The effect of the platelet concentration in platelet-rich plasma gel on the regeneration of bone

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The aim of our study was to investigate the effect of platelet-rich plasma on the proliferation and differentiation of rat bone-marrow cells and to determine an optimal platelet concentration in plasma for osseous tissue engineering. Rat bone-marrow cells embedded in different concentrations of platelet-rich plasma gel were cultured for six days. Their potential for proliferation and osteogenic differentiation was analysed. Using a rat limb-lengthening model, the cultured rat bone-marrow cells with platelet-rich plasma of variable concentrations were transplanted into the distraction gap and the quality of the regenerate bone was evaluated radiologically.

Cellular proliferation was enhanced in all the platelet-rich plasma groups in a dose-dependent manner. Although no significant differences in the production and mRNA expression of alkaline phosphatase were detected among these groups, mature bone regenerates were more prevalent in the group with the highest concentration of platelets.

Our results indicate that a high platelet concentration in the platelet-rich plasma in combination with osteoblastic cells could accelerate the formation of new bone during limb-lengthening procedures.

There is currently an increased interest in the use of platelet-rich plasma for bone regeneration and healing. It contains osteo-inductive growth factors including platelet-derived growth factors, vascular endothelial growth factor, insulin-like growth factor and transforming growth factors.1-5 These cause the proliferation and differentiation of local osteoprogenitor cells into bone-forming cells, leading to mineralisation and the formation of bone matrix.6,7 It has been shown, however, that variations in the concentration of platelets in the platelet-rich plasma have diverse effects on the proliferation and differentiations of the osteoblasts.8-14 Choi et al8 reported that the viability and proliferation of alveolar bone cells were suppressed by a high, and stimulated by a low concentration of platelets in the platelet-rich plasma. Uggeri et al14 found that proteins released from platelet gel stimulated the proliferation of osteoblasts in a dose-dependent manner. In order to provide clear evidence for the clinical use of platelet-rich plasma, it is necessary to determine its direct effect on osteogenic cells at the cellular and molecular level.

Platelet-rich plasma is an autologous preparation and there are therefore no concerns about the transmission of disease or an immunogenic reaction. There have been several clinical trials using a combination of platelet-rich plasma and bone graft or osteoprogenitor cells which have aimed at increasing the rate of osteogenesis and the enhancement of the formation of bone.4-19

We have established a new technique of transplantation using culture-expanded bone-marrow cells and platelet-rich plasma in distraction osteogenesis of the long bones and demonstrated a satisfactory clinical outcome by accelerating the formation of new bone.15-17 To improve our combined bone-marrow cells and platelet-rich plasma cell therapy further, it was necessary to determine the optimal platelet concentration of the plasma for bone regeneration.

Our aim therefore was to evaluate the osteogenic differentiation of rat bone-marrow cells embedded in platelet-rich plasma gels with different platelet concentrations. Using a rat model of limb lengthening, the rat bone-marrow cells and platelet-rich plasma gels were then transplanted into the lengthened femur, and bone formation in vivo was analysed.

Materials and Methods
Preparation of platelet-rich plasma gels with different platelet concentrations. Under general anaesthesia whole blood was withdrawn...
by cardiac puncture from 90 14-week-old male Sprague-Dawley rats (Japan SLC Inc., Shizuoka, Japan). For one series, 40 ml of anticoagulated whole blood was initially centrifuged at 1100 g for ten minutes to precipitate the red blood-cell fraction. The supernatant was again centrifuged to produce precipitation of platelets. The platelet-rich plasma was then divided into three groups (low concentration; medium; and high concentrations) according to the amount of supernatant which had been removed. We used platelet concentrations as suggested by Marx. The supernatant alone was used as platelet-poor plasma. The number of platelets in the high, medium, low concentrate, platelet-poor plasma and whole blood was counted using a Sysmex XE 2100 haematology analyser (Sysmex, Kobe, Japan).

**Bone-marrow cells culture.** Specially-prepared minimum essential medium alpha (Gibco BRL, Carlsbad, California), supplemented with 10% fetal bovine serum (Dainippon Pharmaceutical, Osaka, Japan), 100 μg/ml of penicillin-streptomycin (Gibco-BRL, Life Technologies, Grand Island, New York), 0.2 mM ascorbic acid (Sigma, St Louis, Missouri) 10 mM Na-b-glycerophosphate (Sigma), and 10 μM dexamethasone (Sigma), was used as a growth medium in all the cell-culture experiments.

Rat bone-marrow cells were isolated from 60 four-week-old, male Sprague-Dawley rats weighing between 90 g and 110 g using the technique described by Takamine et al. The harvested cells were cultured with 8 ml of growth medium. The adherent cells were expanded as monolayer cultures in a 5% CO₂/95% air atmosphere at 37°C with medium changes every three days. When the cultures became nearly confluent, the cells were dissociated with 0.25% trypsin/ethylene diamine tetraacetic acid (EDTA) (Gibco BRL) and re-seeded at a density of 3 × 10⁵ cells/dish. Cells passaged twice (P2) were used for all three-dimensional (3-D) cultures.

**3-D gel-embedded culture of rat bone-marrow cells.** A suspension of 8 × 10⁴ rat bone-marrow cells in 150 μl of each platelet derivative was mixed with 50 μl of a thrombin/CaCl₂ solution to obtain platelet gel. The rat bone-marrow cells were also mixed with bovine-dermal-pepsin-solubilised type-I collagen (Koken Co., Ltd, Tokyo, Japan) in gel form at 37°C. The rat bone-marrow cells embedded in gel at a density of 4 × 10⁵ cells/ml were cultured for six days and were then divided into five groups (group P, embedded in the platelet-poor plasma gel; group L, embedded in the low concentration gel; group M, embedded in the medium concentration gel; group H, embedded in the high concentration gel; and group C, embedded in the collagen gel).

**In vitro cell proliferation.** The WST-1 cell proliferation reagent (Roche Diagnostics GmbH, Mannheim, Germany) was used for counting the cells. The WST-1 test measures the mitochondrial activity which corresponds to the number of viable cells. After incubation for one hour at 37°C, the absorption of the medium containing the WST-1 reagent was measured by an enzyme-linked immunosorbent assay (ELISA) Reader (Thermo Fisher Scientific Inc., Waltham, Massachusetts) at 440 nm with a reference wavelength > 600 nm. The WST-1 assay was carried out at intervals of two days after the initiation of the 3-D cultures. Each experiment was repeated twice with 20 samples for each group, resulting in a total of 40 samples per group.

**In vitro osteogenic differentiation.** The cultures were washed in phosphate-buffered saline and the cells were lysed using a UP50H ultrasonic processor (Hielscher Ultrasonics GmbH, Teltow, Germany). Using the alkaline phosphatase-B-test (Wako, Osaka, Japan), the activity of alkaline phosphatase in the cell lysates was measured every two days after embedding of the cells.

After the gels had been dissolved by urokinase (Uronase; Mochida, Tokyo, Japan) and collagenase (Sigma-Aldrich, Tokyo, Japan), the total RNA was isolated every two days using the RNeasy Mini Kit (QIAGEN KK, Tokyo, Japan). For reverse transcription into circular DNA (cDNA) (reverse transcription system; Perkin Elmer, Waltham, Massachusetts), one μg of RNA was used as a template. cDNA was amplified by the polymerase chain reaction with oligonucleotide primers for alkaline phosphatase and glyceraldehyde-3-phosphate dehydrogenase as housekeeping genes.

Quantitative real-time was carried out using a Light Cycler 480 Real-Time System (Roche Diagnostics Corporation, Indianapolis, Indiana) and a Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics Corporation). The cultures were washed in phosphate-buffered saline and the cells were lysed using a UP50H ultrasonic processor (Hielscher Ultrasonics GmbH, Teltow, Germany). Using the alkaline phosphatase-B-test (Wako, Osaka, Japan), the activity of alkaline phosphatase in the cell lysates was measured every two days after embedding of the cells.

All the animal experiments were carried out in compliance with the laws and guidelines for the experimental use and care of animals. Application of an external fixation device to the femora of 91 nine-week-old male Sprague-Dawley rats weighing between
320 g and 380 g was followed by an osteotomy at the level of the diaphysis. Weight-bearing as tolerated was allowed immediately after the operation. Seven days after operation lengthening was initiated at a rate of 0.375 mm twice daily for ten days (7.5 cm of total distraction). Immediately after the completion of lengthening, 150 $\mu$l of each type of gel containing rat bone-marrow cells at a density of $1 \times 10^7$ cells/ml were injected into the distraction callus under fluoroscopic guidance. The rats were divided into five groups, according to the type of injected gel as follows: group P ($n = 16$), group L ($n = 20$), group M ($n = 20$), group H ($n = 20$), and group C ($n = 15$).

**Radiological evaluation.** This was performed using a soft radiograph apparatus (Softex ES/M; Softex Co, Tokyo, Japan). Lateral radiographs were taken at one, two, and four weeks after injection of gel. They were evaluated using image-analysis software (Scion Image for Windows, Scion Co, Frederick, Maryland). The distraction gap was outlined as a quadrilateral region of interest from the outside corners of the two proximal and the two distal cortices. Mineralised new bone was defined as any region with a density equivalent to or greater than the adjacent medullary bone.

**In vivo micro-CT.** Representative specimens of distracted femora (six rats in each group, 30 in total) were examined using a high-resolution micro-CT imaging system (Scanmate-A100S; Comscantecno Co. Ltd, Kanagawa, Japan) four weeks after injection of bone-marrow cells. Volumetric measurements of mineralised new bone were made using 3-D image analysis software (TR1/3D-BON; Ratoc System Engineering Co. Ltd, Tokyo, Japan). The volume of the distraction gap was also recorded and it represented the volume of interest which was mineralised.

**Statistical analysis.** Equality of variances was verified using the Bartlett test (Stat View for Windows version 5.0; SAS Institute Japan Ltd, Tokyo, Japan). One-way analysis of variance was used for comparison of groups, supported by the Bonferroni-type multiple comparison. Statistical significance was set at a p-value $\leq 0.05$. All the results are expressed as the mean and SEM ($\pm$).

**Results**

**Properties of platelet-rich plasma and platelet-poor plasma.** The mean concentration of platelets (platelets/$\mu$l) was $4358 \pm 265 \times 10^3$ in high concentration, $1453 \pm 88 \times 10^3$ in medium concentration, $48 \pm 29 \times 10^3$ in low concentration, $8 \pm 2 \times 10^3$ in platelet-poor plasma and $413 \pm 54 \times 10^3$ in whole blood. The mean concentration of platelets in the low, medium and high concentration groups was 117% (76% to 146%), 352% (263% to 441%) and 1055% (695% to 1233%) of that in whole blood, respectively. There was a ninefold difference in the mean platelet concentration between the low and high concentration groups. There was no significant difference in the concentration of fibrinogen among the groups.

**In vitro cell proliferation.** Group H showed a significant increase in the proliferation of rat bone-marrow cells compared with the remaining groups on days 2, 4 and 6 (analysis of variance [ANOVA], $p < 0.05$). By contrast, groups L and M did not show increased cell proliferation, compared with group P (Fig. 1).

**In vitro osteogenic differentiation.** On day 6, the alkaline phosphatase activity was significantly increased in group C (ANOVA, $p < 0.05$), compared with the other groups (Fig. 2a). Group P L, M and H showed no significant increase in
alkaline phosphatase activity. Similarly, the expression of alkaline phosphatase mRNA was significantly increased in group C (ANOVA, p < 0.05) while there was no significant differences in the other groups (Fig. 2b).

**Radiological evaluation.** The formation of callus was enhanced in a platelet dose-dependent manner among the scaffolds. At four weeks after completion of distraction, radiological union was evident in group H only. In the
remaining groups, the central radiolucent area (fibrous zone) was still present (Fig. 3). In group H, satisfactory formation of callus was seen soon after the injection of the rat bone-marrow cells, resulting in an early bridging of the distraction gap.

Significantly larger areas of mineralised bone were found in group H than in the other groups at four weeks after injection of the rat bone-marrow cells (ANOVA, p < 0.05). There were no significant differences in bone formation among groups P, L, M, and C (Fig. 4).

**Micro CT.** Favourable bone formation was observed in groups H and C (Fig. 5). Quantitative 3-D CT showed that the volume of the distraction gap was largest in group H, although there were no statistically significant differences among any group (Fig. 6).

**Discussion**

We have previously shown a satisfactory outcome in femoral lengthening enhanced by transplantation of culture-expanded bone-marrow cells and platelet-rich plasma into the callus. However, the beneficial effect of cell therapy on osteogenesis was less pronounced at the anteromedial aspect of the tibia. To improve our bone-marrow cells and platelet-rich plasma into the callus. The positive effect on osteogenesis in group H may have resulted from the increased potential for proliferation of the osteoblastic cells within the gels.

Platelet-rich plasma with a higher platelet concentration significantly enhanced the proliferation of rat bone-marrow cells, although the rate of differentiation of osteoblasts was not accelerated. However, the effect of platelet-rich plasma on the proliferation and differentiation of osteoblastic cells is still controversial. Choi et al. using canine platelet-rich plasma and alveolar bone cells, showed that the viability and proliferation of the latter were suppressed by high, but were stimulated by low concentrations of platelet-rich plasma. Kanno et al. noted that human platelet-rich plasma inhibited activity in the osteoblastic cell line during the growth phase, but stimulated it when the cells attained confluence. Graziani et al. showed that moderate platelet concentrations in human platelet-rich plasma stimulated the differentiation of human osteoblasts. Lucarelli et al. stated that 10% platelet-rich plasma promoted the proliferation of stromal stem cells derived from human bone marrow. The results of previous studies may differ from our findings because of the different origins of platelet-rich plasma used or cell types examined. The amount of platelet-rich-plasma-derived growth factors varied depending on the animal species, and the cellular response to these growth factors may also depend on the phenotype of the cells tested. However, similar results were reported by Arpornmaeklong et al. and Ogino et al. who analysed the effect of platelet concentrations in rat platelet-rich plasma on the proliferation and differentiation of rat bone-marrow cells. Arpornmaeklong et al. noted that platelet-rich plasma caused a dose-dependent stimulation of cell proliferation while reducing alkaline phosphatase activity and calcium deposition in the 3-D culture. Ogino et al. showed that platelet-rich plasma stimulated the proliferation but suppressed the differentiation in the monolayer culture system. These studies suggested that platelet-rich plasma could have a beneficial effect on the proliferation of bone-marrow cells, without promoting osteoblastic differentiation.

The quality of the regenerate bone was significantly improved after transplantation of rat bone-marrow cells with a plasma with a higher platelet concentration, although these cells showed no increase in alkaline phosphatase activity in the in vitro 3-D culture, compared with gels with a lower concentration of platelets. We have previously observed elevated levels of alkaline phosphatase activity in rat P2 cells cultured with differentiation medium containing dexamethasone. In the present study, the embedded rat P2 bone-marrow cells used for transplantation could have differentiated into an osteoblastic phenotype during the monolayer culture before transplantation into the callus. The positive effect on osteogenesis in group H may have resulted from the increased potential for proliferation of the osteoblastic cells within the gels.
Rat bone-marrow cells embedded in collagen gels showed the most favourable effect on osteogenic differentiation in vitro, and they also resulted in good bone formation in vivo. Type-I collagen, as a carrier, is considered to maintain the phenotype of osteoblasts, and the combination of cultured osteoblasts and type-I collagen is commonly-used in tissue engineering.\(^{25,26}\) We have previously demonstrated that culture-expanded bone-marrow cells mixed with collagen gel accelerated the maturation of regenerate bone and shortened the consolidation period during distraction osteogenesis in rats.\(^ {20}\) Type-I collagen is, however, typically obtained from bovine hides and has the associated risks of immune reaction and disease transmission. On the other hand, autologous platelet-rich plasma is non-toxic and non-immunoreactive and appears to be safe for clinical use. Bone-marrow cells with a high concentration of platelet-rich plasma showed lower alkaline phosphatase activity and mRNA expression compared with those with collagen gels, but transplantation of bone-marrow cells and high concentration platelet-rich plasma significantly improved the radiological appearance of regenerate bone. Quantitative micro-CT showed the largest bone volume within the distraction gap of group H, but the small sample sizes precluded the determination of any statistically significant difference between groups C and H. Therefore, a platelet-rich plasma with a higher concentration of platelet could be a safe substitute for collagen scaffolds for bone-marrow cell therapy in distraction osteogenesis.

In conclusion, a higher concentration of platelets in the platelet-rich plasma gel stimulated the proliferation of rat bone-marrow cells, but did not promote osteoblastic differentiations. Rat bone-marrow cells with a higher concentration of platelets in the platelet-rich plasma had the most favourable effect on osteogenesis in the rat limb-lengthening model. Our results indicate that in clinical practice platelet-rich plasma with a high concentration of platelets may be a useful adjunct for bone regeneration during distraction osteogenesis.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.
References


