(passage 1). The medium was completely changed every alternate day.

Subculturing

At subconfluency, about 3 days after the initial culture, cells were subcultured by trypsinization with 0·025% trypsin/0·1% EDTA (Clonetics, San Diego, CA, U.S.A.), neutralized using PBS with 10% FBS, centrifuged at 60 *g* for 10 min at 4 °C, then seeded at $2{\cdot}5 \times 10^4$ cells cm^{-2} in new collagen I-coated flasks (passage 2). During passages 2–4, weak trypsinization (0·025% trypsin/0·1% EDTA treatment for 5 min at 37 °C) was first performed on subconfluent cells to remove coexisting fibroblasts, which are easily peeled off. After neutralization and washing, the remaining adherent keratinocytes were cultured. After passage 5, normal trypsinization (0·025% trypsin/0·1% EDTA treatment for 10 min at 37 °C) was performed, and peeled keratinocytes were seeded at $2{\cdot}5 \times 10^4$ cells cm^{-2} in new collagen I-coated flasks.

Immunofluorescence staining

For analysing keratin 14, cells obtained by trypsinization were cultured on collagen type I-coated slide glasses (Iwaki). At confluency, adherent cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Blocking was performed using PBS with 10% FBS for 60 min at room temperature. Firstly, an antimouse keratin 14 monoclonal antibody (1 : 100, a kind gift from Professor E. B. Lane, University of Dundee School of Life Sciences, Dundee, U.K.) was reacted for 60 min at room temperature. After washing with PBS, antimouse IgG secondary antibody (1 : 1000, Alexa 594 conjugated; Molecular Probes, Eugene, OR, U.S.A.) was reacted for 30 min at 4 °C. At the same time, we also employed Hoechst 33342 dye (0·2 μg mL^{-1}; Sigma, St Louis, MO, U.S.A.) nuclear staining in order to permit semiquantitative calculation of the frequency of keratin 14-expressing cells among all the cultured cells in the same field of vision.

For detecting keratin 1, cultured keratinocytes (passage 30) on the slide glass were differentiated by EGF and CT depletion and high calcium concentration (2 mmol L^{-1}) for 48 h. Then an antimouse keratin 1 antibody (1 : 500; Covance, Berkeley, CA, U.S.A.) and antirabbit IgG secondary antibody (1 : 1000, Alexa 594 conjugated; Molecular Probes) with Hoechst 33342 dye were reacted.

Results

In the suspension of isolated epidermal cells, dye exclusion tests indicated a viability of more than 80%. The culturing procedures described above enabled us to purify the basal epidermal keratinocytes after passage 5 and to extend the cultures up to passage 70. Phase-contrast microscopy revealed that the confluent keratinocytes formed a pavement-like monolayer. The population doubling time was about 48 h during passages 5–15. The microscopic appearance of the cells remained almost the same throughout the 70 passages (Fig. 1A–C). We also analysed the ploidy of these keratinocytes using propidium iodide. We found that passage 14 keratinocytes all had normal DNA content, about half of passage 20 keratinocytes had double DNA content, and

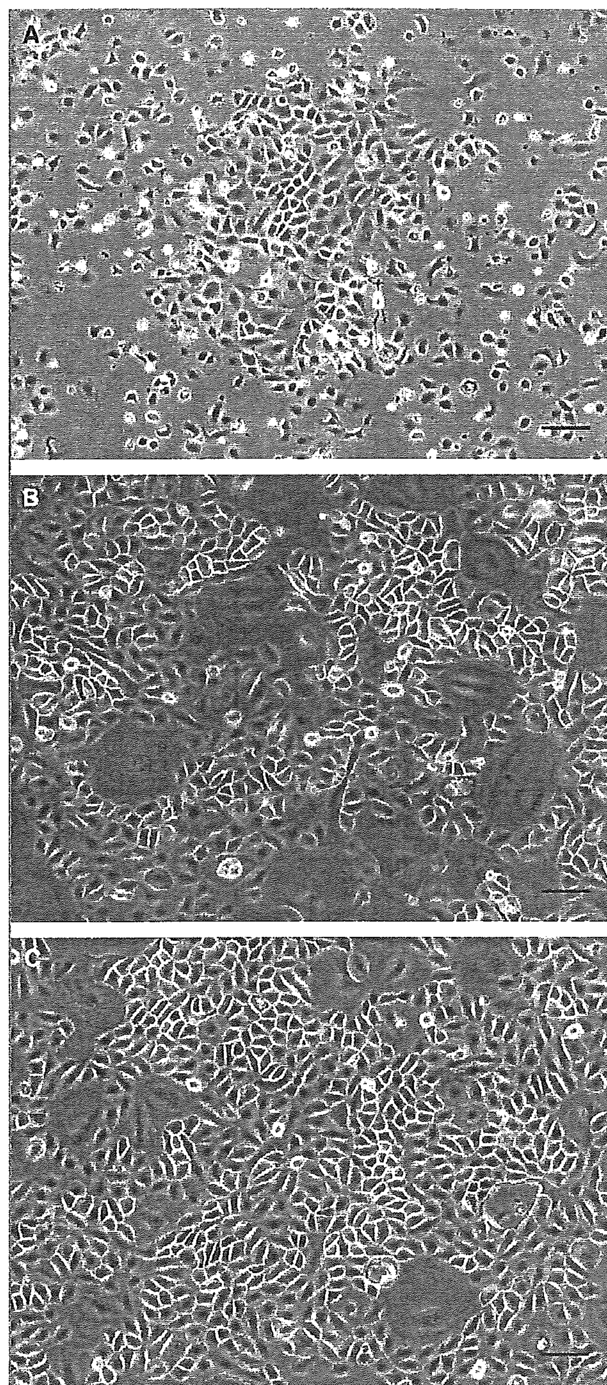

Fig 1. Phase-contrast microscopy of the cultures of murine keratinocytes. Keratinocytes at passages 1 (A), 15 (B) and 70 (C) formed pavement-like monolayers. Scale bar = 100 μm.

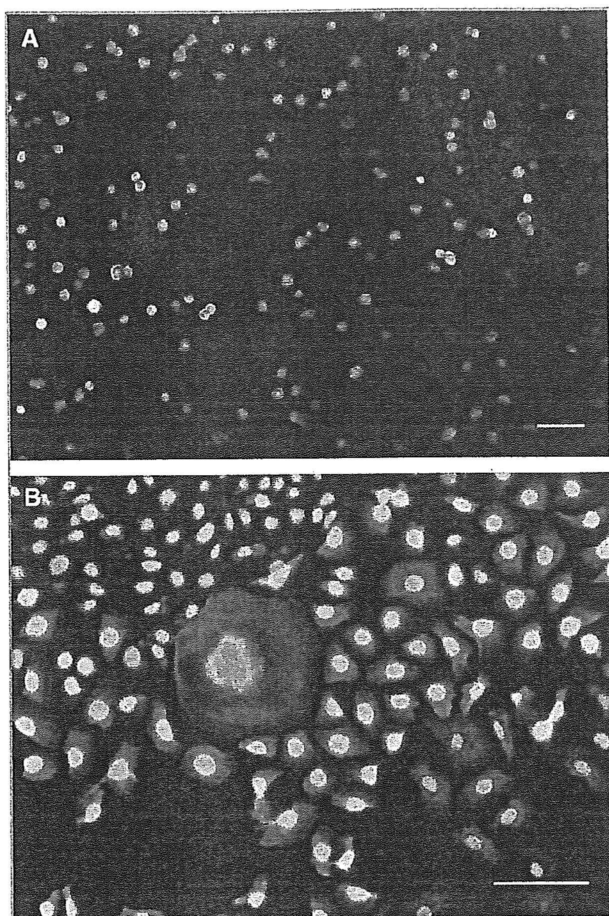

Fig 2. Immunofluorescence studies using an antimouse keratin 14 antibody revealed that the cytoplasm of most freshly isolated (A) and cultured keratinocytes (B, passage 12) stained positively. Scale bar = 100 μm.

almost all of passage 35 and 67 keratinocytes had double DNA content (data not shown).

The expression of keratin 14, which is the basal keratinocyte marker, was strongly positive in the cytoplasm of the cells at passages 15, 30 and 45 (over 95% of all nucleated cells) when compared with the primary scraped cell suspensions (60–70%) and passage 1 (70–80%) (Fig. 2A,B). The differentiated cells tended to show weak staining for keratin 14 (data not shown). These results suggest that we succeeded in obtaining highly purified mouse keratinocytes with the basal keratinocyte phenotype.

We also demonstrated the expression of keratin 1, which is a marker of differentiated keratinocytes, in the cytoplasm of the cells at passage 15 on differentiation (Fig. 3). Undifferentiated cells did not stain for keratin 1 (data not shown). This result suggests that our cultured murine keratinocytes can differentiate under suitable conditions (e.g. high calcium). We further established long-term cultures of epidermal keratinocytes from adult mouse ear skin using the same technique (not shown). Moreover, these cells could be cryopreserved; after thawing, at least 80% of the cells were viable and could be subcultured several times.

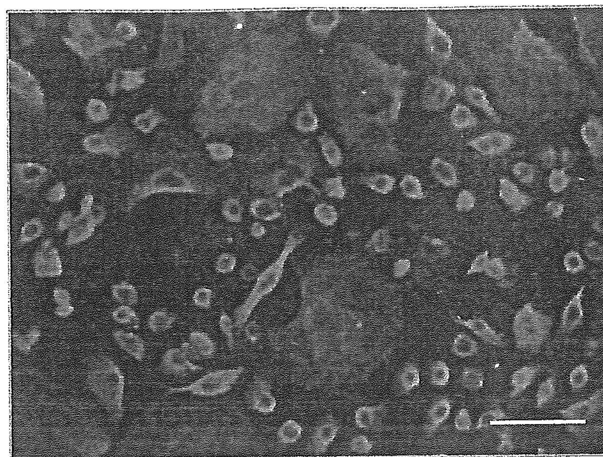

Fig 3. Immunofluorescence studies using an antimouse keratin 1 antibody revealed that the differentiated keratinocytes stained positively in the cytoplasm. Scale bar = 100 μm.

Discussion

In this paper, in order to obtain adult mouse epidermal keratinocytes more easily without FBS, feeder layers, FCM or bovine pituitary extract, we adapted the method of Caldelari *et al.*[3] with minor modifications, after comparing several commercial media and coating dishes. We used adult mouse dorsal skin instead of embryonic skin. We used type I collagen-coated dishes instead of uncoated dishes. We used treatment with trypsin alone for 2 h, while they used trypsin after treatment with dispase for 7 h. Weak and normal trypsinizations were performed to remove coexisting fibroblasts and to grow the remaining keratinocytes. These procedures enabled us to purify keratin 14-positive basal keratinocytes. Our technique was very simple: we used the usual incubator at 5% CO_2 and 37 °C. No special apparatus was needed. Mutation due to DNA ploidy was not seen for at least up to 14 passages of keratinocytes.

The effects of EGF and CT on keratinocyte cultures have been previously well investigated.[7–10] EGF is now frequently used as a supplement in many and various media; however, CT is rarely used to supplement current media. CT causes diarrhoea in the human intestine by elevating intracellular cyclic AMP levels; it is therefore thought to be toxic. However, we observed that adult mouse keratinocytes could not be cultured without CT, suggesting that cyclic AMP plays a crucial role in cellular proliferation. Hirobe utilized dibutyryl cyclic AMP for mouse epidermal melanoblasts in a serum-free medium and suggested that this chemical is required for melanoblast proliferation.[11] Although our protocols require neither feeder layers nor FCM, collagen I-coated flasks are indispensable; when using ordinary polystyrene culture flasks we could not establish either primary cultures or subcultures.

In summary, we report the establishment of long-term adult mouse keratinocyte cultures using ordinary techniques, media and growth factors. We obtained larger numbers of keratinocytes (over 1×10^9 cells per one adult mouse after passage 5)

from dorsal skin than previously reported. The cells obtained were morphologically normal, and the proliferative keratinocytes showed a highly basal phenotype. Our protocols should be very helpful for investigating biological functions of murine epidermal keratinocytes or for handling epidermal cells in rebuilding entire skin and improving skin grafting.

Acknowledgments

This work was supported by the Health Science Research Grant from the Japanese Ministry of Health Labour and Welfare.

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TISSUE ENGINEERING
Volume 11, Number 3/4, 2005
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Human Periodontal Ligament Cell Sheets Can Regenerate Periodontal Ligament Tissue in an Athymic Rat Model

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ABSTRACT

Conventional periodontal regeneration methods remain insufficient to attain complete and reliable clinical regeneration of periodontal tissues. We have developed a new method of cell transplantation using cell sheet engineering and have applied it to this problem. The purpose of this study was to investigate the characteristics of human periodontal ligament (HPDL) cell sheets retrieved from culture on unique temperature-responsive culture dishes, and to examine whether these cell sheets can regenerate periodontal tissues. The HPDL cell sheets were examined histologically and biochemically, and also were transplanted into a mesial dehiscence model in athymic rats. HPDL cells were harvested from culture dishes as a contiguous cell sheet with abundant extracellular matrix and retained intact integrins that are susceptible to trypsin–EDTA treatment. In the animal study, periodontal ligament-like tissues that include an acellular cementum-like layer and fibrils anchoring into this layer were identified in all the athymic rats transplanted with HPDL cell sheets. This fibril anchoring highly resembles native periodontal ligament fibers; such regeneration was not observed in nontransplanted controls. These results suggest that this technique, based on the concept of cell sheet engineering, can be useful for periodontal tissue regeneration.

INTRODUCTION

PERIODONTAL TISSUE functions primarily to attach the teeth to the bone tissue of the jaws and also to establish homeostasis between tissues around the teeth that undergo certain changes with age and is, in addition, subjected to morphologic or functional alterations related to the oral environment.[1] The clinical features of periodontitis are inflammation from gingival pocket areas, and subsequent destruction of periodontal tissues especially the periodontal ligament and alveolar bone. For years, a variety of surgical procedures have been advocated in order to ensure an outcome in which tissue regeneration can occur. However, regardless of the procedures used, the epithelial tissues always proliferate into the defect at a faster rate than the underlying mesenchymal tissues,[2] so that long junctional epithelium attachment to the dentin root surface is established and the original form and functions of periodontal tissues cannot be restored. To elucidate the process of periodontal regeneration, several studies have been performed[3–5] with results strongly suggesting that exclusion of epithelial and gingival connective tissue cells from the healing area might allow cells derived from periodontal ligament to repopulate the root surface and re-

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generate periodontal tissues.[6] This concept has provided the basis for clinical application of the treatment principle termed "guided tissue regeneration" (GTR).

To create an environment suitable for selective cell repopulation and subsequent matrix synthesis, dentin root surfaces have been conditioned with various agents.[7] Applications to root surfaces of some growth and differentiation factors have also been investigated.[8,9] However, further research is needed to reveal the full functions of these factors and the predictability of this procedure. Various grafting materials have also been used and promoted for the filling of defect volumes,[10,11] but are still insufficient to induce periodontal regeneration because their ability to induce production of new cementum and periodontal ligament is limited. For decades, although a number of animal experiments and clinical trials using the regenerative procedures described above have been performed, all evidence supports their inability to attain complete and ideal regeneration of periodontal tissue.[12] One reason may be that all treatments depend on cell proliferation from local, residual tissue. However, this proliferation tends to be influenced by defect morphology and by the amount of residual periodontal ligament tissue, so that repopulation of cells into the defect may not occur completely. If cells that have the potential to create new periodontal attachment apparatus are exploited to rapidly establish effective and sustained contact with the root dentin surface, prevention of epithelial downgrowth into the defect and reconstruction of periodontal tissue without reliance on cell migration from residual periodontal tissue are expected. The cells derived from periodontal ligament tissue (periodontal ligament [PDL] cells) would be an appropriate cell source for periodontal regeneration by cell transplantation for several compelling reasons. First, evidence that PDL cells play an important role in promoting periodontal regeneration is accumulating.[13,14] Actually, cultured PDL cells applied in various scaffolds or in suspension have been shown to be able to induce a new periodontal tissue apparatus on root dentin surfaces and dental implants.[15–18] However, these results show not only the potential of significant periodontal regeneration but also possible unpredictable adverse outcomes, such as ankylosis. Periodontal tissue is highly complex, so that it is inevitable that such direct cell transplantation might require more sophisticated arrangements in the tissue defect.

In the present study, we show an innovative method to improve such cell transplantation. We have previously reported new methods to control cell surface adhesion by exploiting cell culture temperature and a surface-grafted temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm).[19,20] On the grafted surface, various types of cells adhere, spread, and proliferate similarly to those in culture on ungrafted tissue culture polystyrene (TCPS) surfaces at 37°C. However, when culture temperature is lowered below the lower critical solution temperature, cells detach spontaneously to float free as confluent, viable cell sheets because of the change in culture surface aspect from hydrophobic to hydrophilic. These intact contiguous cell sheets recovered only by low-temperature treatment can avoid trypsinization and retain associated extracellular matrix (ECM), cell–cell junctions, and highly differentiated functions compared with similar cells recovered by proteolytic treatment.[21–24] It can be hypothesized that PDL cell sheets transplanted onto denuded root dentin surfaces may generate a new periodontal attachment apparatus. Here, we report an application of this novel cell-harvesting strategy for periodontal regeneration therapy.

MATERIALS AND METHODS

Preparation of cell culture dishes grafted with a thermo-responsive polymer

Specific procedures for the preparation of PIPAAm-grafted cell culture dishes are described elsewhere.[22] Briefly, *N*-isopropylacrylamide (IPAAm; kindly provided by Kohjin, Tokyo, Japan) monomer in 2-propanol solution was spread onto TCPS dishes (Falcon 3001; BD Biosciences Discovery Labware, Franklin Lakes, NJ). The dishes were then subjected to irradiation with an area beam electron processing system (Nissin High Voltage, Kyoto, Japan). The PIPAAm-grafted dishes were rinsed with cold distilled water to remove nongrafted IPAAm and dried in nitrogen gas. Untreated TCPS dishes were used as control surfaces. The PIPAAm-grafted dishes were gas sterilized by ethylene oxide before use in cell culture.

Isolation and culture of human periodontal ligament cells

In accordance with an institutional review board-approved protocol, human PDL (HPDL) cells were isolated from the third molar tooth of a 20-year-old male patient with no periodontitis; the molar was extracted for orthodontic reasons. After extraction, the middle root section of periodontal ligament tissue was scraped away with a scalpel blade. The harvested tissue was placed onto culture dishes and kept in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Culture was maintained at 37°C in a humidified atmosphere at 5% CO_2. Cells migrated out of the tissue and covered the bottom of the culture plate. Cells from passages 3 to 5 were used for all subsequent experiments.

Recovery of HPDL cell sheets from temperature-responsive culture dishes

HPDL cells were plated on PIPAAm-grafted culture dishes at a density of 1×10^5 cells/35-mm dish and cul-

tured at 37°C supplemented with 10% FBS, ascorbic acid phosphate ester (50 μg/mL; Wako, Tokyo, Japan), and antibiotics. After 3 weeks of culture at 37°C, these dishes were transferred to another incubator set at 20°C and cell sheet detachment was monitored under a phase-contrast microscope. After a 2-h incubation at 20°C, all HPDL cells spontaneously detached from temperature-responsive culture dishes and floated in the culture medium. Harvested HPDL cell sheets were fixed with 10% neutral formalin solution. The specimens were then embedded in paraffin blocks and serial sections were cut at a thickness of 5 μm and subjected to hematoxylin and eosin (H&E) or azan staining.

Fluorescence microscopy

Detaching cell sheets were fixed with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) at room temperature for 20 min after short-term incubation at 20°C. They were washed with PBS and blocked with 0.1% bovine serum albumin (BSA) in PBS for 90 min. Samples were then reacted with a rabbit anti-collagen type I polyclonal antibody (diluted 1:10,000; LSL, Tokyo, Japan) overnight at 4°C. This polyclonal antibody was raised against porcine type I collagen and recognizes porcine, canine, bovine, and human type I collagens. Weak cross-reactivity with rat and mouse type I collagen was observed, but no cross-reactivity was detected with types II, III, IV, V, and VI collagen according to the developer's documentation. After three washes with 0.1% BSA in PBS, samples were incubated for 1 h with a 1:1000 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody and again washed three times with PBS. To examine F-actin, cells were stained with rhodamine-conjugated phalloidin. For nuclear staining, the cells were costained with a DNA-binding dye (Hoechst 33258). The stained dishes were observed under a microscope with fluorescence equipment (ET300; Nikon, Tokyo, Japan). Fixed cell sheets were also stained without the primary or secondary antibodies and used as negative control. In the negative control, no fluorescence was detected under the conditions used in this study.

Immunoblotting

HPDL cells were harvested from culture by one of the following three methods: physical scraping with a rubber blade, low-temperature treatment (cell sheet), and trypsin–EDTA treatment. Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then immunoblotting. After removal of culture medium, cells were rinsed three times with prewarmed PBS. In physical scraping, cell layers were harvested from the surface with a rubber

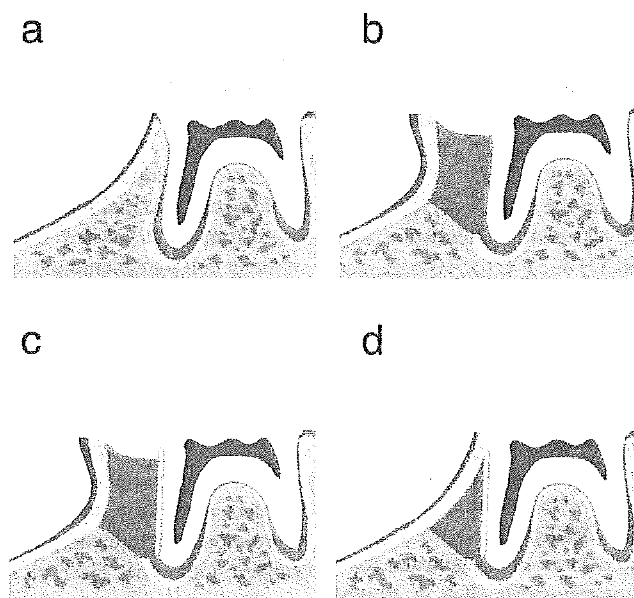

FIG. 1. Schematic illustration of artificial periodontal tissue defects and cell sheet transplantation. A vertical incision was made on the mesiopalatal side of the maxillary first molar tooth on both sides (**a**). Mucoperiosteal flaps were elevated to expose the mesial alveolar bone and the bone was removed at the mesial side of the mesial root. The root surface was curetted to remove all periodontal ligament and cementum (**b**). Harvested HPDL cell sheets were then transplanted to denuded root dentin surfaces (**c**). Finally, the mucoperiosteal flaps were repositioned and sutured (**d**). Right sides were used as cell sheet-transplanted sides, and left sides were control without cell transplantation.

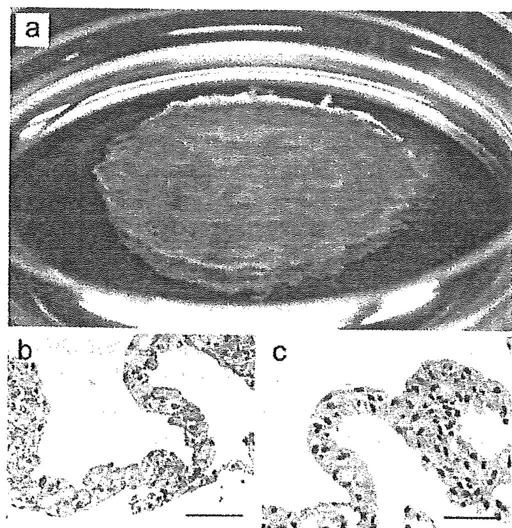

FIG. 2. Harvested HPDL cell sheets. All HPDL cells were detached as a single contiguous cell sheet from temperature-responsive culture dishes and floated up into the culture medium after low-temperature treatment (**a**). Histological sections of harvested HPDL cell sheets stained with azan (**b**) and H&E (**c**). Scale bars: 50 μm.

blade in lysis buffer (300 μL, 20 mM Tris-buffered saline [pH 7.4] containing 1% SDS, 2 M urea, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). In the low-temperature treatment, HPDL cells were allowed to detach from temperature-responsive dishes in 1 mL of PBS containing a protease inhibitor cocktail (Wako) after incubation at 20°C for 90 min. After centrifugation at 1000 $\times$ g for 10 min, cells were collected as pellets. The pellet was dissolved in lysis buffer. In the trypsin–EDTA treatment, HPDL cells were harvested after incubation with 0.25% trypsin–EDTA (GIBCO) at 37°C for 10 min. Harvested cells were washed three times with PBS containing protease inhibitors (0.25% soybean type II-S trypsin inhibitor and 1 mM PMSF) and lysed in lysis buffer. These whole cell lysates were electrophoresed on a 7.5% polyacrylamide gel with a 4.5% stacking gel on top in a discontinuous buffer system. Proteins resolved on the polyacrylamide gel were electrophoretically transferred to a hydrophilic poly(vinylidene difluoride) membrane (Immobilon-P, 80 V for 3 h; Millipore, Bedford, MA). The membrane was incubated at room temperature for 1 h in a blocking solution comprising 5% BSA in 20 mM Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBS–Tween), and then probed with either rabbit anti-bovine fibronectin polyclonal antibody (diluted 1:1000 in TBS–Tween containing 1% BSA; Chemicon International, Temecula, CA), rabbit anti-human β_1 integrin polyclonal antibody (diluted 1:100; Chemicon International), or rabbit anti-human α_5 integrin polyclonal antibody (diluted 1:100; Chemicon International) at room temperature for 1 h. After extensive washing with TBS–Tween, the membrane was further incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody at room temperature for 1 h, and the positive bands were detected by product chemiluminescence from peroxidase reactions, using the ECL system (Amersham, Buckinghamshire, UK).

Preparation of artificial periodontal tissue defects and cell sheet transplantation

All animal procedures complied with the guidelines provided by the Institutional Animal Care Committee (Tokyo Women's Medical University, Tokyo, Japan). The investigation was carried out with six female athymic rats (Fischer 344, 12 weeks old, weighing 190–210 g; Charles River Japan, Yokohama, Japan). Under anesthesia induced by intraperitoneal injection of 8% chloral hydrate (0.1 mL/100 g; Wako), a vertical incision was made on the mesiopalatal side of the maxillary first molar tooth on both sides (bilaterally). Subsequently, mucoperiosteal flaps were elevated to expose the mesial alveolar bone. After manually removing the bone to a level of 2 mm apical from the marginal bone crest and 2 mm palatobuccal at the mesial side of the mesial root, using a chisel (Hu-Friedy, Leimen, Germany; modified), the root surface was carefully curetted in order to remove all periodontal ligament and cementum. Cultured HPDL cell sheets harvested by low-temperature treatment were transferred to denuded root dentin surfaces by means of a dental explorer (Fig. 1). The mucoperiosteal flaps were carefully repositioned, and immediately sutured with previously placed 7-0 nylon sutures so that original positions of the cell sheets were maintained. Right sides were used as cell sheet-transplanted sides, and left sides acted as controls without cell transplantation. Sutures were removed on day 6 after surgery. Three of six animals were killed 1 week after surgery and the rest were killed 4 weeks postsurgery. A part of the mandible beneath the defects was excised and fixed immediately in 10% neutral formalin solution for 3 days. Demineralization was conducted by immersion in 4% EDTA (pH 7.4) aqueous solution for 5 weeks at 4°C with constant stirring. The specimens were embedded in paraffin and serial mesiodistal 5-μm sections were mounted on clean glass slides. These sections were stained with either H&E or azan.

RESULTS

HPDL cells adhered, spread, proliferated, and reached confluency between days 8 and 10 on PIPAAm-grafted dishes, similar to that observed on ungrafted TCPS dishes

at 37°C. After HPDL cells were incubated at 20°C for 2 h, all cells spontaneously detached from PIPAAm-grafted dishes, whereas these cells remained adherent to ungrafted TCPS dishes. All HPDL cells were completely detached as a single contiguous cell sheet and floated up into the culture medium (Fig. 2a). These detached cell sheets shrink because of cell-induced lateral contraction forces sustained from culture. During culture in medium supplemented with ascorbic acid, HPDL cells deposited abundant ECM that stained blue by azan (Fig. 2b). Immunocytochemistry revealed that detaching cell sheets had abundant type I collagen (Fig. 3c). F-actin was well developed in the cytoplasm (Fig. 3d). Remnants of either cells or type I collagen were not observed on culture dishes after cell sheet detachment.

Harvested HPDL cell sheets were then biochemically examined (Fig. 4). Cultured HPDL cells were recovered from temperature-responsive dishes by one of the three methods described: physical scraping with a rubber blade, low-temperature treatment, or trypsin–EDTA treatment. All cells were lysed and subjected to immunoblot analysis (Fig. 4). α_5 Integrin was degraded by trypsin digestion, whereas such degradation was not observed with low-temperature treatment (Fig. 4a). β_1 Integrin was detected as two bands after physical scraping as reported previously.[25] After trypsin digestion, a band with lower mobility disappeared (Fig. 4b), whereas the other band with higher mobility was unchanged. By contrast, after low-temperature treatment two bands were obtained in similar amounts compared with those after physical scraping. These results suggest that trypsin digestion damaged β_1 integrin molecular structure and might hamper cell adhesion. Importantly, such destructive effects were not observed with the cell sheet harvest by reducing temperature in the absence of proteolytic enzymes. After trypsin–EDTA treatment, fibronectin was faintly detected (Fig. 4c), whereas an amount equal to that detected by physical scraping was also detected after low-temperature treatment, results similar to those reported previously using bovine aortic endothelial cells.[21]

These harvested HPDL cell sheets were transplanted into an athymic rat mesial dehiscence model (Fig. 1). Right sides were cell sheet-transplanted sides, and left sides were sham surgery control sites without transplan-

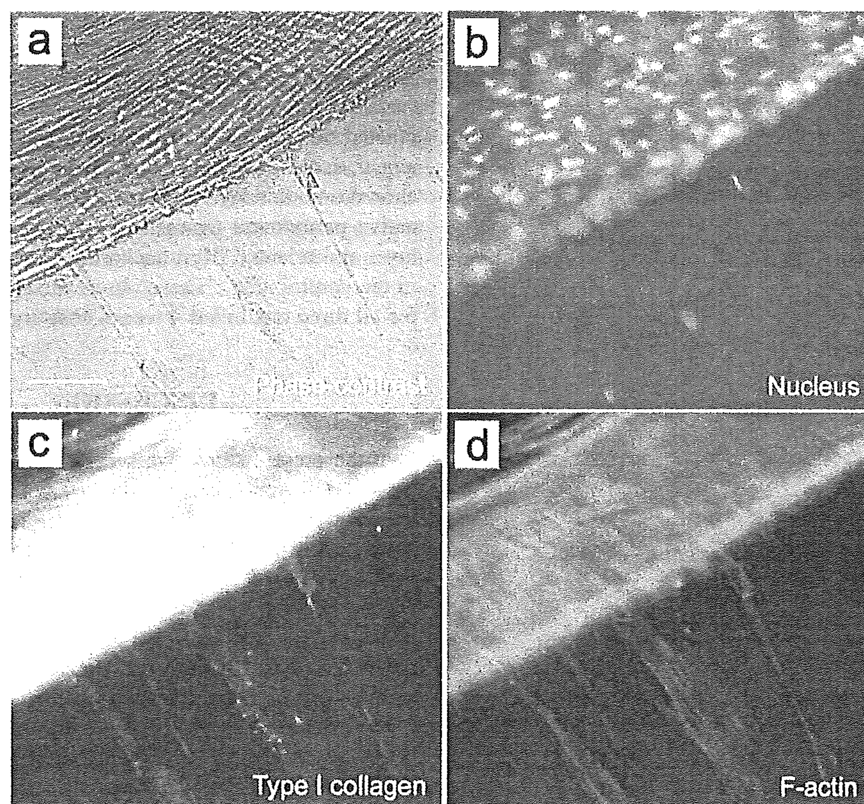

FIG. 3. Detaching HPDL cell sheets. HPDL cell sheets were detached from the periphery of temperature-responsive culture dishes after reducing the temperature. Phase-contrast microscopy (**a**) and fluorescence microscopy after staining of nuclei (**b**), type I collagen (**c**), and F-actin (**d**). All four images were obtained from the same view field. Scale bar: 50 μm.

tation. Throughout the experimental time course, healing of the defects in both transplanted and nontransplanted control sides was completed uneventfully. At 1 week postsurgery, new connective tissue attachment binding to root dentin surfaces and gingival tissues was not observed in either side (Fig. 5). Many inflammatory cells were observed in the gingival connective tissue of both sides. Some putative transplanted cells were observed to attach onto root dentin surfaces (arrowheads in Fig. 5d). Such cell attachment onto root dentin surfaces was not observed in the nontransplanted control sides (Fig. 5c). Gingival tissues of the control sides were detached from root dentin surfaces in histological sections (Fig. 5a and c). Such detachment was observed with all the control sides, but not with any of the transplanted sides. It is plausible that gingival tissue detachment in control sides was an artifact produced during histological preparation, suggesting weak binding between gingival connective tissue and dentin surfaces. At the marginal portion of alveolar bone, new immature bone formation was observed in both sides. Previously mentioned observations were ascertained in all rats of each group killed 1 week postsurgery.

At 4 weeks postsurgery, extensive new bone formation leading to ankylosis with partial root resorption was observed in all control sides (Fig. 6a), consistent with similar observations of dentin surfaces directly contacting bones (e.g., bone graft and tooth replantation).[26] In the corona of the regenerated bone tissue, curetted root surfaces were covered by gingival connective tissue. Azan staining revealed that gingival connective fibers adjacent to the root surfaces were mature but their orientations were parallel to root surfaces (Fig. 6c), implying weak interactions between dentin surfaces and the fibers. As observed 1 week after surgery, gingival tissues were detached from the dentin surfaces in the control sides. In contrast, newly formed immature fibers that obliquely anchored dentin surfaces were a predominant feature in the transplanted sides of all the rats (Fig. 6d), whereas such an observation was not made in any control sides. Amounts of new bone formation were reduced in transplanted sides over that seen in control sides. Ankylosis was observed in only a few histological sections among the semiserial sections made from the entire tissues of all three transplanted sides. Downgrowth of junctional epithelium was observed to a slight degree in both groups. Interestingly, the outermost layer of curetted root dentin surfaces was positively stained blue only in the transplanted sides (Fig. 6d). In the regenerated periodontal ligament tissues, immature thin fibers were obliquely anchored onto this layer. This orientation highly resembles native periodontal ligament fibers. Such a blue-stained layer and oriented fibril anchoring were never observed in the control sides. These observations were consistent for all three rats killed 4 weeks postsurgery.

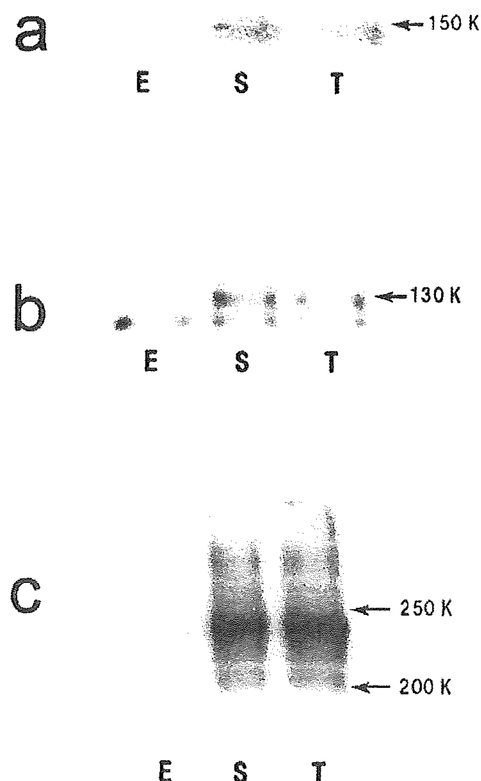

FIG. 4. Immunoblotting of HPDL cells recovered from temperature-responsive culture dishes. Cell lysates obtained by trypsin–EDTA treatment (lane *E*), physical scraping with rubber blade (lane *S*), or low-temperature treatment (lane *T*) were subjected to Immunoblotting with α_5 integrin (**a**), β_1 integrin (**b**), and fibronectin (**c**).

DISCUSSION

In the present study, we successfully regenerated periodontal ligament tissues, using a novel HPDL cell sheet transplantation method. This work represents a starting point for further studies that will explore the time line of regenerative events in more detail. Because the present experiments used a xenogeneic model utilizing cultured human primary cells in athymic rats, the grafted rats were killed 1 or 4 weeks after cell sheet transplantation. For longer term follow-up, autologous or congenic models are now indicated.

Although no definitive studies on the origin of cells repopulating wounded periodontal tissue, especially periodontal ligament and cementum, have been undertaken, the importance of periodontal ligament in time of regeneration is perhaps best demonstrated by the considerable interest that has been generated after the suggestion of

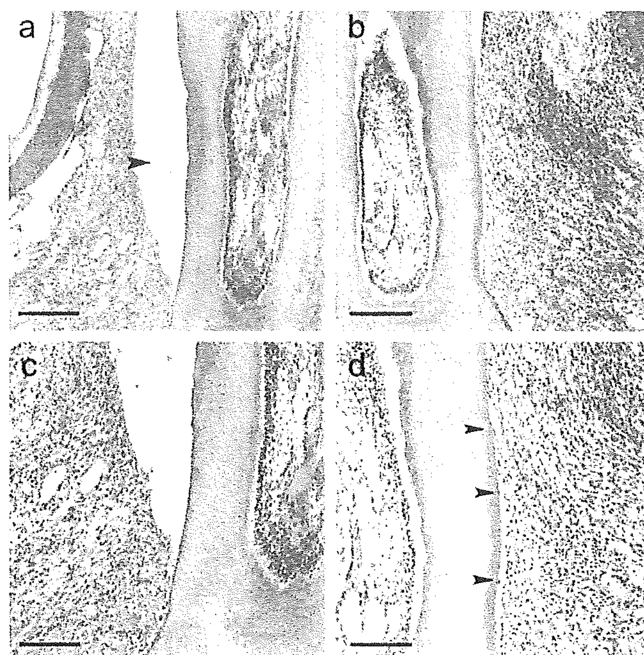

FIG. 5. One week after HPDL cell sheet transplantation. Nontransplanted control sides (*a* and *c*) and transplanted experimental sides (*b* and *d*). Gingival tissues of the control sides were detached from root dentin surfaces in histological sections (arrowhead in **a**). Many inflammatory cells were observed in the gingival connective tissue in both sides. Some putative transplanted cells were observed to attach onto root dentin surfaces (arrowheads in **d**). H&E. Scale bars: (**a** and **b**) 200 μm; (**c** and **d**) 100 μm.

GTR.[27] Some separate investigations using cell kinetic methods also indicate that periodontal ligament fibroblast populations in regenerating tissues are oriented from the residual periodontal ligament and endosteal spaces.[28,29] Periodontal regeneration presumably involves several cell types: fibroblasts for soft connective tissue, cementoblasts for cementogenesis, osteoblasts for bone, endothelial cells for angiogenesis, and epithelial rests of Malassez, which may be related to the control of the regeneration process. During the regenerative process, these cells must interact with a variety of soluble mediators such that the course of regeneration is dictated by a combination of molecule–cell, cell–matrix, and cell–cell interactions.[30] Little is known about the signals that initiate and regulate these interactions *in vivo*. Periodontal ligament fibroblast populations dominate the cells derived from periodontal ligament. They have several other characteristics that may help distinguish them from other connective tissue cells in the body. For instance, they express bone-associated markers including alkaline phosphate and bone sialoprotein and they have the potential for mineralized nodule formation under appropriate conditions.[31,32] These investigations can provide us with information about the possible roles of periodontal ligament fibroblasts in tissue regeneration. From these perspectives, utilizing cells from the periodontal ligament may be a rational method for periodontal regeneration.

In the presence of ascorbic acid, fibroblasts are known to synthesize and secrete collagen, and these cells show a higher proliferative capacity for the making of cell multilayers.[33] Similarly, HPDL cells supplemented with ascorbic acid produce thick ECM, as revealed in the present study. Recovered cell sheets were fragile and inappropriate for transplantation when subjected to cell sheet harvest by low-temperature treatment just after reaching confluency. Consequently, addition of ascorbic acid strengthened the HPDL cell sheets, facilitating cell sheet manipulation. However, ascorbic acid not only has a profound effect on HPDL cell proliferation and migration, but also enhances differentiation to mineralized tissue-forming cells.[34,35] Because tissue mineralization may not be necessarily desirable for periodontal ligament regeneration, appropriate conditions to block this are required.

Confluent HPDL cells were recovered as a contiguous cell sheet from temperature-responsive culture after reducing the culture temperature. In this method, neither proteolytic enzymes nor EDTA is required, so that the HPDL cells could be harvested and handled intact and without alteration of cell–cell contacts or deposited ECM (Figs. 2–4).

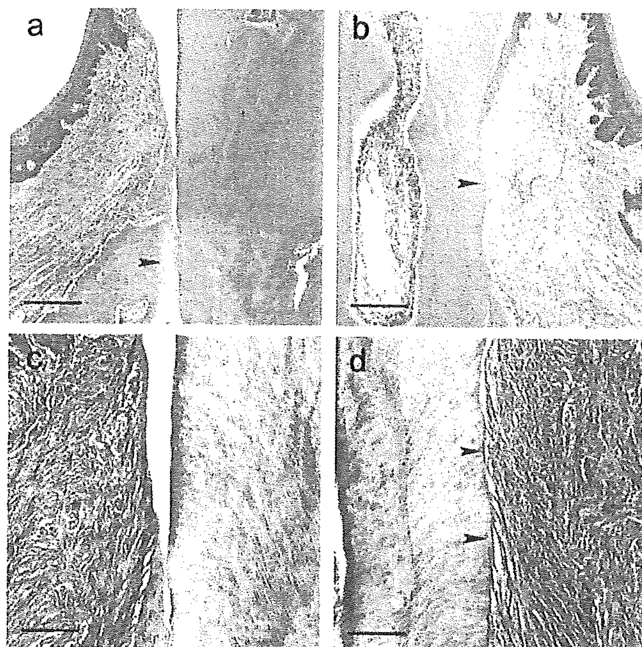

FIG. 6. PDL regeneration at 4 weeks postsurgery. Nontransplanted control sides (**a** and **c**) and transplanted experimental sides (**b** and **d**). Extensive new bone formation and ankylosis with partial root resorption was observed only in control sides. Regeneration of periodontal ligament-like tissue was the predominant feature in the experimental sides. A layer well stained with blue was deposited onto the curetted root dentin surface, and immature thin fibers were obliquely anchored onto this layer (arrows). H&E (**a** and **b**) and Azan (**c** and **d**) staining. Scale bars: (**a** and **b**) 200 μm; (**c** and **d**) 100 μm.

Integrins are a large family of transmembrane proteins that are a primary means by which HPDL cells integrate with ECM for their migration and adhesion.[36] Fibronectin is a major protein that is synthesized, secreted, and incorporated into the extracellular matrix by cultured periodontal ligament cells.[37] The integrity of the fibronectin molecule in the matrix is important for formative or anabolic phases of periodontal regeneration or wound healing.[38] Fibronectin matrix adhering to the basal side of each harvested cell sheet can function as a natural adhesive to attach cell sheets to other surfaces. HPDL cell sheets were therefore predicted to rapidly attach onto planed root dentin surfaces at an early phase of wound healing.

Cementogenesis on tissue sites previously exposed by periodontal disease is a prerequisite for periodontal regeneration.[39] In the experimental animal sites, a layer clearly colored blue with azan stain was observed on root dentin surfaces (Fig. 6d). This stainability may suggest that this layer was comparatively immature and newly deposited onto previously denuded root dentin surfaces. To clarify further the properties of this deposited layer, it is necessary to perform immunohistochemistry. Periodontal tissues are well organized, consisting of epithelium, gingival connective tissue fibers, periodontal ligament fibers, cementum, and alveolar bone.[40] Cementum and alveolar bone exist on either side of the periodontal ligament. Periodontal ligament never calcifies naturally, although it is in close contact with calcified tissues. Reconstructions of both these calcified and noncalcified tissues must be achieved for periodontal regeneration. Although previous studies have suggested the feasibility of using three-dimensional polymer scaffold materials for regeneration of bone and cementum,[41,42] a different approach may be required for periodontal regeneration. Conceptually, the delivery of selected cells to the discretional area in periodontal defects, using appropriate methods, may serve as a viable approach to promote ideal periodontal tissue regeneration. From this perspective, multilayered cell sheets comprising calcified bone-producing cell sheets at both exposed surfaces and nonproducing cell sheets between them might be useful. Such multilayering of several cell sheets has in fact been previously reported.[23] This technique should be directly applicable to periodontal regeneration with advanced pre-tissue-layered, implantable constructs.

ACKNOWLEDGMENTS

We are indebted to Prof. D. Grainger (Colorado State University) for technical criticism. We wholeheartedly appreciate Dr. K. Sena (Periodontology, Tokyo Medical

and Dental University) for technical advice and support, especially in managing the experimental animals, and Dr. A. Kushida (Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University) for valuable comments in the preparation of cell sheets and in their biochemical analyses. This research was supported by grants from the Center of Exellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone (Tokyo Medical and Dental University) and from the Center of Excellence Program for Regenerative Medicine (Tokyo Women's Medical University).

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JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2005.00799.x

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

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Akizuki T, Oda S, Komaki M, Tsuchioka H, Kawakatsu N, Kikuchi A, Yamato M, Okano T, Ishikawa I. Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs. J Periodont Res 2005; 40: 245–251.
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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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Key words: cell sheet; periodontal diseases/therapy; periodontal ligament; tissue engineering

Accepted for publication December 6, 2004

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

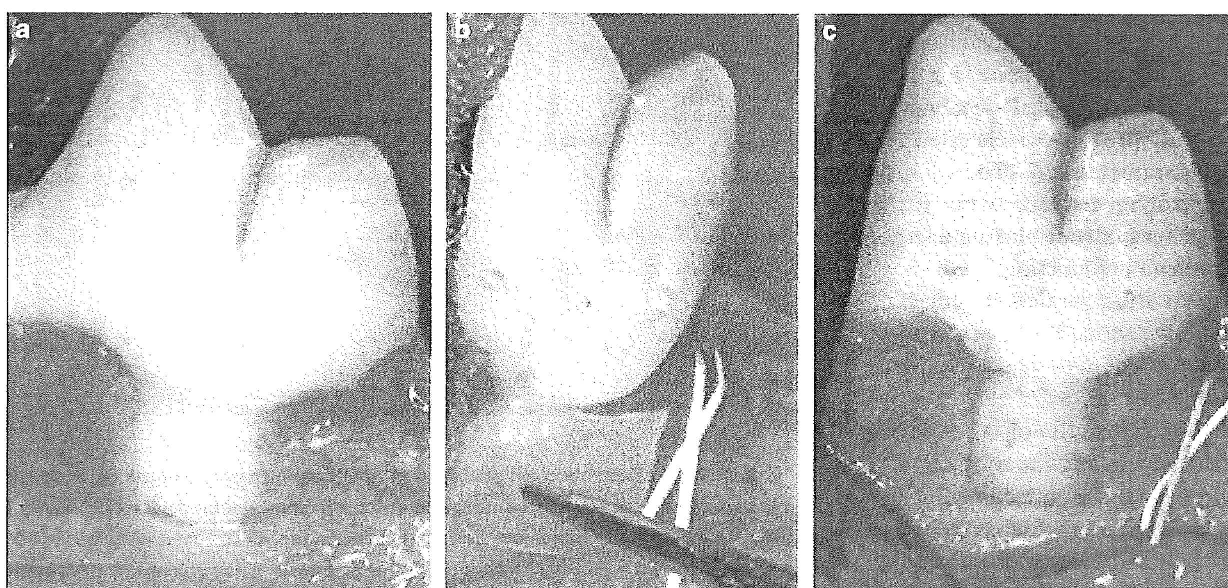

Fig. 1. (a) The dehiscence type of defect was formed on the buccal side of the mesial root of the mandibular first molar. (b) Periodontal ligament cell sheet with the reinforced hyaluronic acid carrier was applied to the defect with tweezers in the experimental defect. (c) Applied periodontal ligament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

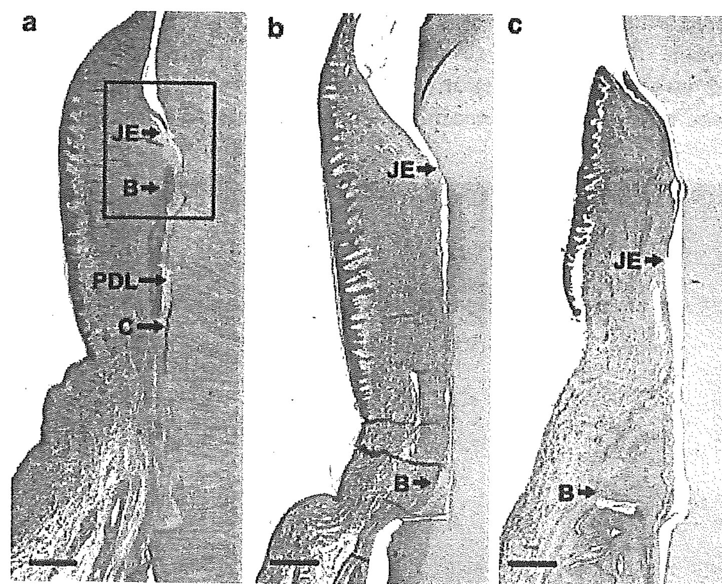

Fig. 2. Histology of the bucco-lingual section in the experimental defects (a–c). A variety of healing processes were observed among the different experimental defects. (a) Newly formed periodontal tissue with bone, cementum and periodontal ligament was observed at the coronal portion of the defect. (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).

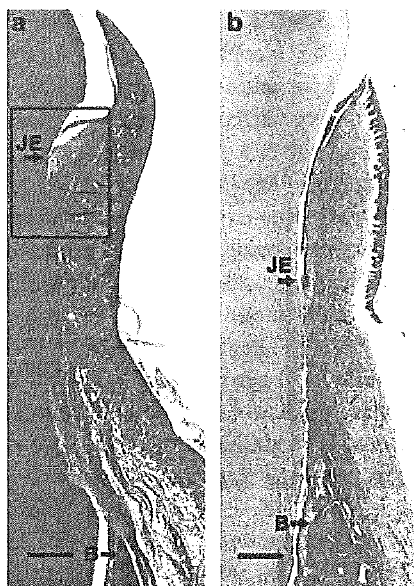

Fig. 3. Histology of the bucco-lingual section in the control defects (a and b). (a) Formation of the periodontal supportive tissue was not observed in the four cases of the control defect. (b) Newly formed bone continuous with the thick host bone plate was observed in one case at the apical portion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

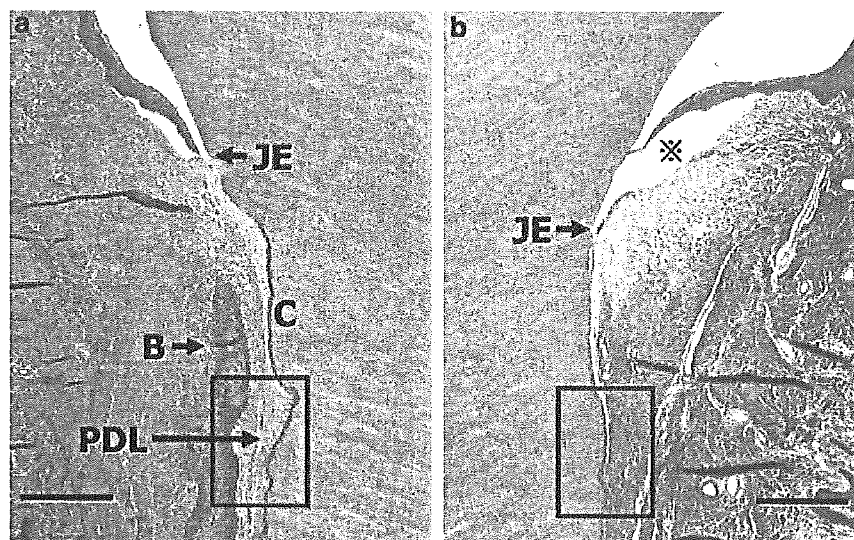

Fig. 4. Higher magnification of the framed area in Figs 2(a) and 3(a). Epithelium downgrowth towards the treated root surfaces was observed at the coronal part of the connective tissue attachment both in the experimental (a) and control defect (b). JE, junctional epithelium; B, bone; PDL, periodontal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

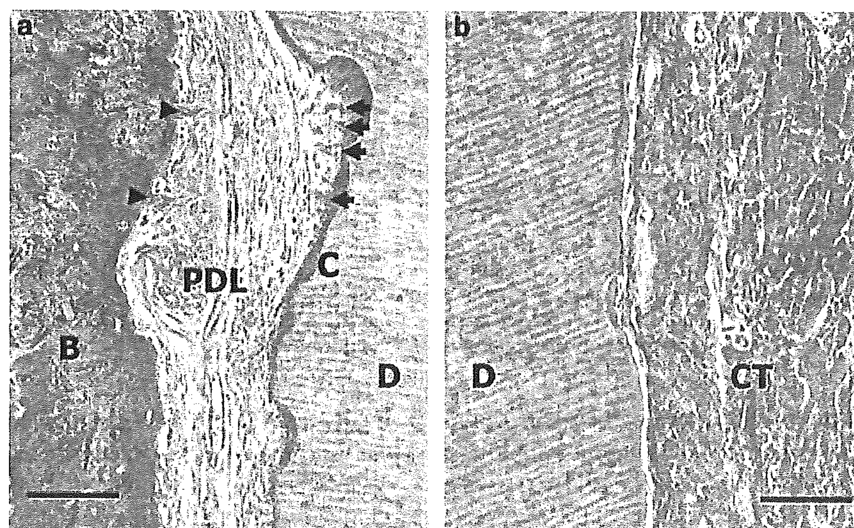

Fig. 5. Higher magnification of the framed area in Figs 4(a and b). (a) Newly formed bone, cementum and periodontal ligament were observed in the experimental defect. (b) This healing process was never observed in the control. Collagen fibers inserted perpendicularly into the newly formed bone (arrowhead) 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

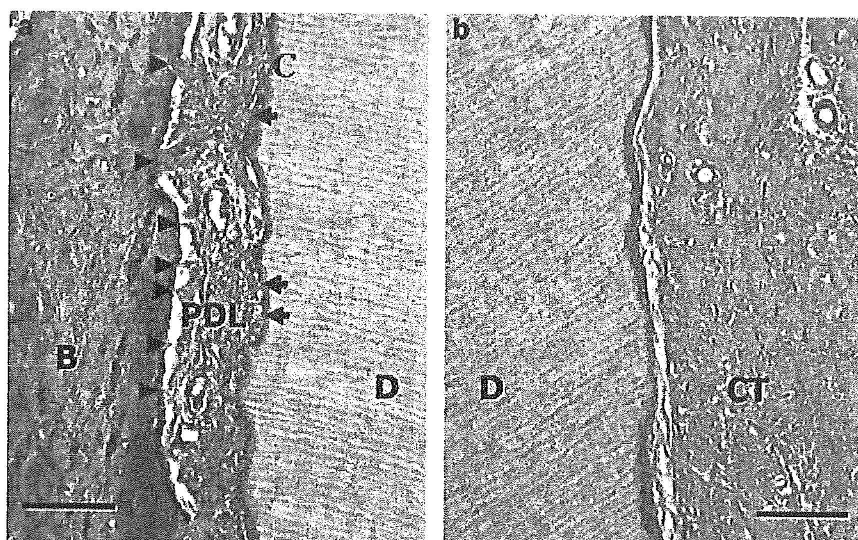

Fig. 6. Higher magnification of the experimental (a) and control defect (b). Well-vascularized periodontal ligament was observed between the newly formed bone and cementum. Collagen fibers inserted perpendicularly into the newly formed bone (arrowhead) and cementum (arrow). B, bone; PDL, periodontal 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.

Acknowledgements

The authors thank our colleagues Drs Atsuhiro Kinoshita, Shigenari Kikuchi, Kohji Mizutani, Chie Hayashi, and Aristeo Atsushi Takasaki for their assistance. This study was also supported by Seikagaku Kogyo, Japan for the hyaluronic acid sheets. This research was supported by the grant for Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone in Tokyo Medical and Dental University.

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