

Periodontal Tissue Regeneration with Adipose-Derived Stem Cells

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ABSTRACT

A number of surgical techniques have been developed to promote periodontal tissue regeneration. Bone marrow–derived stem cells have also been shown to promote periodontal tissue regeneration. In this study, we sought to determine whether adipose-derived stem cells (ASCs) can promote periodontal tissue regeneration as well. ASCs were isolated from a Wistar rat, passaged twice, mixed with platelet-rich plasma (PRP) obtained from inbred rats, and implanted into the periodontal tissue defect that had been generated in the test rats. Tissue specimens were harvested after 2, 4, and 8 weeks for histological analysis. Rats that received PRP only or were not implanted served as controls. A small amount of alveolar bone regeneration was observed 2 and 4 weeks after ASC/PRP implantation. Moreover, 8 weeks after implantation, a periodontal ligament-like structure was observed along with alveolar bone. These observations suggest that ASCs can promote periodontal tissue regeneration *in vivo*. Because large amounts of human lipoaspirates are readily available, and their procurement induces only low morbidity, ASCs may be useful in future clinical cell-based therapy for periodontal disease.

INTRODUCTION

THE IMMUNE-INFLAMMATORY RESPONSE that develops in the gingival and periodontal tissues in response to the chronic presence of plaque bacteria results in destruction of structural components of the periodontium, leading, ultimately, to clinical signs of periodontitis.¹ This condition induces the breakdown of the tooth-supporting structures until teeth are lost.² To stop the progression of the disease and to regenerate the lost tissue, it may be necessary to intervene surgically. A number of surgical techniques have been developed to regenerate periodontal tissue, including guided tissue regeneration,^{3–5} bone grafting,^{6,7} and the use of enamel matrix derivative.^{8–13} However, recent developments in the field of tissue engineering and regeneration suggest that it may eventually be possible to replace or repair damaged tissue.^{14,15}

It has been demonstrated that bone marrow–derived mesenchymal stem cells (BSCs) efficiently regenerate periodontal tissue in the canine model.^{16–18} Moreover, a recent clinical report indicated that periodontal tissue could be successfully regenerated using autologous BSCs in combination with platelet-rich plasma (PRP).¹⁹ The fate of the implanted BSCs has also been well documented.¹⁸ However, bone marrow procurement is painful for donors, so general anesthesia is often required, and yields low numbers of BSCs.

A cell population that is similar to BSCs has been demonstrated to exist within human adipose tissue.²⁰ This cell population, adipose-derived stem cells (ASCs), may be a promising source of cells for tissue-engineering strategies because adipose tissue is available in large quantities and is relatively easy to obtain. Moreover, liposuction procedures have minimal donor site morbidity and induce limited

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patient discomfort. Therefore, in this study, we sought to determine whether ASCs would be useful for periodontal tissue regeneration *in vivo*.

MATERIALS AND METHODS

Experimental animals

After receiving approval from the Committee of Research Facilities for Laboratory Animal Science, Nippon Medical School, 10-week-old male Wistar rats (Saitama Experimental Animals Supply Corporation Ltd, Saitama, Japan) and green fluorescent protein (GFP)-transgenic rats [Crj:Wistar-Tgn(CAG/GFP)] (Health Science Research Resources Bank, Osaka) weighing 330 to 380 g were used for the following experiments. The Animal Ethical Committee of Nippon Medical School approved all animal experiments (Approval number: 17-052), which were performed according to the Guidelines for Regulation of Animal Experiment of Nippon Medical School.

Harvest and primary culture of ASCs

Adipose tissue obtained from a 10-week-old inbred male Wistar rat was processed to isolate the ASC population, as described previously.²¹ Briefly, under general anesthesia with pentobarbital sodium at 1 mg/100 g, the inguinal fat pads of the rat were harvested, washed extensively with phosphate buffered saline (PBS; Gibco-BRL, Grand Island, NY), minced for 10 min with fine scissors, and enzymatically digested at 37°C for 40 min with 0.1% collagenase (Wako, Osaka, Japan). An equal volume of control medium (Dulbecco's modified Eagle medium (DMEM, Gibco-BRL) containing 10% fetal bovine serum (FBS; Gibco-BRL) and 1% antibiotic/antimycotic (Gibco-BRL)) was then added to neutralize the collagenase. The cell suspension was centrifuged at 1300 rpm (260 g) for 5 min to obtain a high-density ASC pellet, which was resuspended in control medium. After being counted using trypan blue, the cells were plated at a concentration of 5×10^5 cells per 100-mm² tissue culture dishes (Becton-Dickinson, Franklin Lakes, NJ) and maintained in control medium at 37°C in 5% carbon dioxide. The culture medium was changed every 3 days. Confluent ASC cultures (approximately 80% confluence) were passaged at a ratio of 1:3 in trypsin/ethylenediaminetetraacetic acid (Gibco-BRL).

PRP preparation and analysis

PRP preparation was performed as described by Koike *et al.*¹³ Thus, 10 mL of whole blood from two 10-week-old inbred male Wistar rats was drawn preoperatively via cardiac puncture with an 18-gauge needle (TERUMO, Tokyo, Japan) into tubes containing 3.8% sodium citrate. The blood was first centrifuged in a standard laboratory centrifuge machine (Kubota 3740, Tokyo, Japan) for 10 min at 2400 rpm

(450 g). The supernatant plasma was collected along with the buffy coat, which consists of platelets and leukocytes, into a neutral tube with a long cannula. A second centrifugation at 3600 rpm (850 g) was performed for 15 min to concentrate the platelets. The infranatant containing the buffy coat was resuspended with 1.3 mL of the residual plasma to prepare the final PRP product. The final concentration of platelets in the whole blood and PRP was then analyzed in an automatic counter LC-152 (Horiba, Kyoto, Japan). PRP gelation was activated with a 10% calcium chloride solution immediately before the administration *in vivo*. PRP preparations were made from each of the 2 rats and were used for the subsequent experiment in a blind fashion.

Constitution of ASC/PRP admixture

After the primary culture in control medium and expansion to 2 passages, 1×10^7 ASCs/mL were mixed with 1 mL of PRP before implantation. The ASC/PRP admixture was then activated with a 10% calcium chloride solution, which induced gelation.

Preparation of the rat periodontal defect model and ASC implantation

A periodontal tissue defect was created according to a modification of a previous technique¹² using the palatal side of the upper first molar. Thus, the rats were anesthetized with diethyl ether and 0.3 mg/kg of pentobarbital sodium. In the supine position, a mucosal incision was made from the gingival sulcus of the second molar mesial palatal side to the first molar mesial palatal side, and an approximately 5-mm incision was made continuously in the mesial direction from the first molar mesial side. After mucosal flap elevation, the periodontal tissue, including the cementum, alveolar bone, and periodontal ligament, was excised bilaterally at the first molar palatal side under irrigation using a dental round bar (ISO standard 010) so that the dimensions of the defect were approximately $1 \times 1 \times 1$ mm (Fig. 1). After irrigation with physiological saline solution, the ASC/PRP admixture was injected into the defect (ASC/PRP group, $n = 24$). Finally, the mucosal flaps were placed back using 6-0 absorbable surgical suture BioSorbC (Alcon, Tokyo, Japan). Periodontal tissue defects into which nothing or PRP gel lacking ASCs was injected served as controls, called the no-implantation and PRP groups, respectively ($n = 24$ each).

Tissue preparation and histological analysis

At 2, 4, and 8 weeks, the rats were euthanized with an overdose of an intraperitoneal injection of pentobarbital sodium ($n = 8$ at each time point in each group). The specimens were harvested, fixed in 10% buffered formalin, decalcified (K-CX, Falma Co., Tokyo, Japan), dehydrated using graded ethanol, cleared with xylene, and embedded in paraffin.

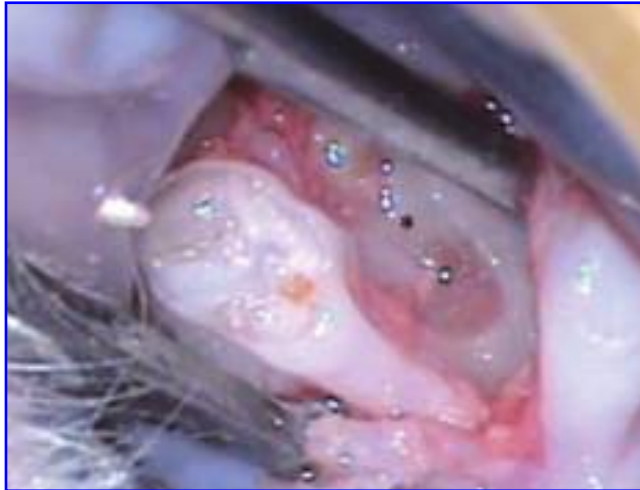


FIG. 1. Macroscopic view of the periodontal tissue defect in a rat model. Periodontal tissue including the cementum, alveolar bone, and periodontal ligament of the first upper molar palatal side was excised. *defect site. Color images available online at www.liebertpub.com/ten.

Serial sections (5 μm) were performed in the buccal–palatal plane. The sections were stained with hematoxylin and eosin according to standard procedures and observed under an Olympus AX80 light microscope.

Immunohistochemistry

In a separate set of experiments, ASCs were isolated from a GFP rat so that the origin of the cells that contributed to periodontal tissue regeneration could be discerned. Thus, periodontal tissue defects were generated in 4 Wistar rats as described above and then filled with GFP rat-derived ASC/PRP admixture. The tissue specimens were harvested 8 weeks after implantation for immunohistochemical staining of GFP, osteocalcin, osteopontin, and type I collagen. Serial sections of the same specimens were used for the subsequent immunohistostaining. Thus, the tissue specimens were incubated with a polyclonal antibody specific for GFP (1:500 dilution, Medical Biological Laboratories, Tokyo, Japan) for 1 h at room temperature, then washed with PBS and incubated at room temperature for 10 min with biotin-conjugated goat anti-rabbit immunoglobulin (Ig)G secondary antibody. The secondary antibody was visualized using the Histofine simple stain kit rat MAX-PO (MULTI) (Nichirei Bioscience, Tokyo, Japan) according to the manufacturer's specifications. Finally, the sections were counterstained with Meyer's hematoxylin for 3 min. In addition to GFP immunohistochemistry, osteocalcin, osteopontin, and type I collagen activity was examined using standard procedures. Briefly, 5- μm tissue sections were deparaffinized in xylene and in graded alcohols. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min, after which the sections were rinsed with

TRIS/hydrochloric acid (HCL) buffer for 5 min 3 times, blocked with normal swine serum for 30 min, and incubated with anti-osteocalcin monoclonal antibodies (1:200 dilution; Takara Bio Inc., Shiga, Japan), anti-osteopontin monoclonal antibodies (1:100 dilution; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan), and anti-type I collagen monoclonal antibodies (1:40 dilution; Southern Biotech., Birmingham, AL), respectively, for 60 min at room temperature. After being washed with TRIS/HCL buffer, the sections were incubated at room temperature for 30 min with biotin-conjugated goat anti-rabbit IgG secondary antibody. Visualization of secondary antibody and counterstaining was performed as described above. Photographs were taken with an Olympus AX80 light microscope.

RESULTS

Primary culture of ASCs

Digestion of the inguinal fat pads of a Wistar rat yielded approximately 5×10^5 nucleated cells that were used in primary cultures. The cells expanded rapidly *in vitro* and formed a heterogeneous population that morphologically resembled fibroblast-like cells, as described in previous reports (Fig. 2).

Platelet concentration of the PRP

The 2 whole blood samples had, on average, $157.5 \times 10^3/\text{mm}^3$ ($162 \times 10^3/\text{mm}^3$ and $153 \times 10^3/\text{mm}^3$) platelets, whereas the mean platelet count in the resulting PRPs was $1150 \times 10^3/\text{mm}^3$ ($1190 \times 10^3/\text{mm}^3$ and $1110 \times 10^3/\text{mm}^3$) (i.e., concentrated more than 700% relative to whole blood). This

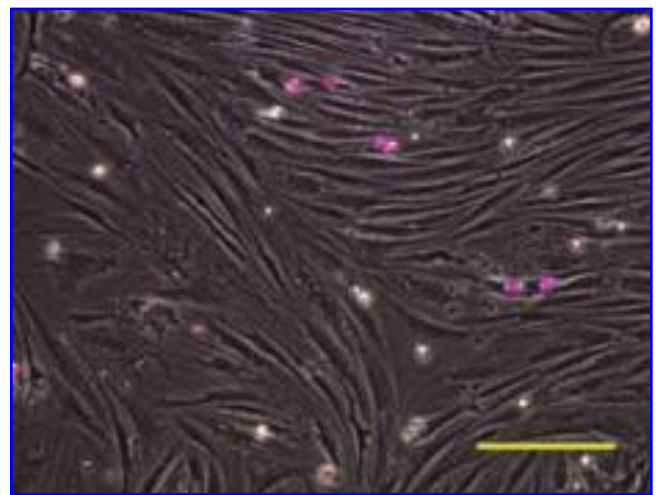


FIG. 2. Microscopic view of primary cultured adipose-derived stem cells (ASCs) harvested from the inguinal fat pads of a Wistar rat. The ASCs have an elongated fibroblast-like cell appearance (scale bar: 200 μm). Color images available online at www.liebertpub.com/ten.

indicates that the PRP preparing process sufficiently concentrated the platelets.

Implantation of ASCs induces periodontal tissue regeneration

Two weeks after ASC/PRP was implanted into the periodontal tissue defect, a small amount of bone regeneration from the residual bone was evident, and a cell mass had accumulated adjacent to the regenerated bone. However, periodontal ligament was not seen. High magnification of the defect demonstrated the presence of the cell mass and dense collagen on the surface of regenerated bone, which was connected to the alveolar bone. These cells were columnar in shape and stretched in various directions (Fig. 3). In contrast, no implantation group showed any regeneration of bone or periodontal tissue. In addition, inflammatory cell infiltration was seen in the periodontal defect site, which represents a characteristic of the inadequate healing process that marks periodontal disease (Fig. 3).

Four weeks after ASC/PRP implantation into the periodontal tissue defect, bone regeneration was also observed on the surface of the alveolar bone. More interestingly, isolated bone formation surrounded by fibrous connective

tissue was observed in many specimens (Fig. 4). The extracellular matrix around the fibrous tissue stained positive for Alcian Blue, a staining of mucopolysaccharides, which indicates higher activity of bone formation (data not shown). In contrast, periodontal tissue and regenerated bone were not observed in the periodontal tissue defect in the no-implantation group (Fig. 4).

Eight weeks after ASC/PRP implantation into the periodontal tissue defect, regenerated bone and the other periodontal tissues were observed in the defect (Fig. 5). A cementum-like structure was noted on the superficial surface of the dentin, and the regenerated bone exhibited alveolar crista. Moreover, a periodontal ligament-like structure was located perpendicularly between the cementum and alveolar bone. In contrast, neither the PRP group nor the no-implantation group had any periodontal tissue, except for a small amount of alveolar bone (Fig. 5).

Immunohistochemistry

In a separate set of experiments, the ASCs were harvested from a GFP rat so that the origin of the cells that contributed to periodontal tissue regeneration could be discerned. Eight weeks after the GFP ASC/PRP admixture was implanted

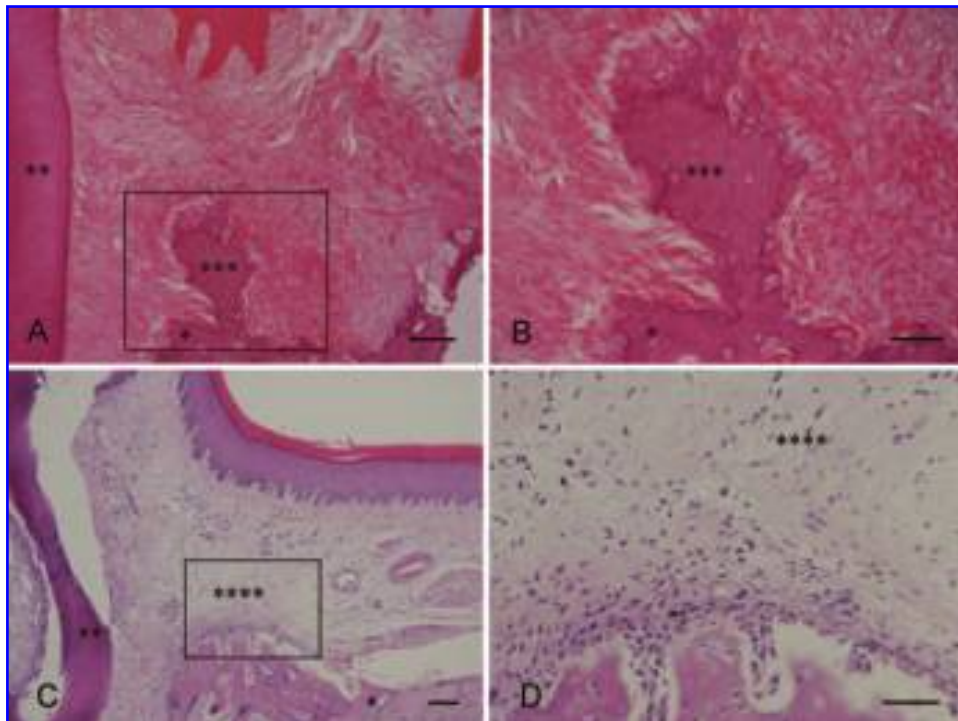


FIG. 3. Hematoxylin and eosin-stained histological cross-section of the periodontal tissue defects 2 weeks after implantation. (A) The defects in the adipose-derived stem cell/platelet-rich protein (ASC/PRP) group show a small amount of alveolar bone regeneration (scale bar: 100 μ m). (B) High magnification of the defects in the ASC/PRP group reveal that the columnar-shaped cells were found on the surface of the regenerated bone (scale bar: 50 μ m). (C) No alveolar bone regeneration was found in the no-implantation group (scale bar: 100 μ m). (D) High magnification of the defects in the no-implantation group show the presence of inflammatory cellular infiltration (scale bar: 50 μ m). *alveolar bone, ** dental root, *** regenerated bone, **** inflammatory cellular infiltration. Color images available online at www.liebertpub.com/ten.

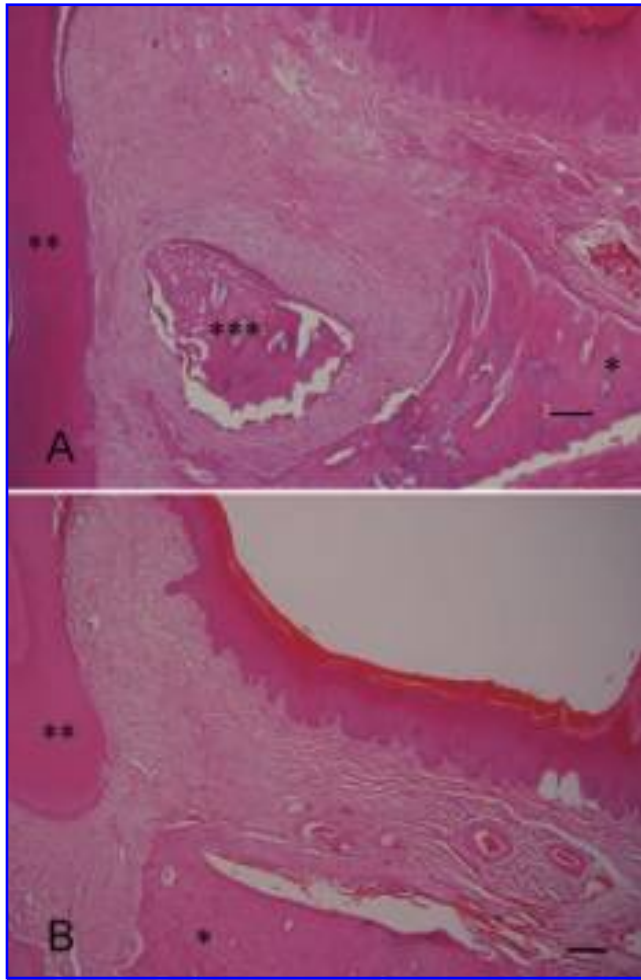


FIG. 4. Hematoxylin and eosin-stained histological cross-section of the periodontal tissue defects 4 weeks after implantation. (A) In the adipose-derived stem cell/platelet-rich protein group, bone regeneration on the surface of alveolar bone and isolated bone structure surrounded by fibrous connective tissue was observed (scale bar: 100 μ m). (B) No alveolar bone regeneration was found in the no-implantation group (scale bar: 100 μ m). *alveolar bone, ** dental root, *** isolated bone. Color images available online at www.liebertpub.com/ten.

into the periodontal defects in 4 Wistar rats, tissue specimens were analyzed using immunohistostaining for GFP, osteocalcin, osteopontin, and type I collagen. GFP-positive cells occurred particularly frequently on the surface of the regenerated alveolar bone crista and in the periodontal ligament-like structure (Fig. 6A, B). In the serial section of GFP immunohistochemistry, cells that stained positive for osteocalcin were also observed in the regenerated bone (Fig. 6C, D). In addition to osteocalcin, cells that stained positive for osteopontin were present particularly in the center of the regenerated bone (Fig. 6E). Finally, the immunohistostaining with type I collagen showed that the activity was positive in almost all area of regenerated bone and periodontal ligament-like structure (Fig. 6F). These findings suggest that

some of the osteocytes and potential periodontal ligament cells differentiated from implanted ASCs.

DISCUSSION

Periodontal disease results in loss of connective tissue and bone support and is a major cause of tooth loss in adults.² The immune-inflammatory response that develops in the gingival and periodontal tissues in response to the chronic presence of plaque bacteria results in destruction of structural components of the periodontium, leading, ultimately, to clinical signs of periodontal disease.¹ Because current therapeutic options are still limiting, a number of surgical approaches have been developed to regenerate periodontal tissue.⁸⁻¹³ Although these treatments have been reported to effectively regenerate periodontal tissue, candidates for such treatments are limited, and the amount of tissue that is regenerated cannot be predicted.²² Therefore, periodontal tissue regeneration using autologous stem cells may be promising as a future cell-based therapy for periodontal diseases.²³ This method is based on tissue engineering¹⁴ and involves the morphogenesis of new tissue using biocompatible scaffolds, isolated stem cells, and growth factors. Candidate cells for tissue regeneration include embryonic stem cells, adult stem cells, and progenitor cells. Because ethical concerns and potential problems with cell regulation limit the use of embryonic stem cells,²⁴⁻²⁶ adult stem cells and progenitor cells are more suitable for use in clinical situations. With regard to adult stem cells, the use of mesenchymal stem cells is also problematic, because they must be isolated from the bone marrow, which has been shown to contain pluripotent self-renewing cells.^{15,27} Recently, it was shown that human adipose tissue contains cells that are similar to BSCs.²⁰ These cells, ASCs, may be a promising source of cells for many tissue engineering strategies, because many studies have shown that ASCs can differentiate into adipogenic, osteogenic, chondrogenic, myogenic, and neurogenic lineage cells; moreover, they have been used successfully to regenerate various tissues.^{20,28-32} Adipose tissue is available in large quantities and is relatively easy to obtain. Moreover, liposuction procedures have minimal donor site morbidity and induce less patient discomfort than bone marrow procurement. Thus, where bone marrow-derived mesenchymal stem cells have been shown to be useful in the field of regenerative medicine, ASCs are likely to be capable of the same functions.

In our study, some of the osteocytes in the regenerated alveolar bone and the potential periodontal ligament cells stained positive for GFP in immunohistochemical analysis. This indicates that ASCs isolated from GFP transgenic rats differentiate directly into mature osteocytes and periodontal ligament cells.

Some arguments may have been raised with regard to immunological concerns regarding the origin of ASCs in this study. ASCs isolated from a Wistar rat were implanted

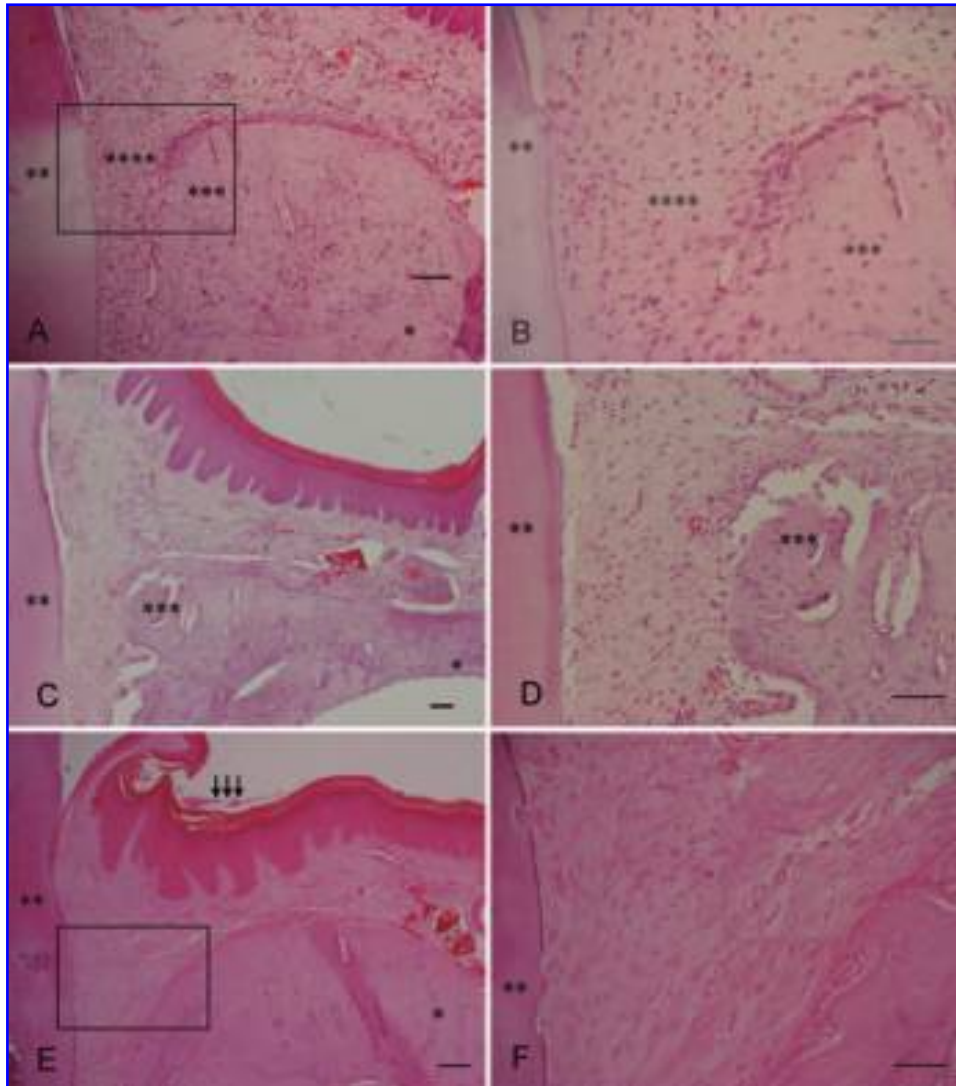


FIG. 5. Hematoxylin and eosin–stained histological cross-section of the periodontal tissue defects 8 weeks after implantation. (A) In the adipose-derived stem cell/platelet-rich protein (ASC/PRP) group, a cementum-like structure and alveolar bone with alveolar crista had regenerated (scale bar: 100 μ m). (B) High magnification of the defects in the ASC/PRP group showed a periodontal ligament-like structure located perpendicularly between the cementum-like structure and the alveolar bone (scale bar: 50 μ m). (C) Alveolar bone regeneration was observed at the low level, although alveolar crista was not seen in the PRP group (scale bar: 100 μ m). (D) A perpendicular, periodontal ligament-like structure was not seen between the alveolar bone and the dentin surface in the PRP group (scale bar: 100 μ m). (E) Little bone regeneration or alveolar crista was noted in the no-implantation group. In addition, the volume of the gingival was relatively decreased (arrow) (scale bar: 100 μ m). (F) In the no-implantation group, dense collagen fibers and granulation tissue occupied the space between the dentin surface and alveolar bone. A perpendicular structure was not detected (scale bar: 50 μ m). *alveolar bone, ** dental root, *** regenerated bone, **** periodontal tissue-like structure. Color images available online at www.liebertpub.com/ten.

in the periodontal tissue defect made in the inbred rats. Although the fate of ASCs from inbred animals remains unclear, several previous studies have demonstrated that the mesenchymal stem cells isolated from inbred animals are capable of mature tissue regeneration with no immunological rejection.^{33,34} Therefore, we do not believe that our experimental model raises any immunological concerns.

In addition to ASCs, the periodontal defect was implanted with a PRP gel, which served as a cytokine cocktail

or a cell delivery carrier. PRP is known to contain several growth factors (e.g., isomers of platelet-derived growth factor, transforming growth factor β 1 and β 2, insulin-like growth factor alpha and beta, and vascular endothelial growth factor) that not only promote bone regeneration, but may also help the differentiation of pluripotent cells.³⁵ These growth factors have been shown to be mitogenic for osteoblasts and to stimulate the migration of mesenchymal progenitor cells.³⁶ Furthermore, the gel property of PRP

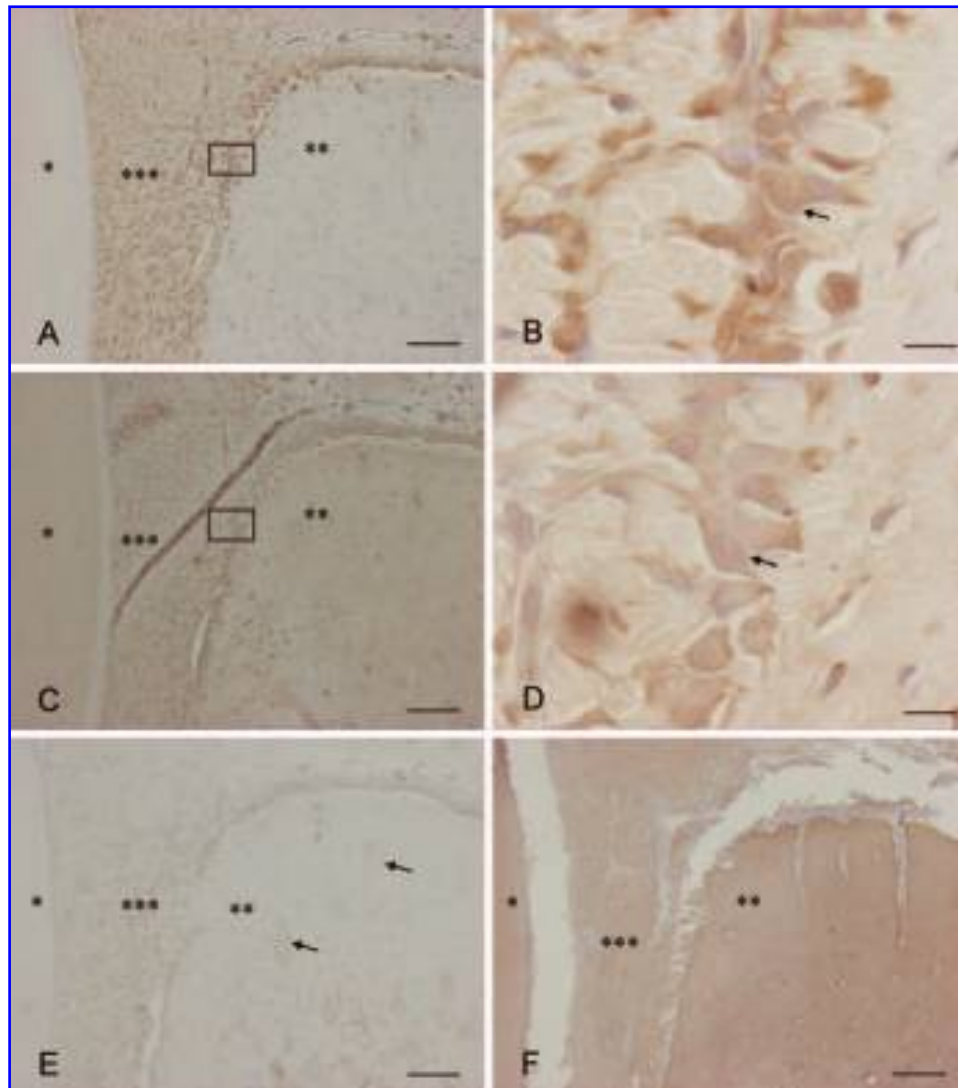


FIG. 6. Immunohistochemical analysis for green fluorescent protein (GFP), osteocalcin, osteopontin, and type I collagen expression in periodontal tissue defects 8 weeks after implantation with GFP adipose-derived stem cell/platelet-rich protein (ASC/PRP). (A) GFP-positive cells were found in the surface of regenerated bone and periodontal ligament-like structure. Most of these cells were detected in the alveolar crista (scale bar: 50 μ m). (B) High magnification of the regenerated alveolar bone. Cells on the surface of the regenerated bone stained positively for GFP (arrow) (scale bar: 5 μ m). (C) Cells that stained positive for osteocalcin were found in the regenerated bone on the serial section of A (scale bar: 50 μ m). (D) Higher magnification of the defect shown in C. Arrow indicates where the cell stained positive for anti = GFP and anti = osteocalcin antibodies (scale bar: 5 μ m). (E) Cells stained positive for osteopontin were found particularly in the center of the regenerated bone (arrow) (scale bar: 50 μ m). (F) Immunohistostaining with type I collagen showed that the activity was positive in almost all areas of regenerated bone and periodontal ligament-like structure (scale bar: 50 μ m). *dental root, ** regenerated bone, *** periodontal ligament-like structure. Color images available online at www.liebertpub.com/ten.

allows ASCs to be injectable and to stay in the implanted site. However, the efficacy of PRP in bone regeneration remains unclear, in particular because PRP does not contain bone morphogenetic protein (BMP). BMP is the most potent osteoinductive protein that promotes stem cell differentiation into the osteoblastic lineage and the only known growth factor that can induce ectopic bone formation.³⁷ Sarkar *et al.* concluded in their study that PRP has no effect on bone defect healing.³⁸ On the other hand, many other studies have reported that PRP has a positive influence on bone regen-

eration with or without any stem cells.^{5,13,17,19,39–41} Our results showed that PRP with or without ASCs induced alveolar bone regeneration, although only the ASC/PRP group successfully demonstrated regeneration of other periodontal tissues, including the cementum and periodontal ligament-like structure. We did not find epithelial invasion or ingrowth, which is characteristic of the inadequate healing process that marks periodontal disease, especially in ASC/PRP and PRP groups at all time points. We speculated that this phenomenon may be due to the strong connection

between the defect and the apical root side so that epithelial ingrowth could be completely prevented,⁴² which is similar to guided tissue regeneration method, in which a membrane was placed under the flap.³⁻⁵ Therefore, PRP gels containing stem cells such as ASCs may be useful clinically for periodontal tissue regeneration.

The goal of periodontal tissue regeneration is the treatment of periodontal disease with or without alveolar bone resorption. Complete regeneration of damaged cementum, periodontal ligament, and alveolar bone could be a possibility with this technique. Our observations suggest that human ASCs may be highly useful in periodontal tissue regeneration.

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