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Platelet-rich plasma/osteoblasts complex induces bone formation via osteoblastic differentiation following subcutaneous transplantation

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Background and Objective: Platelet-rich plasma (PRP) has been shown to enhance the maturation of bone grafts following local application and to have biological effects on osteoblasts *in vitro*. However, PRP is not applied by itself clinically as a result of its poor benefits in large bone defects. The present study was undertaken to develop a clinical alternative to autologous bone, by investigating the application of PRP in combination with osteoblastic cells and evaluating its effects after transplantation.

Material and Methods: PRP and platelet-poor plasma (PPP) were prepared from blood, obtained from ddY mice, by two centrifugation steps. MC3T3-E1 cells were labeled with fluorescent carbocyanine just before transplantation. The combination of labeled cells and PRP gel was subcutaneously transplanted into the back of severe combined immunodeficient (SCID) mice, and the transplants were evaluated radiographically and immunohistologically after 4 wk. The effects of PRP were assessed by alkaline phosphatase (ALP) staining and von Kossa staining, and the expression of bone-related markers was analyzed by reverse transcription–polymerase chain reaction before transplantation.

Results: Before transplantation, PRP enhanced the expression of Osterix and bone sialoprotein mRNAs compared with PPP. Furthermore, PRP elevated ALP activity and induced the formation of mineralized nodules. After transplantation, the combination of labeled cells and PRP gel formed mineralized tissue, and the transplanted cells visualized in the tissue using fluorescence microscopy expressed osteocalcin and type I collagen.

Conclusion: These results suggest that the application of a PRP/osteoblasts complex has beneficial effects for transplanting engineered cells into bone defects through the promotion of osteoblastic differentiation.

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Platelets contain a variety of autologous growth factors, including platelet-derived growth factor (PDGF), transforming growth factor β1 and β2 (TGF-β1 and TGF-β2), insulin-like growth factor (IGF) and epidermal growth factor (EGF), as well as a growth factor for hepatocytes (1,2).

The local application of platelet-rich plasma (PRP) has been reported to enhance the maturation of bone grafts (3–6). The use of PRP in cardiovascular surgery has increased over the last decade, leading to better postoperative outcomes, as a result of the availability of easily accessible sources of autologous growth factors to support bone and soft tissue healing (7,8). Periodontal regeneration is a multifactorial process that requires an orchestrated sequence of biological events, including cell adhesion, migration, multiplication and differentiation (9). A recently developed procedure for regeneration in the periodontal area has been used to combine PRP with autologous bone or bone substitutes or guided tissue regeneration in humans (10–12). PRP was found to have mitogenic effects and differentiation-inducing actions on osteoblasts during *in vitro* studies (13–16). Recently, the use of PRP, in combination with cancellous bone grafts, has been reported to enhance bone healing for mandibular reconstruction in goats, as evaluated both histomorphometrically and radiographically (17–19). Although PRP appears to have beneficial effects on osteoblasts *in vitro*, it is not applied by itself clinically, because some previous reports concluded that little or no benefit was gained from using PRP with sinus lift grafts, cranial defects or ridge augmentations (20–22). Therefore, it is necessary to develop alternative approaches for bone regeneration.

The present study was undertaken to estimate bone formation, induced by a combination of PRP gel and osteoblastic cells, before and after subcutaneous transplantation in severe combined immunodeficient (SCID) mice.

Material and methods

Preparation of PRP and platelet-poor plasma (PPP)

The protocol for PRP preparation was adopted from Yamada *et al*. (23) and modified for use with abdominal blood obtained from ddY mice (7–9 wks of age; throughout the experiments, 21 ddY mice were used). Briefly, mouse blood was obtained from the abdominal aorta, from mice under diethyl ether anesthesia, using 4-ml vacutainer tubes containing 7.2 mg of EDTA 2K or 3.8% sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA). The blood was pooled and centrifuged at 150 ***g*** at room temperature for 10 min, resulting in the separation of three basic fractions. Subsequently, almost all the plasma containing buffy coats was transferred to a new tube and centrifuged again at 250 ***g*** for 5 min. Next, the upper half of the preparation was transferred and designated PPP, while the lower half, in which the resulting platelet pellet was resuspended, was designated PRP.

PRP, PPP and blood from the mice were sent to SNBL Inc. (Kagoshima, Japan) for measurement of the platelet, red blood cell and white blood cell counts using a hematology system (ADVIA120; Bayer Medical Inc., Tokyo, Japan). Next, a thrombin/$CaCl_2$ solution was created by dissolving 100 units of thrombin from mouse plasma (Sigma, St Louis, MO, USA) in 1 ml of 10% calcium chloride, and 100 μl of PRP or PPP was gelled with 5 μl of thrombin/$CaCl_2$.

Preparation of PRP gel supernatant (PRPGS) and TGF-β1 assay

PRP gel or PPP gel was incubated at 37°C in a water bath for 40 min and then allowed to clot at 4°C overnight (24) before being homogenized and centrifuged at 9100 ***g*** The supernatant was collected and a sample removed for determining the concentration of TGF-β1 by using an enzyme-linked immunosorbent assay (Quantikine®; R & D systems Inc., Minneapolis, MN, USA). The detectable range of TGF-β1 in this assay was 1.56–3.24 ng/ml. The remaining supernatant was transferred to a new tube and stored at −80°C until use.

Cell culture

Mouse osteoblastic MC3T3-E1 subclone 4 cells were purchased from the ATCC (Manassas, VA, USA). The cells were grown in α-modified minimum essential medium (α-MEM; Sigma) containing 10% fetal bovine serum (FBS; Moregate Biotech, Bulimba, Australia), 2 mM L-glutamine (ICN Biomedicals Inc., Aurora, OH, USA), 100 units/ml penicillin G and 100 μg/ml streptomycin (Wako, Osaka, Japan) in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The medium was changed every 2–3 d until the cells reached subconfluence. Next, the cells were passaged using 0.12% trypsin (Gibco Laboratories, Paisley, UK)/0.02% EDTA (Nacalai Tesque Inc., Kyoto, Japan) in phosphate-buffered saline (PBS).

In vitro studies

Alkaline phosphatase (ALP) staining, and assay for mineralization in vitro – ALP staining and mineralization of MC3T3-E1 cells were analyzed in 24-well plates using a histochemical assay kit (Alkaline Phosphatase, Leukocyte; Sigma) and von Kossa staining, respectively. After reaching subconfluence, cells (2×10^4 cells/well) were grown in α-MEM supplemented with 0.5% FBS, 2 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin, in the presence or absence of 0.5% PRPGS, for up to 21 d (25). The medium was changed every 1–2 d.

Scanning electron microscopy (SEM) of PRP/cells complexes – A total of 5×10^5 cells were mixed with 50 μl of PRP gel. After culture for 2 d, the cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1 h, rinsed in the buffer, and post-fixed in 1% osmium tetroxide for 1 h. After dehydration through a graded ethanol series, the specimens were transferred to t-butyl alcohol, subjected to critical point drying, and then

sputter-coated with gold. Subsequently, the cells were examined under a scanning electron microscope (JSM-5510; JEOL, Tokyo, Japan) at 5 kV.

Reverse transcription–polymerase chain reaction (RT–PCR) analysis of cells embedded in PRP gel or PPP gel (PRP/cells complex or PPP/cells complex) – Before transplantation, the expression of bone-related markers in the embedded cells in PRP gel or PPP gel was analyzed by RT–PCR. Briefly, a total of 1×10^6 cells embedded in 100 µl of PRP gel or PPP gel were cultured in α-MEM containing 10% FBS for 7 d. Next, the PRP/cells complex or PPP/cells complex was transferred to a new plate and washed several times with PBS. Total cellular RNA was extracted using Sepasol-RNA I Super (Nacalai Tesque Inc.). Contaminating genomic DNA was eliminated by digestion with DNAse I (Invitrogen Corp., Carlsbad, CA, USA) at 37°C for 30 min, followed by heating to 75°C for 5 min to inactivate the enzyme. cDNA was reverse-transcribed from 300 ng of total RNA by Superscript II RNase H- (Invitrogen Corp.) at 37°C for 30 min, followed by heating to 75°C for 5 min to inactivate the enzyme. DNA amplification was performed using a Taq Qiagen PCR core kit (Qiagen GmbH, Hilden, Germany) with gene-specific primers (Table 1) in accordance with the manufacturer's protocol. The PCR conditions were: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C or 60°C (Osterix: Osx) for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 3 min. The amplified products were separated in a 5% agarose gel and visualized by ethidium bromide staining.

Statistical analysis – Quantitative values were collected in three sets and expressed as the mean ± standard deviation (SD). Statistically significant differences were analyzed using two-tailed Student's *t*-tests for unpaired samples, and *p*-values of < 0.05 were considered significant.

Cell labeling – Before transplantation, the cells were labeled with fluorescent carbocyanine (SP-DiOC$_{18}$; Molecular Probes Inc., Eugene, OR, USA), which acts as a lypophilic tracer. Cells were incubated with the fluorescent carbocyanine tracer (4 µg/ml) for 5 min at 37°C, washed once with PBS, and then embedded in PRP gel. After fixation with acetone, the cells were observed using fluorescence microscopy.

In vivo studies

Transplantation – Female SCID mice (10 wk of age; $n = 7$; Clea Japan Inc., Tokyo, Japan) were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal Injection®; Dainippon Pharmaceutical Co. Ltd, Osaka, Japan), and their dorsal hair was removed with hair clippers. A total of 1×10^6 labeled cells were gently mixed in 100 µl of PRP or PPP, followed by the addition of thrombin/$CaCl_2$. Immediately, 100 µl of the PRP/cells complex, PPP/cells complex, PBS/cells, PRP alone, PPP alone or PBS alone was injected subcutaneously into the skin on the back using a Tuberculin syringe® (Terumo Medical Corp., Tokyo, Japan). The lesions were allowed to develop for 4 wk before the mice were killed by diethyl ether anesthesia. The transplants were examined by soft X-ray analyses.

Preparation of tissue samples and histological observation – Following fixation with 10% paraformaldehyde, the tissue samples containing radiographically recognizable lesions were decalcified in 5% EDTA, embedded in paraffin according to a routine method and sectioned. Next, the 4-µm sections were deparaffinized, hydrated, and stained with hematoxylin and eosin. Some of the sections were observed under a fluorescence microscope to detect the labeled cells.

Immunohistochemistry – Osteoblastic differentiation of the labeled cells after subcutaneous transplantation was examined immunohistochemically. Specifically, 4-µm sections were incubated with either a rabbit anti-mouse osteocalcin (OCN) immunoglobulin G (IgG) (Cosmo Bio LSL, Tokyo, Japan) or a rabbit anti-mouse type I collagen IgG (Biogenesis Ltd, Poole, UK) for 1 h at room temperature. Control

Table 1. Gene-specific primer sequences, annealing temperatures (Tm) and thermal cycles in polymerase chain reaction (PCR) amplification

Target gene	Primer sequence (5′–3′)		Fragment size (bp)	Tm	cycles
Runx2	Forward:	ACTTTCTCCAGGAAGACTGC	380	55	35
	Reverse:	GCTGTTGTTGCTGTTGCTGT			
Osx	Forward:	CTGGGGAAAGGAGGCACAAAGAAG	497	60	35
	Reverse:	GGGTTAAGGGGAGCAAAGTCAGAT			
ALP	Forward:	GCCCTCTCCAAGACATATA	372	55	35
	Reverse:	CCATGATCACGTCGATATCC			
BSP	Forward:	GAGCCAGGACTGCCGAAAGGAA	653	55	35
	Reverse:	CCGTTGTCTCCTCCGCTGCTGC			
OCN	Forward:	CAGCTTGGTGCACACCTAGC	243	55	35
	Reverse:	AGGGTTAAGCTCACACTGCTCC			
GAPDH	Forward:	TGAAGGTCGGTGTGAACGGATTTGGC	983	55	35
	Reverse:	CATGTAGGCCATGAGGTCCACCAC			

ALP, alkaline phosphatase; BSP, bone sialoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OCN, osteocalcin; Osx, Osterix.

sections were incubated with a mouse isotype-control IgG. Immunoperoxidase labeling was performed with an LSAB® kit (Dako, Kyoto, Japan) using diaminobenzidine (DAB) precipitation for detection.

Results

The mouse platelet count in the whole blood was $635 \pm 141 \times 10^3/\mu l$ [mean ± standard deviation (SD)]. The concentrated mouse platelet count in the PRP was $1844 \pm 206 \times 10^3/\mu l$, whereas the decreased count in the PPP was $92 \pm 33 \times 10^3/\mu l$ (Table 2). Therefore, the platelet concentration was 20-fold higher in PRP than in PPP. TGF-β1 was principally found in the supernatant of the PRP gel (590 ± 55 ng/ml), but was present only in small amounts (35 ± 4 ng/ml) in the supernatant of the PPP gel (data not shown).

Effects of PRPGS on osteoblastic differentiation and mineralization

We tested whether the addition of PRPGS induces osteoblastic differentiation and mineralization of MC3T3-E1 cells, using ALP staining and von Kossa staining, respectively (Fig. 1). PRPGS enhanced ALP staining of MC3T3-E1 cells after 21 d of culture, compared with only slight ALP staining in the absence of PRPGS, indicating that PRPGS is effective for promoting osteoblastic differentiation. Furthermore, PRPGS promoted the formation of mineralized nodules after 21 d of culture.

Ultrastructural observation of cells embedded in PRP gel

SEM was used to confirm how embedding MC3T3-E1 cells in PRP gel affects their morphological changes (Fig. 2). PRP gel without embedded cells appeared to have an almost homogenous structure, consisting of multiple fibrin fibers (Fig. 2A, inset). MC3T3-E1 cells cultured in PRP gel showed a spindle-shaped morphology on the surface of the gel (Fig. 2B).

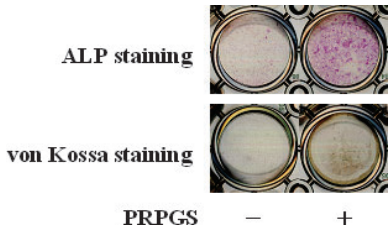

Fig. 1. Alkaline phosphatase (ALP) staining and von Kossa staining of MC3T3-E1 cells after 21 d of culture in the presence or absence of platelet-rich plasma gel supernatant (PRPGS). After reaching subconfluence, cells (2×10^4 cells/well) were grown in α-minimal essential medium (MEM) supplemented with 0.5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin, in the presence or absence of 0.5% PRPGS, for up to 21 d. The medium was changed every 1–2 d. ALP staining and von Kossa staining of MC3T3-E1 cells were determined in 24-well plates. The experiment was repeated more than three times, and one representative result is shown.

Expressions of bone-related marker mRNAs by cells embedded in PRP gel or PPP gel

Cells embedded in PRP gel or PPP gel expressed Runx2, Osx, ALP and bone sialoprotein (BSP) mRNAs (Fig. 3). In particular, the PRP gel appeared to enhance the expression of Osx and BSP mRNAs compared with the PPP gel. OCN mRNA was weakly expressed in both the PRP gel and the PPP gel.

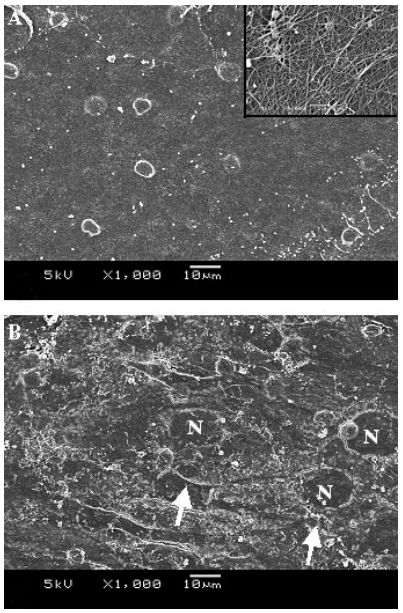

Fig. 2. Scanning electron microscopy (SEM) of platelet-rich plasma (PRP)/cells complexes. A total of 5×10^5 cells were mixed with 50 μl of PRP gel. After 2 d of culture, the cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1 h and observed by scanning electron microscopy (SEM). PRP gel alone consists of multiple fibrin fibers (A, inset) and some blood cells can be observed (A). MC3T3-E1 cells display a spindle-shaped morphology on the surface of the gel (B). (N, nucleus) Original magnification ×1000 (A: inset: ×8000). The experiment was repeated three times and one representative result is shown.

Transplantation

MC3T3-E1 cells were labeled using fluorescent carbocyanine and then embedded in PRP gel or PPP gel (Fig. 4). The presence of labeled cells was confirmed by analysis under a fluorescence microscope (Fig. 4B). The gel/cells complexes were then transplanted subcutaneously in the skin at sites on the right side of the back (Fig. 5A). Soft X-ray analysis clearly showed the presence of mineralized deposits at sites injected with a PRP/cells complex, while no such deposits were observed at other sites (Fig. 5B–D). The mineral deposits were detected in all the PRP/cells complex sites. Respectively, the ratio of detected sites of newly mineralized deposits per

Table 2. Platelet, red blood cell and white blood cell counts in platelet-rich plasma (PRP), platelet-poor plasma (PPP) and blood

	Platelet (10^3 cells/μl)	Red blood cell (10^6 cells/μl)	White blood cell (10^3 cells/μl)
PRP	1844 ± 206	0.01 ± 0.01	0.17 ± 0.10
PPP	92 ± 33	0.01 ± 0.01	0.02 ± 0.00
Blood	635 ± 141	8.10 ± 0.28	3.73 ± 0.46
PRP:PPP ratio	20	1	8.5
PRP:blood ratio	2.90	0.001	0.046

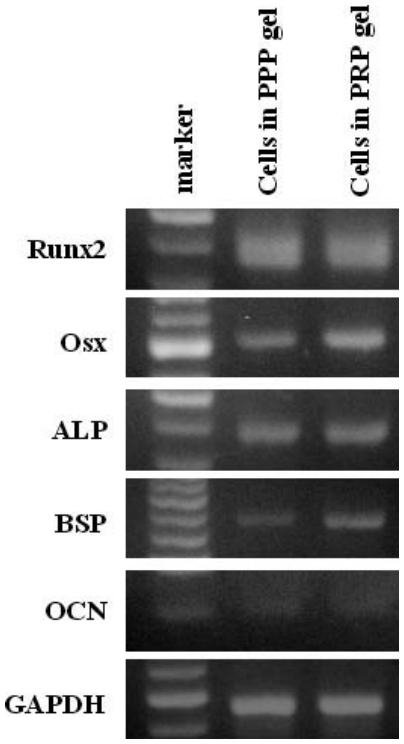

Fig. 3. Reverse transcription–polymerase chain reaction (RT–PCR) analysis for the expression of bone-related markers in MC3T3-E1 cells embedded in platelet-rich plasma (PRP) gel or platelet-poor plasma (PPP) gel. A total of 1×10^6 cells, embedded in 100 μl of PRP gel or PPP gel, were cultured in α-minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) for 7 d. Next, each PRP/cells complex or PPP/cells complex was transferred to a new plate and washed several times with phoshate-buffered saline (PBS). Total RNA was extracted from each complex and subjected to RT–PCR analysis to determine the expression of Runx2 (380 bp), Osterix (Osx; 497 bp), alkaline phosphatase (ALP; 372 bp), bone sialoprotein (BSP; 653 bp) and osteocalcin (OCN; 243 bp) mRNAs. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) product (983 bp) in each sample is shown as an internal control. The experiment was repeated three times and one representative result is shown.

injected sites of PRP/cells complex, PPP/cells complex, PBS/cells, PRP alone, PPP alone and PBS alone was 3/3, 0/2, 0/2, 0/3, 0/2 and 0/2.

Immunohistochemistry

The mineralized tissue was carefully removed and tissue sections were examined in detail. The periphery of the mineralized tissue was surrounded by adipose tissue (Fig. 6A,B). The mineralized tissue included the labeled cells, indicating that they had formed the mineralized deposits (Fig. 6C,D). Furthermore, the labeled cells expressed OCN (Fig. 7B), indicating that the transplanted cells had differentiated into mature osteoblasts. Moreover, the mineralized tissue contained type I collagen, which represents the main bone matrix protein (Fig. 7D).

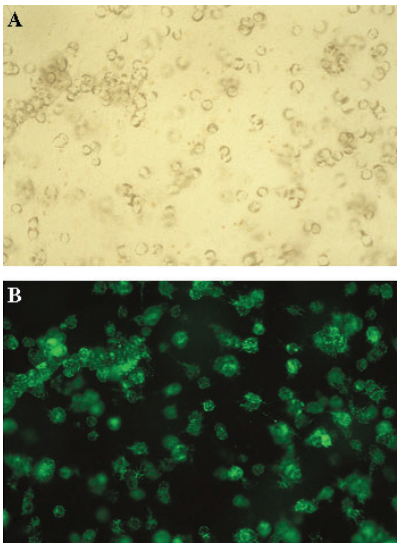

Fig. 4. Micrograph of cells labeled with fluorescent carbocyanine and embedded in platelet-rich plasma (PRP) gel (PRP/cells complex). Cells were incubated with the fluorescent carbocyanine tracer (4 μg/ml) for 5 min at 37°C, washed once with phosphate-buffered saline (PBS) and then embedded in PRP gel. After fixation with acetone, the cells were observed under a fluorescence microscope. (A) Photograph of the cells under a light microscope. Original magnification ×200. (B) Photograph of the cells under a fluorescence microscope. The labeled MC3T3-E1 cells embedded in the PRP gel are highly fluorescent (green). Original magnification ×200. The experiment was repeated three times and one representative result is shown.

Discussion

We have shown that PRP gel directly promotes osteoblastic differentiation followed by bone formation after subcutaneous transplantation in SCID mice. These results suggest that PRP gel produces a beneficial effect on MC3T3-E1 cells *in vivo*, as evaluated histologically, and further suggest the possibility of its use, in combination with autologous biomaterials, in transplantation.

Local application of PRP to enhance the maturity of bone grafts for repairing bone defects of the maxillofacial region was originally recommended by Marx *et al.* (3). Since then, PRP has been used, in combination with bone graft materials or autologous bone, for clinical application, as a result of its poor benefit in large bone defects when applied alone (17–19,26). However, bone graft materials have limitations to their quality, while autologous bone grafts cause increased morbidity, as harvesting the autologous bone requires additional surgery. To minimize these risks, we attempted to develop an application involving PRP gel combined with osteoblasts for large bone defects and to evaluate the beneficial effects after subcutaneous transplantation into SCID mice.

In the present study, we needed to confirm the degranulation of platelets in PRP or PPP by thrombin before conducting biological activity and transplantation experiments. Degranulation of platelets causes them to release a variety of growth factors, such as PDGF, TGF-β2, IGF and EGF. Therefore, TGF-β1 was quantified as an indicator of degranulation of platelets. The TGF-β1 content in PRP (590 ± 55 ng/ml) and PPP (35 ± 4 ng/ml) in the present study indicated that platelets in PRP and PPP were degranulated by thrombin (data not shown). Growth factors derived from platelets influence cell activity differently and raise the possibility that combining factors may enhance the biological activity of cells (27).

In a previous study, PRP was found to increase the proliferation of osteoblasts derived from osteosarcoma, such as HOS and SaOS-2 cell lines (15), and immortalized osteoblasts (hFOB1.19), depending on the PRP concentration (28). Graziani *et al.* also reported that primary human bone cells showed a marked increase of proliferation in PRP containing 2.3-fold concentrated platelets compared with baseline blood (29).

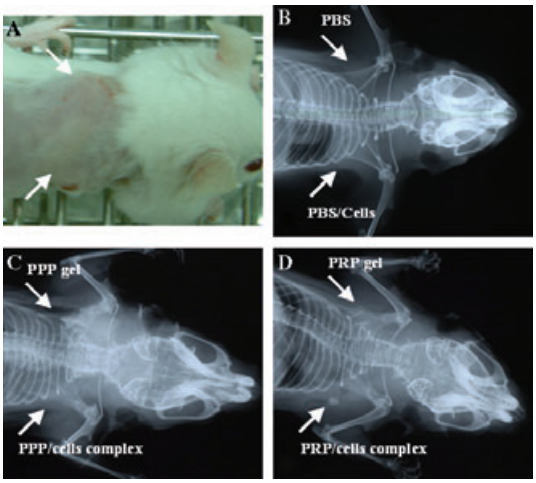

Fig. 5. Soft X-ray radiographs obtained 4 wk after transplantation. Severe combined immunodeficient (SCID) mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Phosphate-buffered saline (PBS) or PBS/cells ($n = 2$), platelet-poor plasma (PPP) gel or PPP/cells complex ($n = 2$) and platelet-rich plasma (PRP) gel or PRP/cells complex ($n = 3$) were injected into the right or left sites on the back of the SCID mice (A). Note that a radiographic region in the skin (arrow) is only found at sites injected with a PRP/cells complex. (B) Injection with PBS or PBS/cells. (C) Injection with PPP gel or PPP/cells complex. (D) Injection with PRP gel or PRP/cells complex.

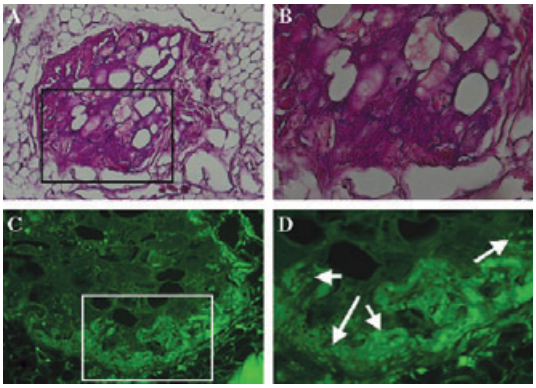

Fig. 6. Histological features of the mineralized tissue 4 wk after transplantation. Cross-sections were stained with hematoxylin and eosin (A,B) or observed under a fluorescence microscope (C,D). Note that the mineralized tissue contains the MC3T3-E1 cells labeled with fluorescent carbocyanine before the transplantation. (A,C) Original magnification ×100; (B,D) high magnification ×200.

However, PRP containing highly concentrated platelets seems to induce a decreased proliferation of alveolar bone cells (30). PRP used in the present study contained 2.9-fold concentrated platelets (Table 2). In our preliminary study, PRP induced the increase in proliferation of MC3T3-E1 cells (data not shown). These studies suggest that an optimal concentration of platelets would need to be established before PRP could be used clinically in humans.

In the present study, PRPGS enhanced the ALP activity in MC3T3-E1 cells and slightly promoted mineralized nodule formation (Fig. 1). These results suggest that PRP directly promotes osteoblastic differentiation *in vitro*, consistent with the results of previous studies (15,16,31,32). Calcification in MC3T3-E1 cells *in vitro* is also induced by polyphosphate (33). However, ALP may not be involved in polyphosphate metabolism in MC3T3-E1 cells (34). Accordingly, the effect of PRP seems to be different from that of polyphosphate in ALP activity.

We transplanted PRP/cells complexes subcutaneously in the skin to evaluate clinically the effect of PRP application on bone formation. In addition, we used SCID mice to avoid immunological rejection after transplantation of the allogeneic cells and PRP. The gelatinization of PRP made it easy to transplant the cells into the subcutaneous tissue as a fibrin glue, which mainly consists of fibrinogen and fibronectin and has been used as a three-dimensional matrix in previous studies (35–37). Before transplanting the cells, the expression of bone-related markers was confirmed by RT–PCR analysis after culturing the PRP/cells complexes for 7 d (Fig. 3). Interestingly, PRP gel enhanced the mRNA expression, including that of Osx and BSP, in MC3T3-E1 cells. In contrast, PRP gel did not enhance the level of OCN mRNA in the present study. Therefore, PRP gel appears to enhance osteoblastic differentiation of MC3T3-E1 cells, but does not induce their final differentiation.

In the transplantation experiment, the cells were labeled with fluorescent carbocyanine in advance in order to clarify the presence of transplanted cells. Although we could not identify bulges that may have been hard tissue at any of the injected sites, we were able to distinguish the presence of mineralized deposits radiographically and found that these deposits corresponded to the sites injected with a PRP/cells complex.

The mineralized deposits were examined histologically to confirm the existence of, and to characterize, transplanted cells in the deposits. We confirmed the presence of transplanted cells using fluorescence microscopy. Labeled cells were observed within the mineralized tissue, indicating that the transplanted cells had formed the deposits in the skin (Fig. 6). The sections were then investigated immunohistochemically for the degree of differentiation of the transplanted cells *in vivo*. The labeled cells showed positive staining for OCN, indicating that the transplanted cells had begun to synthesize this molecule during the

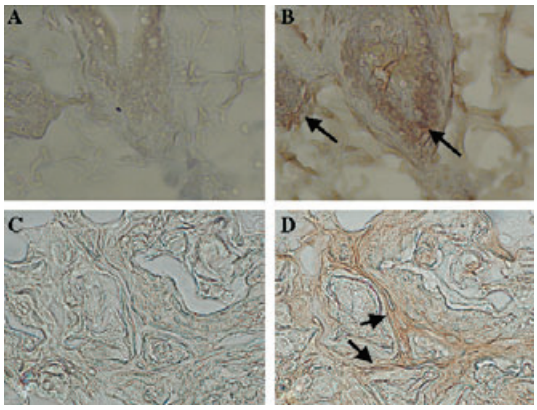

Fig. 7. Immunohistochemical staining of osteocalcin (OCN) and type I collagen in the mineralized tissue 4 wk after transplantation. (B) OCN (arrows) is detected in the mineralized tissue and the cells present in the mineralized tissue express OCN. (D) Type I collagen (arrows) is observed in the mineralized tissue. (A,C) Serial sections stained with control immunoglobulin G (IgG). (A–D) Original magnification ×400.

process of osteoblastic differentiation and mineralization in the skin after transplantation (Fig. 7).

Thus, PRP gel appears to have played an important role as a delivery matrix for transplanting cells in the present study, as fibrin glue has the same functions for cell transplantation and as a matrix (35,37,38). The application of PRP gel with mesenchymal stem cells (MSCs) has been reported for tissue-engineered bone regeneration (23,39). Kotobuki *et al*. has reported that MSCs can be harvested from an aspiration of fresh bone marrow and subcultured under osteogenic conditions, after which they can differentiate into osteoblasts (40). Thus, our procedure for transplantation of PRP/osteoblasts complexes can be used for treating various types of bone defects caused by periodontitis.

In conclusion, we have demonstrated that the application of a PRP/osteoblasts complex has beneficial effects for promoting cell differentiation and bone formation. Therefore, the application of PRP gel in combination with osteoblasts may provide clinically beneficial effects for the transplantation of genetically engineered cells into bone defects.

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