side (cell sheet side) facing the root surface. The temperature-responsive polymer was covalently coated on the culture dish and never detached from the culture dish.

Surgical protocol

All surgical procedures were performed under general and local anesthesia in sterile conditions. Medetomidine hydrochloride (Domitor®, Orion Corporation, Espoo, Finland) was administered intramuscularly as a premedication (0.05 ml/kg). General anesthesia was achieved using intravenous sodium thiopental injection (0.005 ml/kg: RAVONAL®, Tanabe Inc., Osaka, Japan) and spontaneous breathing of the animals was maintained. Local anesthesia was performed with 2% lidocaine hydrochloride containing epinephrine at a concentration of 1 : 80,000 (Xylocaine®, Fujisawa Inc., Osaka, Japan).

Defect preparation and application of the periodontal ligament cell sheet

Dehiscence defects were surgically prepared on the mesial roots of bilateral mandibular first molars. An intracrevicular incision was made on the buccal aspect, from mesial of the second molar to the mesial of the first molar. Following elevation of the buccal mucoperiosteal flap, a square-shaped dehiscence defect, 5 mm × 5 mm (width × length), was prepared on the mesial root using round and fissure burs with sterile saline coolant (Fig. 1a). Root planing was performed using Gracey curettes and chisels. The cementum was completely removed. Periodontal ligament cell sheet with the hyaluronic acid carrier was then applied to the defect in the experimental group (Figs 1b and c). Only the hyaluronic acid carrier was applied to the contralateral defect, which served as a control. The periodontal ligament cell sheet with hyaluronic acid carrier or hyaluronic acid carrier alone was placed on the denuded root surface the same size as the defect. However, the grafted material was not sutured to the surrounding tissue. The mucoperiosteal flap was repositioned and sutured tightly at the cemento-enamel junction covering the grafted materials (periodontal ligament cell sheet with hyaluronic acid carrier) with Gore-Tex suture (Gore-Tex Suture® CV-5, W. L. Gore and Associates, Inc., Flagstaff, AZ, USA).

Postsurgical care

All dogs used in this experiment received antibiotics (penicillin G, 2×10^6 units) intramuscularly daily for 3 days after the surgery. The dogs were fed a soft diet (DKM®, Oriental Yeast Co. Ltd, Tokyo, Japan) for 2 weeks, in order to reduce potential mechanical interference. As for plaque control, 2% solution of chlorhexidine gluconate (Hibitane® concentrate, Sumitomo Inc., Osaka, Japan) was used four times a week for 8 weeks. Sutures were removed 2 weeks after the surgery.

Histological processing

Eight weeks after surgery, the animals were killed with an overdose of sodium

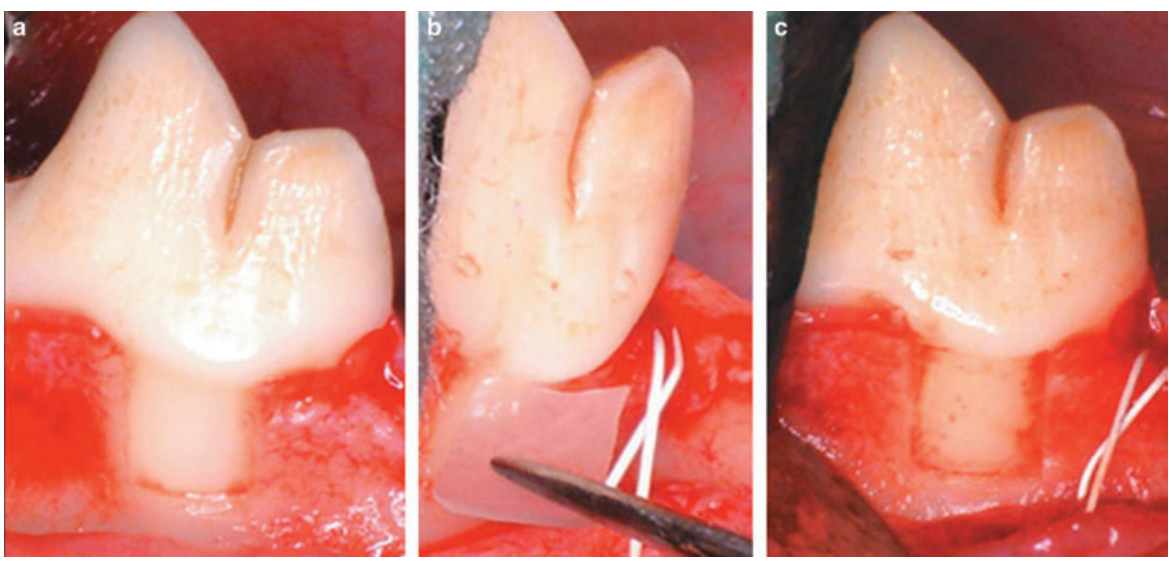

Fig. 1. (a) The dehiscence type of defect was formed on the buccal side of the mesial root of the mandibular first molar. (b) Periodontal ligament cell sheet with the reinforced hyaluronic acid carrier was applied to the defect with tweezers in the experimental defect. (c) Applied periodontal ligament cell sheet with the reinforced hyaluronic acid carrier.

thiopental. All defects in the experimental and control groups were dissected along with the surrounding soft and hard tissues. Block sections were fixed in 10% buffered formalin for 14 days. The specimens were demineralized with Plank-Rychro solution, trimmed, dehydrated, and embedded in paraffin (HISTPREP 568, Wako Pure Chemical Industries Ltd, Osaka, Japan). Serial sections of 6 μm thickness were prepared in the bucco-lingual plane. Sections were stained with hematoxylin–eosin or Masson trichrome-stain at intervals of 60 μm (HM360, Microme International GmbH, Heinberg, Germany).

Histological observation and histometric analysis

All specimens were analyzed histologically and histometrically under a light microscope (Eclipse E800, Nikon Inc., Tokyo, Japan) equipped with a computerized image system (Image-Pro Plus™ Version 3.0.1 for Power Mac, Media Cybernetics, L.P., Silver Spring, MD, USA). One examiner, who was well trained to observe the histology, performed the assessments and he was blinded to the nature of the specimens during the assessment. Instead of placing the notch at the most apical portion of the defect, we removed cementum inside the defect and used the most apical portion of denuded dentin surface as a reference point for analyses. Three histological sections of the central area were selected to measure parameters as follows:

1. defect height: distance between the apical extent of root planing and the cementoenamel junction;
2. new cementum formation: length of the newly formed cementum and cementum-like deposit on the denuded root surface;
3. new bone: length of the newly formed bone along the root;
4. connective tissue attachment: distance between the apical extent of the junctional epithelium and the apical extent of the root planing.

The mean value was calculated from these three specimens in each defect. Means and the deviations for each parameter were calculated for the experimental and control groups. Differences between two groups were analyzed using Student *t*-test for paired observations ($n = 5$).

Results

Clinical observations

Healing occurred uneventfully. Eight weeks after surgery, no visible adverse reactions, such as root exposure, infection, or suppuration, were observed. The initial inflammation immediately after surgery was comparable in both control and experimental groups. No intense inflammatory reaction was observed during the healing period.

Histological observations

Histological observation was performed for all defects in five animals (two defects each for five animals). No signs of acute inflammation were observed in any defects. Transplanted hyaluronic acid carriers had been absorbed completely in all 10 defects. In both the experimental and control groups, migration of the epithelium stopped at the most coronal part of the defects (Figs 2 and 3).

In the experimental group, periodontal tissue healing with bone, cementum and periodontal ligament formation was observed in three out of five defects. In one defect, newly formed periodontal tissue with bone was observed only at the coronal portion of the defect. In this defect, there was no continuity between the newly formed bone and the original bone at the apical portion of the defect (Fig. 2a). The down growth of the junctional epithelium stopped at the coronal portion of the defect and the newly formed bone, periodontal ligament and cementum were observed (Fig. 4a). Figures 5(a) and 6(a) show a higher magnification of the experimental defect, exhibiting the collagen fibers inserted perpendicularly into the newly formed bone and cementum. The root surface exhibited lacunae. The newly formed cementum was observed on the previously denuded dentin surface. No sign of continuous root absorption was observed on the root surface. Newly formed

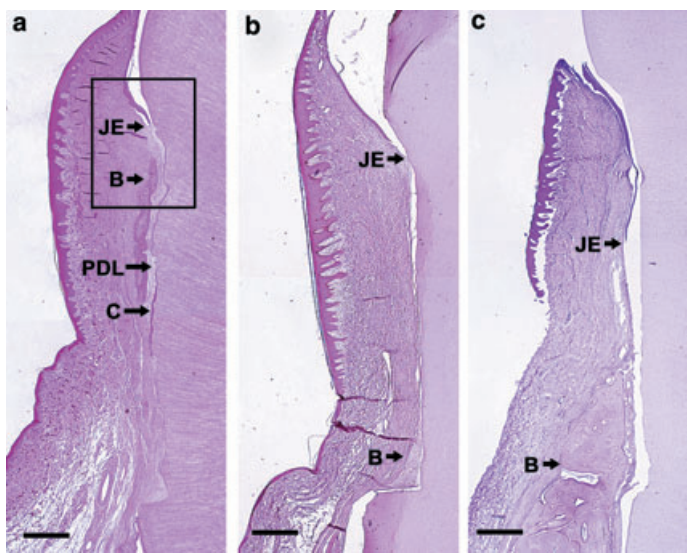

Fig. 2. Histology of the bucco-lingual section in the experimental defects (a–c). A variety of healing processes were observed among the different experimental defects. (a) Newly formed periodontal tissue with bone, cementum and periodontal ligament was observed at the coronal portion of the defect. (b) Thin newly formed bone at the apical side of the defect was observed in the defect with thin host bone plate. (c) Newly formed bone continuous with the host bone plate was observed at the apical portion of the defect in the defect with the thick host bone plate. JE, junctional epithelium; B, bone; PDL, periodontal ligament; C, cementum (bar, 500 μm; hematoxylin–eosin stain).

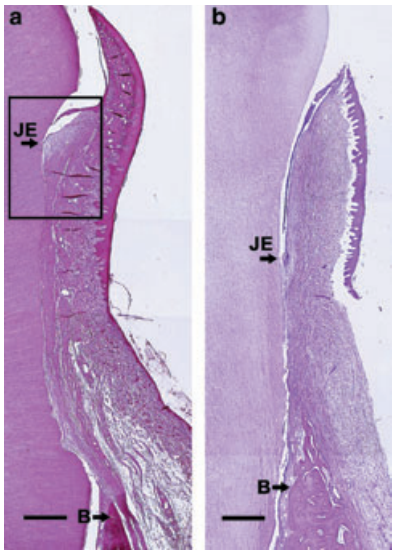

Fig. 3. Histology of the bucco-lingual section in the control defects (a and b). (a) Formation of the periodontal supportive tissue was not observed in the four cases of the control defect. (b) Newly formed bone continuous with the thick host bone plate was observed in one case at the apical portion of the defect. JE, junctional epithelium; B, bone (bar, 500 μm; hematoxylin–eosin stain).

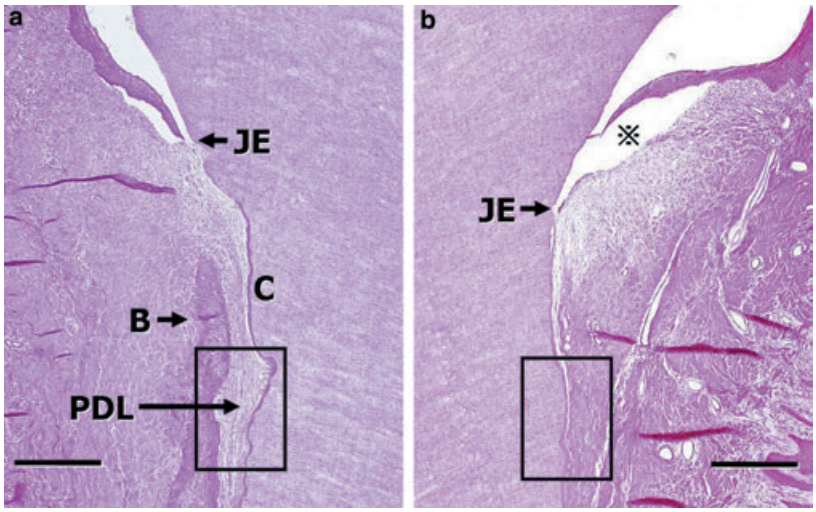

Fig. 4. Higher magnification of the framed area in Figs 2(a) and 3(a). Epithelium downgrowth towards the treated root surfaces was observed at the coronal part of the connective tissue attachment both in the experimental (a) and control defect (b). JE, junctional epithelium; B, bone; PDL, periodontal ligament; C, cementum (bar, 200 μm).

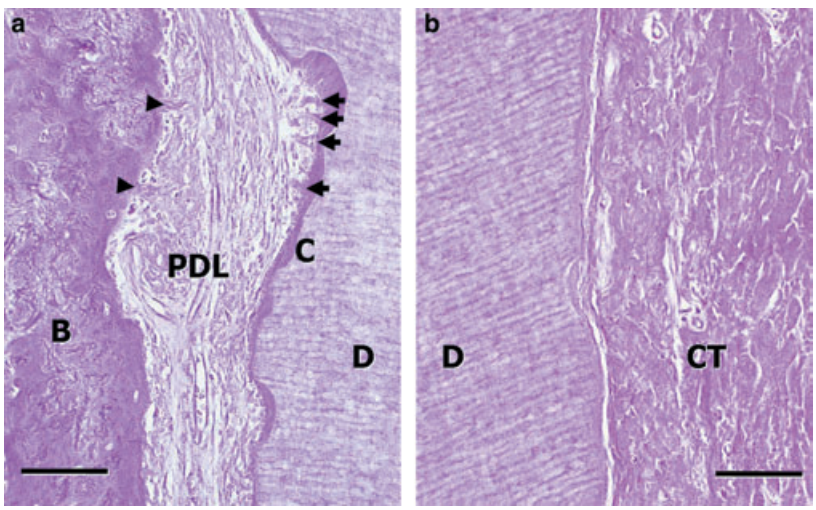

Fig. 5. Higher magnification of the framed area in Figs 4(a and b). (a) Newly formed bone, cementum and periodontal ligament were observed in the experimental defect. (b) This healing process was never observed in the control. Collagen fibers inserted perpendicularly into the newly formed bone (arrowhead) and cementum (arrow). B, bone; PDL, periodontal ligament; C, cementum; D, dentin; CT, connective tissue (bar, 50 μm; hematoxylin–eosin stain).

periodontal ligament with rich capillary vessels was observed between the newly formed bone and cementum (Figs 5a and 6a). In the specimens of another defect, thinner bone was newly formed at the apical side of the defect in the defect with thin host bone plate (Fig. 2b). In the specimens of another defect, new bone was formed continuously from the thick host bone plate (Fig. 2c). Signs of ankylosis were observed in some specimens. In the rest of the defects (two out of five), histological findings were similar as those of the control.

In the control group, neither bone nor cementum was formed in four defects (Fig. 3a). In these control specimens, connective tissue existed adjacent to the denuded root surface. Orientation of the connective tissue adjacent to the root surface was parallel to the root surface (Figs 5b and 6b).

There was only one defect showing new bone formation in the control defects (Fig. 3b). In this defect, the newly formed bone was conducted from the base of the thick host bone plate.

Histometrical analysis

Table 1 shows summary of histometric analyses of the periodontal defects after surgery. Histometric analysis revealed that the formation of new cementum in the experimental group was significantly higher than that in the control group. Histometric analysis also indicated that the new bone formation was much higher in the experimental group. However, significant difference between the experimental and the control groups was not observed.

Discussion

The objective of this study was to evaluate the periodontal healing after application of periodontal ligament cell sheets to surgically created periodontal defects in beagle dogs. Melcher first showed that only cells from the

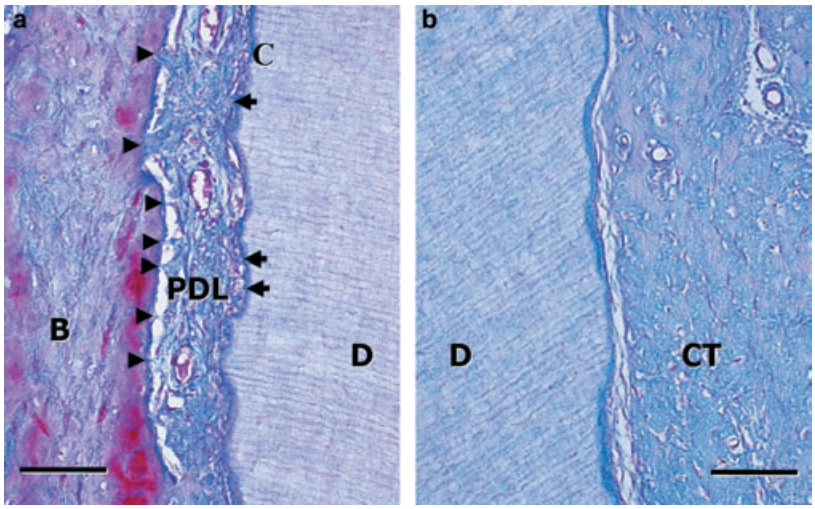

Fig. 6. Higher magnification of the experimental (a) and control defect (b). Well-vascularized periodontal ligament was observed between the newly formed bone and cementum. Collagen fibers inserted perpendicularly into the newly formed bone (arrowhead) and cementum (arrow). B, bone; PDL, periodontal ligament; C, cementum; D, dentin; CT, connective tissue (bar: 50 μm; Masson trichrome stain).

Table 1. Histometric analyses of the periodontal tissue formation after surgery (mean ± SD, mm; Student's t-test for paired observations)

	Periodontal ligament cell sheet ($n = 5$)	Control ($n = 5$)	p-value
Defect height	4.42 ± 0.25	4.39 ± 0.25	0.81
New bone	0.70 ± 0.79	0.26 ± 0.58	0.16
New cementum	2.42 ± 1.29	1.78 ± 0.93	0.045*
Connective tissue attachment	3.59 ± 0.36	3.50 ± 0.35	0.17

*$p < 0.05$ when compared to the control (experimental number).

periodontal ligament have the potential to regenerate the periodontal tissue (1). Moreover, it was recently reported that periodontal ligament contained stem cells that had the potential to generate cementum and periodontal ligament like tissue *in vivo* (21). We assume that grafting of periodontal ligament cells into periodontal defects would be a direct and efficient procedure for periodontal regeneration.

We prepared the periodontal ligament cell sheet from canine periodontal ligament. The single layered canine periodontal ligament cell sheet fabricated using the temperature-responsive culture dish was fragile by itself and required a scaffold for support. The scaffold material should be biocompatible and biodegradable so that it can be replaced by host tissues (22, 23). Hyaluronic acid, the source of the hyaluronic acid carrier, is a non-sulphated glycosaminoglycan consisting of a linear sequence of D-glucuronic acid and *N*-acetyl-D-glucosamine. It has been reported that hyaluronic acid is associated with the tissue repair process and plays a prominent role in the wound-healing process (24, 25). Thus, we used hyaluronic acid as a reinforced carrier in order to compensate for the fragility of the cell sheet. Our findings showed that the reinforced hyaluronic acid carrier was completely degraded without any signs of inflammation within 8 weeks after surgery.

In the experimental group, application of the periodontal ligament cell sheet with reinforced hyaluronic acid carrier showed newly formed bone and cementum. Periodontal ligament with Sharpey's fiber-like structure was also seen in the experimental group. However, application of hyaluronic acid carrier alone did not induce bone, cementum or periodontal ligament formation (control group). Newly formed periodontal tissues were observed in three defects of the experimental group. The cell sheets could serve as a resource of the cells necessary or suitable for regeneration. In this experiment, we could not obtain perfect regeneration as we expected. Rather the histology varied among the defects evaluated. The reason might be the stability of periodontal ligament cell sheet applied on the denuded root surface, i.e. incomplete attachment of cell sheet on the root surface may have impeded the expected healing with periodontal tissue after graft of the cell sheet. The cell sheet in the two animals with unexpected results in the experimental group might have detached from the treated root surface. To obtain favorable healing with the cell sheet, improvement of techniques that increase the stability of periodontal ligament cell sheet on the root surface should be considered.

Histometric analyses revealed that the formation of cementum in the experimental group was significantly higher than that in the control group. The various periodontal ligament cells and the extracellular matrices preserved in the periodontal ligament sheet might be effectively applied to regenerate the periodontium, as reported by Hasegawa *et al.* (20). Histometric analysis also indicated new bone formation was much higher in the experimental group; however, statistical significance was not obtained. This may be due to the small number of animals and the lack of stability of the periodontal ligament cell sheet. In order to clarify the efficacy of the treatment, further studies using a critical size of the defect, a larger number of animals and histometrical analysis have to be performed.

An important issue in this kind of cell transplantation study is the fate of the implanted cells. In this study, we did not perform the labeling of the implanted cells, thus the destiny of the cell sheet after the operation was not clear. However, we checked the viability of the periodontal ligament cell sheet *in vitro* and found that the periodontal ligament cells

succeeded to adhere to the normal culture dish and proliferate for another 2 weeks (data not shown). Currently, a study is going on to determine the fate of the implanted cells using green fluorescence protein-labeled cell sheet to investigate the fate of the grafted cell sheet.

Previous studies showed that multi-layered or even patterned viable tissue-like cell sheets could be fabricated and transplanted in tissue-regeneration models (26–28). In a future study, application of the multi-layered cell sheet to the periodontal defect could be a beneficial and potential procedure.

In conclusion, the periodontal ligament cell sheet applied in a dehiscence-type defect resulted in regeneration of periodontal tissues in beagle dogs. The periodontal ligament cell sheet has a potential to regenerate periodontal tissue and could be a novel regenerative therapy in periodontics.

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