9. 現在の後療法

術後 12 時間はベッド上で荷重をかけずに臥床安静とし，以後半硬性胸腰仙椎装具を用いて離床する。以前は 72 時間の非荷重安静としていたが，2010 年秋より早期硬化型 CPC が使用可能となり，術後安静期間は 12 時間に短縮している。術後 3 ヵ月は少なくとも装具固定とする。

症例 1 82 歳・女性　L 1 椎体圧潰

椎体圧潰が高度で椎体は扁平椎化していた。寝起きの動作時に最も強い激しい腰痛が主訴であった。仰臥位で撮像した脊髄造影 CT では，圧潰椎体内に vacuum cleft が生じて整復されていた。後壁損傷を認め，脊柱管内突出骨片が脊髄円錐部レベルを軽度圧迫するも，膀胱直腸障害をはじめ下肢の神経症状を認めなかった(図 8)。本例に CPC 椎体形成術を行った。術後 12 時間で体幹装具装着下に離床し，腰痛は著明に減少して歩行を開始できた。術後 2 週時の CT でも CPC 塊に fragmentation を認めなかった。このような扁平椎では，椎体前側壁の cortical shell が完全に破綻しており，術後それが再建されるまでの間，椎体内部に充填された CPC が主として荷重を担うことになる(図 9)。現在術後 1 年を経過したが CPC に fragmentation などの不具合を認めない。

まとめ

骨粗鬆症性椎体圧潰の病態は新鮮圧迫骨折のそれとは明らかに異なり，病態に応じた適切な対応が必要である。骨粗鬆症性椎体圧潰に対する CPC 椎体形成術は，急性期圧迫骨折を対象とし，完全に標準化された BKP 術式と比べると，多少複雑できめ細かい配慮が必要であるが，いくつかのチェックポイントさえ押さえていれば，安全かつ有効であり，逆に自由度が高く，術者の意図が結果に反映されやすい術式である。

【文献】

1. 武政龍一(2010)：骨粗鬆症性椎体骨折の病態 –骨折急性期から骨癒合不全および遅発性神経麻痺発症の病態まで–. 関節外科 29: 10–17.
2. 武政龍一，谷　俊一(2010)：骨粗鬆症性椎体骨折癒合不全に対する CPC 椎体形成術の長所と短所. *J Spine Res* 1: 1260–1266.
3. 武政龍一(2006)：骨粗鬆症性椎体骨折に対する椎体形成術 –リン酸カルシウムセメント(CPC)を用いた修復術– 日本整形外科学会企画・監修　整形外科卒後教育研修用ビデオ FXD–53.

4. 武政龍一(2011):骨粗鬆症性椎圧潰に対するCPC椎体形成術. 馬場久敏·他(編) Instruction 18 腰椎の手術 ベーシックからアドバンスまで必須テクニック, メジカルビュー社(東京) *OS NOW* pp 140–146.
5. 喜安克仁, 武政龍一, 谷 俊一·他(2007):血液存在下における注入充填方法の違いがリン酸カルシウム骨セメント硬化体の圧縮強度に与える影響 –椎体形成術モデルを用いた検討–. 中部整災誌 50: 61–62.

Fresh Bone Marrow Introduction into Porous Scaffolds Using a Simple Low-Pressure Loading Method for Effective Osteogenesis in a Rabbit Model

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ABSTRACT: Recent advances in tissue engineering techniques have allowed porous biomaterials to be combined with osteogenic cells for effective bone regeneration. We developed a simple low-pressure cell-loading method using only syringes and stopcocks, and examined the effect of this method on osteogenesis when applied to the combination of highly porous β-tricalcium phosphate (β-TCP) and fresh autologous bone marrow. Both block and granule β-TCP scaffolds were used to prepare implants in three different ways: without bone marrow as a control, with bone marrow that was allowed to penetrate spontaneously under atmospheric pressure (AP group), and with bone marrow that was seeded under low pressure (ULP group). These implants were transplanted into rabbit intramuscular sites, and the samples were examined biologically and histologically. The penetration efficiency of the block implants after marrow introduction was significantly higher in the ULP group than in the AP group. In the transplanted block samples, alkaline phosphatase activity was significantly higher in the ULP group at 2 weeks after implantation, and significantly more newly formed bone was observed in the ULP group at both 5 and 10 weeks compared with the AP group. Similar results were observed even in the experiment using β-TCP granules, which are smaller than the blocks and frequently used clinically. Because of its convenience and safety, this low-pressure method might be a novel, effective treatment to promote osteogenesis with bone marrow in clinical bone reconstruction surgeries. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:1–7, 2009

Keywords: low pressure; bone marrow; porous scaffold; osteogenesis; clinical application

Autologous bone is the ideal graft material for use in reconstructive orthopaedic surgery. However, its harvesting is closely associated with donor site morbidity,[1] and it is available in limited amounts. Given these problems, calcium phosphate ceramics have been increasingly used as bone substitutes.[2–5] Most have proven both biocompatible and osteoconductive; however, the materials themselves have little osteoinductive properties.[3,5] Therefore, biologically active tissue-inducing substances or cells are preferable for use with the ceramics.

Bone marrow has a good osteogenic ability when combined with porous ceramics,[6–9] and has the clinical merits of being autologous and intraoperatively available, with harvesting done by a simple, safe aspiration.[10,11] However, porous ceramics have small pores, and a large amount of remaining air can prevent marrow penetration. Bone marrow is highly viscous, which can also impair sufficient penetration. To achieve more effective bone regeneration, the efficiency with which bone marrow is introduced into porous biomaterials must be improved. Attempts to augment osteogenesis by concentrating bone marrow have been reported,[12–14] but to our knowledge, the influence of marrow loading efficiency on osteogenesis has been rarely investigated.

The importance of cell-loading efficiency has been discussed in tissue engineering using in vitro expanded bone marrow derived stromal cells (BMSCs) with porous scaffolds.[15,16] Researchers previously reported that a low-pressure environment enhances cell-loading efficiency and bone formation.[17,18] We have further developed an ameliorated low-pressure cell-loading procedure[19] in which cell soaking is conducted under a low-pressure environment, whereas previous methods used low pressure after cell soaking to remove remaining air. This "under low pressure" (ULP) loading method is more effective for loading efficiency and bone formation of in vitro expanded BMSCs when compared with conventional low-pressure methods.[19] We hypothesized that this method would also be beneficial for enhancing osteogenesis using fresh bone marrow combined with porous scaffolds.

We modified the ULP method to use only syringes and stopcocks, making it more simple and useful for clinical application. In a rabbit ectopic transplantation model, the effect of the ULP method on osteogenesis was examined using fresh autologous bone marrow with highly porous β-tricalcium phosphate (β-TCP), which is bioabsorbable with good osteoconductive properties.[5]

MATERIALS AND METHODS

This study used 28 Japanese white rabbits (Saitama Animal Laboratory, Japan), all 4 months old and weighing 2.8 to 3.2 kg. The rabbits were kept and treated according to the guidelines of the Tokyo Medical and Dental University for the care and use of laboratory animals.

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Preparation of the Implants

Three milliliters of bone marrow were aspirated percutaneously from the bilateral iliac crests of each rabbit using an 18-gauge needle and a heparinized 10-mL syringe. The harvested marrow was immediately introduced into highly porous β-TCP (Osferion®: Olympus Biomaterial, Japan), which had a 3D structure with 70% porosity with interconnecting macropores (200–400 μm) and micropores (<5 μm); this introduction was done under atmospheric pressure (AP group) or under low pressure (ULP group). The ULP method is a quite simple procedure modified from the described method,[19] using disposable syringes and stopcocks, which are available in any operating room. Briefly, the β-TCPs are set in the 20-mL syringe at the 1-mL level (Fig. 1A), and the inner cylinder is pulled to 20 mL after closing the stopcock (Fig. 1B) to create low pressure. The stopcock is then turned to seed the prepared bone marrow into the porous implants under low pressure (Fig. 1C). After tapping carefully to allow for sufficient cell penetration and removal of the surrounding air bubbles, the low pressure is released (Fig. 1D). This procedure is conducted in a completely closed system and can be completed within a minute.

In 20 rabbits, 12 β-TCP blocks (4.0 × 4.0 × 4.0 mm) blocks were prepared for each animal. Four blocks were used without bone marrow as a control group, four were used with autologous bone marrow penetrated spontaneously under atmospheric pressure (AP group), and four were used with marrow seeded under low pressure (ULP group). In the other eight rabbits, 12 granule implants were prepared in the same manner as the block β-TCPs: four as a control group, four as an AP group, and four as an ULP group. Each implant consisted of seven granule β-TCPs, which are smaller than the blocks at 2.0 mm in diameter.

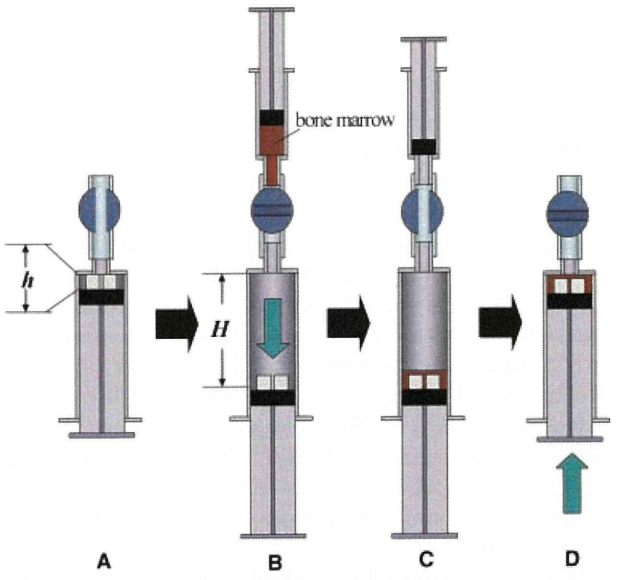

Figure 1. (A) Implants are set in a 20-mL syringe at the 1-mL level. (B) The inner cylinder is pulled to 20 mL after closing the stopcock to create low pressure. (C) The stopcock is turned to seed the prepared bone marrow into porous implants under low pressure. (D) After tapping carefully, the inner cylinder is put back to the set position. The theoretical low pressure at bone marrow introduction can be calculated as below:

$$P = AP \times V_{\min}/(\pi r^2 H - V_i(1 - P_{\text{pore}}) - V_{\text{BM}}),$$
$$V_{\min} = \pi r^2 h - V_i(1 - P_{\text{pore}})$$

where P is the theoretical low pressure after bone marrow penetration; AP is the atmospheric pressure; $V_{\min}$ is the minimum volume before pulling the inner cylinder; r is the half of the syringe diameter; V_i is the volume of the implant; P_{pore} is the porosity of the implant; V_{BM} is the volume of penetrated bone marrow; H is the height after pulling the cylinder; and h is the height before pulling the cylinder. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Evaluation of the Implants

To evaluate bone marrow penetration efficiency, a portion of the prepared block implants ($n = 6$) were not transplanted, and were examined histologically. The samples were fixed in 10% formalin immediately after seeding and were stained using the Villanueva method. Samples were embedded in methylmethacrylate (Wako Pure Chemical Industries, Japan) without decalcification, and were cut into 5 μm-thick sections using a microtome. Quantification of bone marrow penetration into the block implants was calculated according to a previously described method.[18,19] Briefly, the block β-TCPs were divided into five sections at equal intervals. The area of bone marrow penetration within the macropores was manually selected and measured using image-editing software (Photoshop, Adobe Systems, San Jose, CA) and image analysis software (Scion Image, Scion Corp., Frederick, MD). Bone marrow penetration efficiency was defined as the ratio of bone marrow penetrated area per whole sectional area, including pores and β-TCP. The average ratio of the five sections was used for analysis.

To assess implant microstructure, scanning electron microscopic (SEM) analysis was performed. The prepared block implants were also examined by SEM. The block implants penetrated with PBS served as a control, and the block samples in the AP and ULP groups were fixed with 2.5% glutaraldehyde in a 0.1-M sodium cacodylate buffer (pH 7.4) and were then cut at the center and postfixed with 0.1% osmium tetroxide. After fixing the samples onto stubs, they were sputtercoated and examined using an Hitachi S-4500 microscope.

Transplantation

The rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (25 mg/kg) and medetomidine hydrochloride (0.1 mg/kg). Prophylactic antibacterial treatment consisted of cefazolin at a dose of 25 mg/kg. Once anesthetized, the rabbits were aseptically draped, and skin incisions were made at the midsagittal line of the lower back. The fascias were cut at 12 sites with a 1-cm length, the muscles were split, and the prepared implants were randomly transplanted into each site. After the fascia and the skin were closed, rabbits were returned to their cages and allowed unlimited movement. No functional impairments were observed postoperatively. Eight of the 20 rabbits with block implants were sacrificed after 2 or 4 weeks, and the samples harvested for biochemical analysis. The remainder were sacrificed after 5 or 10 weeks and examined histologically to evaluate bone formation.

Biochemical Analysis

To evaluate osteogenic capability in early differentiation phases of the progenitor cells, the alkaline phosphatase (ALP) activities in transplanted block samples of each group were analyzed. ALP was quantified by a modification of a total enzyme activity method.[20] The harvested samples were first crushed with a forceps, homogenized in 500 μL 0.2% Triton X-100, and then sonicated to destroy cell membranes. After centrifugation at 3000 rpm for 5 min at 4°C, 20 μL of the supernatant was added to 200 μL of substrate buffer (10 mM disodum *p*-nitrophenylphosphate hexahydrate, 0.056 M

2-amino-2-methyl-1,3-propanediol, and 1 mM $MgCl_2$) in a 96-well plate. After incubation of the mixtures at 37.0°C for 30 min, absorbance at 405 nm was measured on a microplate reader. ALP activity was determined as millimoles of *p*-nitrophenyl released per implant after a 30-min incubation using a standard curve, employing the reaction of 20 μL of a *p*-nitrophenyl solution (Wako, Kyoto, Japan) and 200 μL of substrate buffer for 30 min. The average data of the four samples in each rabbit were used for analysis.

Histological Analysis

The harvested samples were fixed in 10% formalin and decalcified in KCX solution (Falma, Japan), dehydrated, embedded in paraffin, cut into 5-μm sections, and stained with H&E stain. Specimens were examined under light microscopy. Newly formed bone in the block and granule implants was quantified by the same procedure as the bone marrow penetration efficiency. The average data of the four samples in each rabbit were used for analysis. The center sections in the block samples were also evaluated to show bone formation. Bone formation ratio in each group was also evaluated in both block and granule samples.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using paired *t*-test or one-way repeated ANOVA, and the Tukey-Kramer test was used for post hoc pairwise comparisons. Friedman test and Steel-Dwass' post hoc test were used for nonparametric data. The confidence interval was set at 95%; the significance level at $p < 0.05$.

RESULTS

Bone Marrow Penetration Efficiency

Histological features of the block implants in the ULP group immediately after bone marrow introduction showed that marrow cells penetrated through the whole scaffold, whereas penetration was insufficient and inhomogeneous in the AP group, especially centrally (Fig. 2A and B). In the ULP group, the area of bone marrow penetration into the macropores of the block implants was significantly higher than in the AP group ($p < 0.01$; Fig. 2C). SEM analysis of the ULP group showed dense nets of fibrin even in the micropores, where the size was <5 μm, such that cells could not be induced (Fig. 2F). On the other hand, there were fewer fibrin nets induced into the micropores in the AP group (Fig. 2E).

In Vivo Osteogenesis

ALP activity in the transplanted samples of the ULP group was higher than in the control or AP groups

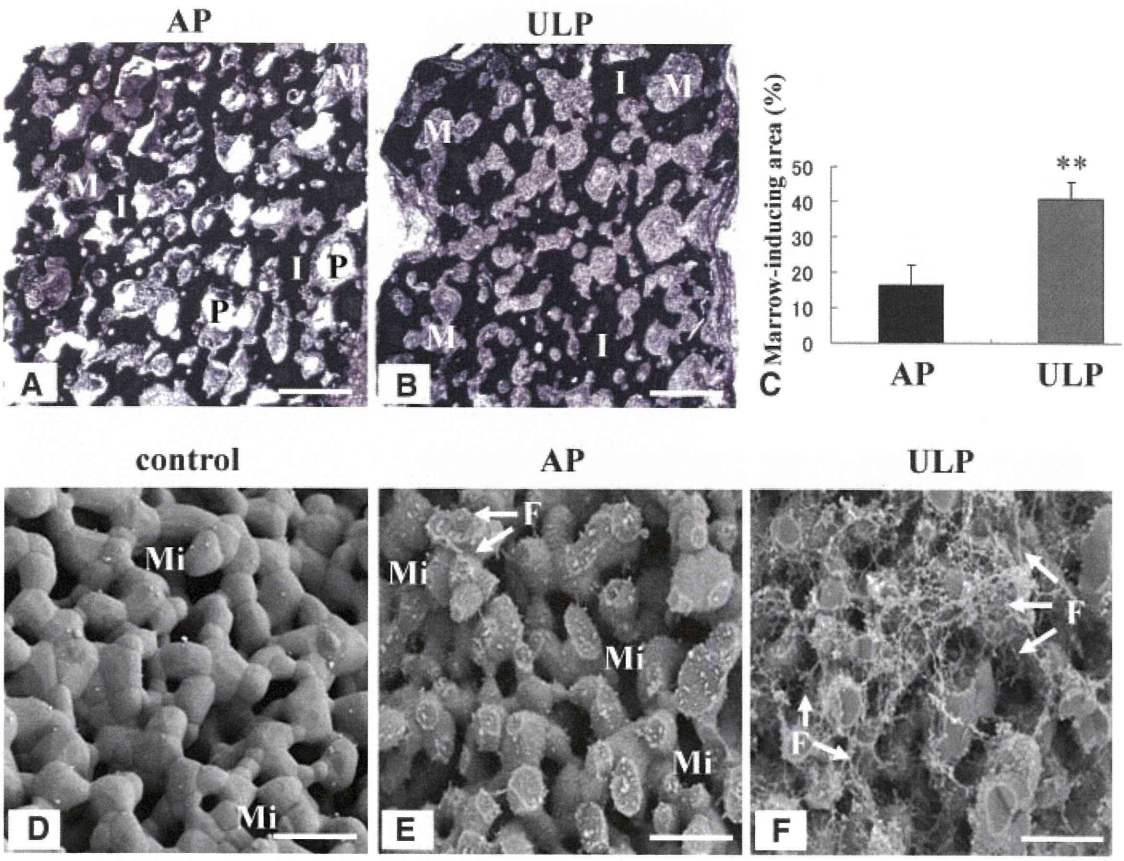

Figure 2. Villanueva staining of the center sections in the block β-TCP after marrow introduction (original magnification 12.5×): (A) AP group, (B) ULP group; scale bar = 1 mm; P, macropores; I, implant area; M, marrow inducing area. (C) The graph presents the mean marrow-inducing area with the SD bars ($n = 6$); $^{**}p < 0.01$. SEM analysis shows the micropore structures in the center of the block β-TCP penetrated with PBS as a control (D), in the AP group (E) and ULP group (F) (original magnification 5000×); scale bar = 6 μm; F, fibrin nets; Mi, micropores. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 3H), especially at 2 weeks ($p < 0.05$). Histological analysis showed new bone formation in most AP samples and in almost all ULP samples; however, no bone formation was detected at either 5 or 10 weeks in any control samples without bone marrow introduction (Table 1). In the block implants, bone was spread over almost the entire implant in the ULP group (Fig. 3B and E), whereas bone formation was limited to the peripheral sites in the AP group (Fig. 3A and D) at 5 weeks. The implants had begun to be absorbed at 10 weeks, but much bone was preserved over the whole area in the ULP group (Fig. 3G). On the other hand, a smaller amount of bone was detected in the AP group (Fig. 3F). In the quantitative analysis, significantly more newly formed bone was detected in the ULP group compared with the AP group at both 5 and 10 weeks ($p < 0.01$; Fig. 3I), and the difference between the groups was more pronounced centrally (Fig. 3J).

Similar results were observed in the experiment using β-TCP granules, which were smaller than the blocks. In the granule implants, significantly more bone formation was detected in the ULP group compared with the AP group at both 5 ($p < 0.05$) and 10 weeks ($p < 0.01$) (Fig. 4A–H). Furthermore, the formed bone tissue was

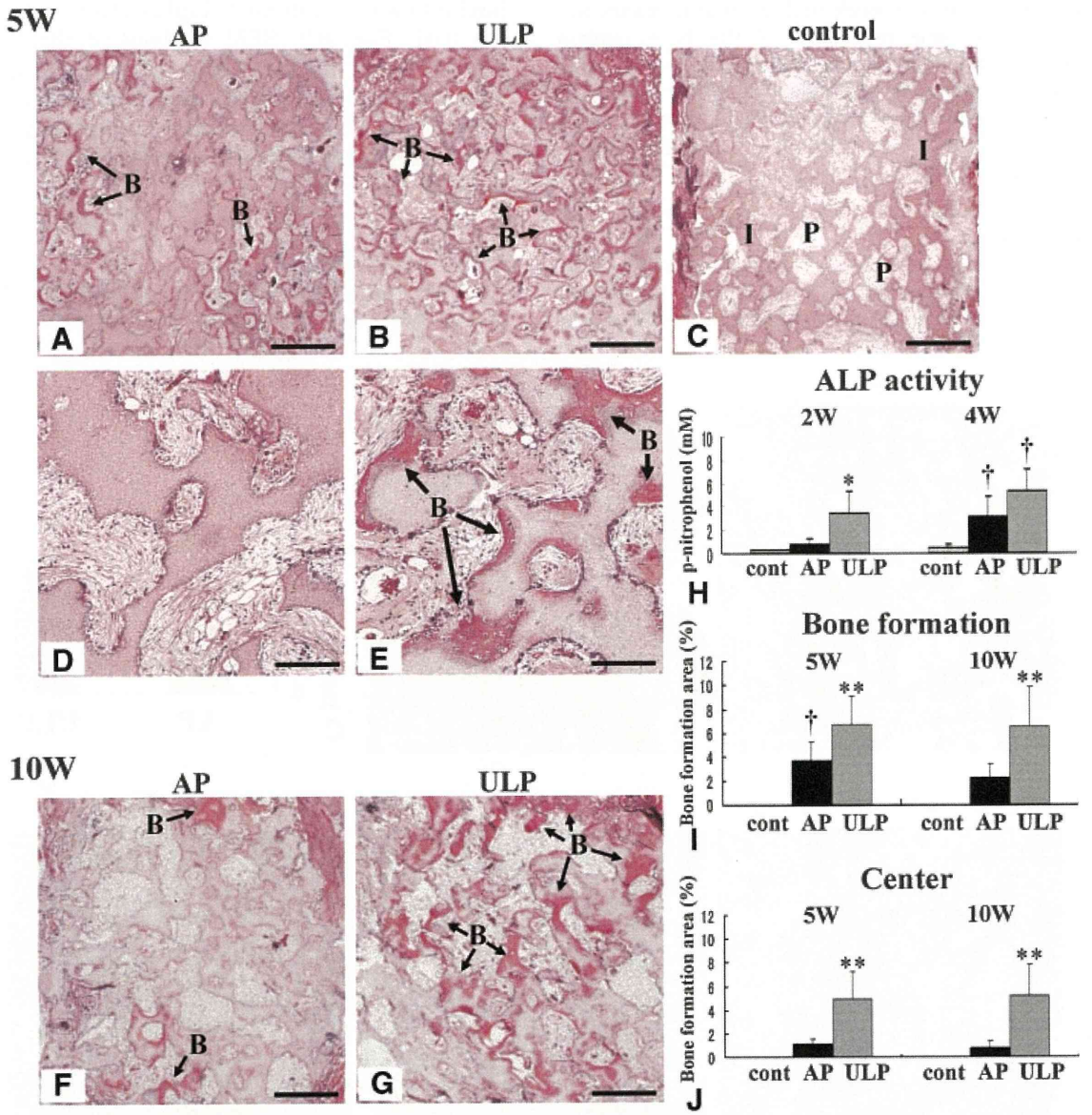

Figure 3. Representative histological specimens of block implants at 5 weeks (original magnification 12.5×): (A) AP group, (B) ULP group, (C) control; high-magnification views (original magnification 100×): (D) AP group, (E) ULP group; the histological sections at 10 weeks (original magnification 12.5×): (F) AP group, (G) ULP group. Little bone formation was observed at the periphery in the AP group, whereas significant bone formation was evident centrally in the ULP group both at 5 and 10 weeks; scale bar = 1 mm (A–C), high-magnification 125 μm (D, E); B, newly formed bone; P, macropores; I, implant area. ALP activity per block at 2 weeks or 4 weeks ($n = 4$) is indicated as the mean ± SD (H); $^{*}p < 0.05$ versus control, AP group, $^{\dagger}p < 0.05$ versus control. The quantitative analysis of newly formed bone in the total area (I) and the central area (J); the graphs present the mean bone formation area ±SD ($n = 6$); $^{\dagger}p < 0.05$ versus control, $^{**}p < 0.01$ versus control, AP group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1. Bone Formation Ratio at 5 Weeks and 10 Weeks (per Total Samples in Each Group)

	Group	5 Weeks	10 Weeks
Block	Control	0/24	0/24
	AP	21/24	19/24
	ULP	22/24	23/24
Granule	Control	0/16	0/16
	AP	14/16	14/16
	ULP	16/16	15/16

limited to the periphery of each granule implant in the AP group, whereas more bone was observed over the whole area in the ULP group.

DISCUSSION

Bone marrow aspiration is a simple, safe, effective means of harvesting osteogenic progenitors,[21] and the method of aspiration has been studied and defined.[10,11] Bone marrow has an osteogenic capability in both orthotopic[22] and heterotopic sites,[23] and its combination with adequate scaffolds can enhance bone formation.[6–9,12,13] In addition, these methods have clinical merits of being autologous, so no concern exists about immunologic responses.

Despite its clinical utility, the population of osteoprogenitor cells in bone marrow is limited,[21] and thus the bone formation capability of fresh marrow may be limited. Attempts have been made to augment the efficacy of bone marrow cells in bone regeneration. One approach for effective osteogenesis using BMSCs is to expand them in vitro with osteogenic supplements.[24,25] Indeed, numerous studies showed the efficacy of in vitro expanded BMSCs when combined with porous scaffolds in bone regeneration.[26–28]

As tissue engineering techniques have advanced, the efficacy of cell-loading procedures into porous scaffolds has also been studied. Because seeding with simple droplets of a cell suspension onto porous scaffolds is inefficient, primarily due to interference by air remaining in the pores, several improved cell-loading methods have been examined. Bone formation is enhanced by methods that create a low-pressure environment to remove the remaining air using a vacuum desiccator after seeding cells into ceramics.[17,18] However, these and many of the other methods require special devices that

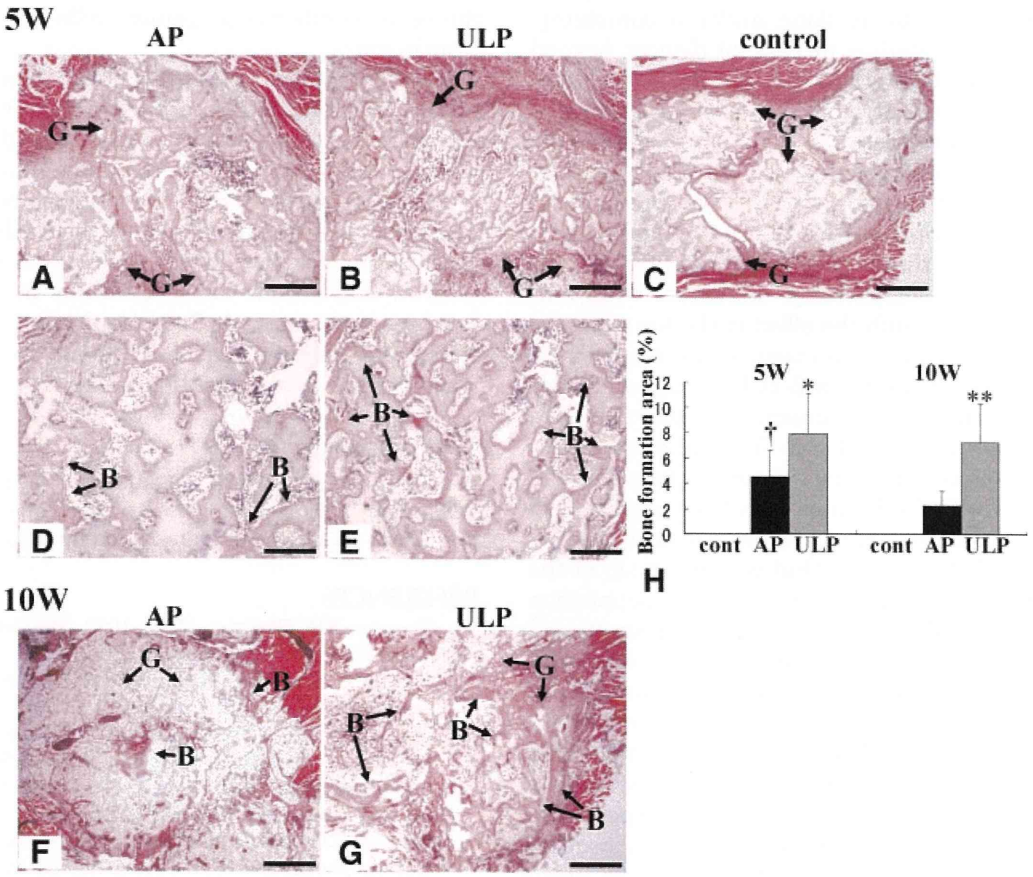

Figure 4. Representative histological specimens of the granule implants at 5 weeks (original magnification 12.5×): (A) AP group, (B) ULP group, (C) control; high-magnification views (original magnification 40×): (D) AP group, (E) ULP group; the histological sections at 10 weeks (original magnification 12.5×): (F) AP group, (G) ULP group. More bone formation was detected over the whole area of each granule in the ULP group compared with the AP group both at 5 and 10 weeks; scale bar = 1 mm (A–C), high-magnification 312.5 μm (D, E); G, granule implant; B, newly formed bone. The quantitative analysis of newly formed bone (H); the graphs present the mean bone formation area ±SD ($n = 4$); $^{\dagger}p < 0.05$ versus control, $^{*}p < 0.05$ versus control, AP group, $^{**}p < 0.01$ versus control, AP group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

complicate the process. We devised a simple low-pressure cell-loading method to ameliorate these problems.[19] This method differs from other procedures in that cell soaking is performed under low pressure, whereas the low pressure is applied after cell soaking in the other conventional procedures. This ULP method enhances the cell-loading efficiency and in vivo bone formation capability of expanded BMSCs with porous scaffolds nearly twofold compared with the conventional low-pressure methods.

Given these facts, we extended this tissue engineering technique in this study to osteogenesis using fresh bone marrow to exploit the optimal osteogenic potential of the fresh bone marrow. This ULP loading method has advantages when applied to fresh bone marrow. Because fresh bone marrow possesses a viscosity and forms clots immediately after harvesting, low pressure after seeding can lead to a lower effect in removing the residual air due to the higher surface tension. On the other hand, the low pressure environment is maintained before and during seeding in this ULP method, and the introduction is completed within a minute, which is considered to be more useful for effective marrow introduction.

In the present study, the ULP method was further modified to simplify it for clinical use by enabling the marrow introduction to be done under a completely closed system that requires no special devices beyond syringes and stopcocks. In the β-TCP blocks, as expected, the bone marrow was introduced almost twofold more efficiently under low pressure compared with simple droplets. The marrow penetration efficiency directly affected in vivo osteogenesis. Both the biochemical activity of the osteoprogenitor cells in the early differentiation phases and the observed new bone formation in the implants were significantly enhanced with low-pressure loading. Although the effect of the low-pressure loading method might have been assumed to be less for the granule β-TCPs than the block β-TCPs because of their smaller sizes, this method also enhanced bone formation in the granule materials, which are frequently used clinically. These results suggest that this technique is effective for osteogenesis with fresh autologous bone marrow and porous scaffolds for use in clinical bone reconstruction.

Connolly et al.[12] described that concentration of the bone marrow cells using density gradient separation resulted in increased bone formation. Muschler et al.[13] demonstrated an effective method to prepare composite grafts with an increased proportion of progenitor cells by letting autologous bone marrow pass through materials at a controlled rate. This selective cell retention procedure can be performed intraoperatively with a commercially available device, and can be expected to enhance bone formation.[14] However, the cell concentration method might not be effective enough by itself, and may be more effective for osteogenesis when combined with a bone marrow clot.[13,14] Becker et al.[9] reported that whole bone marrow showed a greater in vivo osteogenic capability rather than concentrated monouclear cells, also suggesting the possibility that marrow clot consisting of fibrin networks are important for osteogenesis.

We used fresh, whole bone marrow with the highly porous β-TCP, in which interconnecting macropores allowed for good in-growth of blood vessels and other tissues, and the micropores retained a great deal of proteins including osteogenic cytokines within their large surface areas. In the macropores, fresh bone marrow (osteoprogenitor cells and autologous plasma) forms fibrin clots and can serve as a 3D scaffold for attachment and growth of cells.[29,30] In addition, SEM of the samples seeded with the ULP method showed the presence of enriched fibrin nets even in the micropores where the cells cannot be introduced. Some growth factors, including fibroblast growth factor-2 and vascular endothelial growth factor, are known to bind to fibrin and to potentiate endothelial cell proliferation when bound.[31,32] Thus, the fibrin nets in the micropores may also work to retain growth factors present in the bone marrow or delivered by the degranulation of platelets, and may have a potential effect on vascularization.[33] Although the role of the fibrin in osteogenesis needs further investigation, the dense fibrin nets within the micropores introduced by this low-pressure method also contribute to the increased bone formation despite the small number of progenitor cells contained in fresh bone marrow.

This study used relatively small biomaterials in a rabbit ectopic bone formation model to clearly observe the influence of bone marrow and its loading method on in vivo osteogenesis. Further examination may be required to demonstrate the clinical benefits of this method. However, this study represents the possibility of improved osteogenesis with a combination of fresh bone marrow and porous biomaterials by using the "under low-pressure" method. Because this method is simple, safe, rapid, and inexpensive, it should have use in a wide variety of clinical applications.

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REFERENCES

1. Younger EM, Chapman MW. 1989. Morbidity at bone graft donor sites. J Orthop Trauma 3:192–195.
2. Perry CR. 1999. Bone repair techniques, bone graft, and bone graft substitutes. Clin Orthop 360:71–86.
3. Giannoudis PV, Dinopoulos H, Tsiridis E. 2005. Bone substitutes: an update. Injury 36(Suppl 3):S20–S27.
4. Finkemeier CG. 2002. Bone-grafting and bone-graft substitutes. J Bone Joint Surg Am 84:454–464.
5. LeGeros RZ. 2002. Properties of osteoconductive biomaterials: calcium phosphates. Clin Orthop 395:81–98.
6. Ohgushi H, Goldberg VM, Caplan AI. 1989. Heterotopic osteogenesis in porous ceramics induced by marrow cells. J Orthop Res 7:568–578.
7. Vuola J, Goransson H, Bohling T, et al. 1996. Bone marrow induced osteogenesis in hydroxyapatite and calcium carbonate implants. Biomaterials 17:1761–1766.

8. Louisia S, Stromboni M, Meunier A, et al. 1999. Coral grafting supplemented with bone marrow. J Bone Joint Surg Br 81:719–724.
9. Becker S, Maissen O, Ponomarev I, et al. 2006. Osteopromotion by a beta-tricalcium phosphate/bone marrow hybrid implant for use in spine surgery. Spine 31:11–17.
10. Muschler GF, Boehm C, Easley K. 1997. Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. J Bone Joint Surg Am 79:1699–1709.
11. Muschler GF, Nitto H, Boehm CA, et al. 2001. Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. J Orthop Res 19:117–125.
12. Connolly J, Guse R, Lippiello L, et al. 1989. Development of an osteogenic bone-marrow preparation. J Bone Joint Surg Am 71:684–691.
13. Muschler GF, Nitto H, Matsukura Y, et al. 2003. Spine fusion using cell matrix composites enriched in bone marrow-derived cells. Clin Orthop 407:102–118.
14. Muschler GF, Matsukura Y, Nitto H, et al. 2005. Selective retention of bone marrow-derived cells to enhance spinal fusion. Clin Orthop 432:242–251.
15. Bancroft GN, Sikavitsas VI, van den Dolder J, et al. 2002. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci USA 99:12600–12605.
16. Zhao F, Ma T. 2005. Perfusion bioreactor system for human mesenchymal stem cell tissue engineering: dynamic cell seeding and construct development. Biotechnol Bioeng 91:482–493.
17. Dong J, Uemura T, Kikuchi M, et al. 2002. Long-term durability of porous hydroxyapatite with low-pressure system to support osteogenesis of mesenchymal stem cells. Biomed Mater Eng 12:203–209.
18. Wang J, Asou Y, Sekiya I, et al. 2006. Enhancement of tissue engineered bone formation by a low pressure system improving cell seeding and medium perfusion into a porous scaffold. Biomaterials 27:2738–2746.
19. Torigoe I, Sotome S, Tsuciya A, et al. 2007. Novel cell seeding system into a porous scaffold using a modified low pressure method to enhance cell seeding efficiency and bone formation. Cell Transplant 16:729–739.
20. Hughes FJ, Aubin JE. 1998. Culture of cells of the osteoblast lineage. In: Arnett TR, Henderson B, editors. Methods in bone biology. London: Chapman-Hall Ltd.; p 37.
21. Majors AK, Boehm CA, Nitto H, et al. 1997. Characterization of human bone marrow stromal cells with respect to osteoblastic differentiation. J Orthop Res 15:546–557.
22. Connolly JF. 1995. Injectable bone marrow preparations to stimulate osteogenic repair. Clin Orthop 313:8–18.
23. Friedenstein AJ, Petrakova KV, Kurolesova AI, et al. 1968. Heterotopic of bone marrow. Analysis of precursor cells for steogenic and hematopoietic tissues. Transplantation 6:230–247.
24. Derubeis AR, Cancedda R. 2004. Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. Ann Biomed Eng 32:160–165.
25. Pittenger MF, Mackay AM, Beck SC, et al. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.
26. Bruder SP, Kraus KH, Goldberg VM. 1998. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 80:985–996.
27. Petite H, Viateau V, Bensaid W, et al. 2000. Tissue-engineered bone regeneration. Nat Biotechnol 18:959–963.
28. Dong J, Uemura T, Shirasaki Y. 2002. Promotion of bone formation using highly pure porous beta-TCP combined with bone marrow-derived osteoprogenitor cells. Biomaterials 23:4493–4502.
29. Bensa W, Triffitt JT, Blanchat C, et al. 2003. A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. Biomaterials 24:2497–2502.
30. Tajima N, Sotome S, Marukawa E, et al. 2007. A three-dimensional cell-loading system using autologuous plasma loaded into a porous β-tricalcium-phosphate block promotes bone formation at extraskeltal sites in rats. Mater Sci Eng C 27:625–632.
31. Sahni A, Sporn LA, Francis CW. 1999. Potentiation of endothelial cell proliferation by fibrin(ogen)-bound fibroblast growth factor-2. J Biol Chem 274:14936–14941.
32. Sahni A, Francis CW. 2000. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. Blood 96:3772–3778.
33. Mosesson MW. 2005. Fibrinogen and fibrin structure and functions. J Thromb Haemost 3:1894–1904.

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Bone Regeneration with Autologous Plasma, Bone Marrow Stromal Cells, and Porous β-Tricalcium Phosphate in Nonhuman Primates

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To potentiate the bone formation capability of bone marrow stromal cell (BMSC)/β-tricalcium phosphate (β-TCP) constructs, we devised an autologous plasma-based construct. We tested its effectiveness and investigated the effects of its components on a monkey ectopic bone formation model. The autologous plasma (platelet-rich plasma, PRP, or platelet-poor plasma, PPP)/BMSC/β-TCP construct (R group or P group) showed significantly more bone formation at 3 and 6 weeks after implantation than a conventional BMSC/β-TCP construct using a culture medium (M group). There was no significant difference between the P and R groups. Moreover, the P group constructs with a 10-fold lower cell concentration yielded equivalent bone formation to the M group at 5 weeks after implantation. To elucidate the effect of fibrin and serum contained in the plasma, five constructs were prepared using the following cell vehicles: autologous serum+fibrinogen (0, 1, 4, or 16 mg/mL) or phosphate-buffered saline+fibrinogen (4 mg/mL). The serum+fibrinogen (4 mg/mL, physiological concentration of monkeys) construct showed the most abundant bone formation at 3 weeks after implantation, though at 5 weeks no statistical difference existed among the groups. Autologous plasma efficiently promoted osteogenesis of BMSCs/porous β-TCP constructs, and both fibrin and serum proved to play significant roles in the mechanism.

Introduction

The supply of autologous bone grafts is insufficient to repair large bone defects. Harvesting these bone grafts has risks of infection, nerve damage, and cosmetic disability.[1,2] Despite the development of various porous ceramics with osteoconductivity,[3] including hydroxyapatite (HA) and tricalcium phosphate (TCP), by themselves, these ceramics have little osteoinductivity[4,5] and are insufficient to repair massive bone defects. To overcome these problems, bone tissue engineering methods have been developed that combine bone marrow stromal cells (BMSCs) with porous ceramic scaffolds. BMSCs are easily isolated from bone marrow and have a proliferative capability and multilineage differentiation potential, including the capacity for osteogenic differentiation.[6,7] There are many reports that demonstrate the *in vivo* bone formation capability of BMSC/porous ceramic constructs.[8–14]

Although a variety of strategies to prepare BMSC/porous ceramic constructs have been contrived, practical methods have still not been established. In well-accepted techniques, after suspended in culture medium, BMSCs were seeded into porous ceramics, and the cells were cultured on porous ceramics for a few hours,[8–10] 1 week,[12,13] or 2 weeks[11,14] to attach the cells to the scaffolds and induce osteogenic differentiation of the cells. In these constructs, it is probable that BMSCs proliferated two-dimensionally by creeping along the surface of the pores, and the nutrient was supplied from the culture medium at the early stage of implantation. Considering that the range of nutrient diffusion from surrounding tissue is limited within the surface area of cell/scaffold construct,[15–18] BMSC survival after implantation could be impaired at the center of the constructs.

To provide preferable environments to BMSCs seeded into porous ceramics, we devised a construct that utilized autologous plasma of either platelet-poor plasma (PPP) or

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platelet-rich plasma (PRP) type. We expected the fibrin and serum in plasma to have supportive effects on BMSCs, and we tested this using a rat heterotopic bone induction model.[19] A fibrin network, which is formed by fibrinogen (fibrin conversion in a blood clotting reaction), supports migration, proliferation, and differentiation of cells;[20–22] induces angiogenesis;[22–24] and plays a crucial role in the normal wound-healing process.[22] It is also well known that serum is essential for *in vitro* expansion and differentiation of BMSCs. Further, in PRP, the promotive effects of growth factors derived from platelets, which promoted osteogenesis,[25–27] are expected. Hence, viability of seeded BMSCs and effects after implantation would be augmented by a fibrin network, serum supply, and/or growth factors from platelets. As a result, osteogenesis by seeded BMSCs would be promoted in the autologous plasma/BMSC/β-TCP construct. Our study using rats showed the promotion of bone formation by the PPP/BMSC/β-TCP construct compared with a BMSC/β-TCP construct prepared using a culture medium and a PRP/BMSCs/β-TCP construct, in spite of reports that PRP enhances bone formation by growth factors released from platelets.[25–27] Although the effect of growth factors released from platelets is still controversial, it was revealed that fibrin and/or serum in plasma promoted bone formation in rats.

Although the usefulness of the tissue-engineered constructs on bone regeneration has been revealed in many animal species, including mice,[28] rats,[8,11,13] rabbits,[29] canines,[9,30] goats,[12,13,17,31] and sheep,[10] there have been few reports examining this phenomenon in nonhuman primates.[32] It is generally known that there are large differences of bone formation capability among animal species, and monkeys are most similar to humans in this regard.[33] For clinical application, *in vivo* bone formation capability should be examined in nonhuman primates.

The purposes of this study were as follows: (1) to confirm *in vivo* bone formation capability of the autologous plasma (PPP or PRP)/BMSC/β-TCP construct compared with a BMSC/β-TCP construct using culture medium; (2) to examine the influence of cell concentration on the BMSCs/β-TCP construct using culture medium or autologous plasma; and (3) to elucidate the effect of fibrin and serum on *in vivo* bone formation by the BMSCs/β-TCP construct using a ectopic bone formation model in nonhuman primates.

Materials and Methods

Animals

All animal procedures were performed in accordance with the guidelines of Tokyo Medical and Dental University for the care and use of laboratory animals. Ten skeletally mature male cynomolgus monkeys (*Macaca fascicularis*, aged 5–6 years, weight 2.8–3.8 kg) were used for this study. After the collection of bone marrow and venous blood, the transplantation and harvest of implants were performed under general anesthesia by intramuscular injection of 10 mg/kg ketamine hydrochloride and 0.1 mg/kg medetomidine hydrochloride. No monkeys were killed during experiments.

Isolation and culture of BMSCs

Bone marrow was aspirated from both the bilateral posterior iliac crests and the bilateral greater trochanters of the femurs of each monkey using a bone marrow biopsy needle (Cardinal Health, Dublin, OH) and collected into 30 mL of Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical, St. Louis, MO) containing 200 IU sodium heparin. The total volume of the bone marrow aspirated from each site was 5 mL. The bone marrow suspension was centrifuged at 1000 *g* for 5 min and resuspended in the culture medium: DMEM containing 10% fetal bovine serum (Sigma Chemical), 1% antibiotic–antimycotic (100 units/mL Penicillin G sodium, 100 μg/mL Streptomycin sulfate, and 0.25 μg/mL Amphotericine B; Invitrogen), and 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO). Aliquot of the suspension was used to count the number of nucleated cells (NCs) after hemolysis, and the bone marrow suspension was plated at 1×10^6 NCs/cm^2 into 75 cm^2 culture flasks (BD Biosciences, Franklin Lakes, NJ) and cultured at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was changed twice a week thereafter. Upon subconfluence of adherent BMSCs, BMSCs were detached with 0.25% trypsin containing 1 mM EDTA (Invitrogen) and reseeded into 75 cm^2 flasks at a density of 4×10^3 BMSCs/cm^2. Four days after the subculture, the culture medium was changed to an osteogenic medium consisting of the culture medium, 50 μg/mL ascorbic acid phosphate (Wako, Osaka, Japan), and 2 mM β-glycerophosphate (Sigma-Aldrich). After 3 days' osteogenic induction, the BMSCs were used for subsequent implantation experiments.

Preparation of autologous plasma and autologous serum

For preparation of autologous plasma (PPP and PRP), whole blood was collected into a sterile tube containing citrate phosphate dextrose (CPD) solution (2.63% sodium citrate, 0.327% citric acid, 2.32% dextrose, and 0.251% sodium dihydrogen phosphate; TERUMO, Tokyo, Japan) with one-ninth volume of the collected blood as an anticoagulant. The blood was centrifuged at 800 *g* for 10 min, and the upper layer, including plasma and the buffy coat (mostly white blood cells and platelets), was transferred to another tube except for the bottom layer that included red blood cells. To spin down the buffy coat, further centrifugation of the transferred layers was performed at 1500 *g* for 10 min, the bottom layer was collected as PRP (one-tenth volume of the whole blood), and the remainder was collected as PPP (half volume of the whole blood). The number of platelets in the whole blood, PPP, and PRP was counted using Coulter Counter® (Beckman Coulter, Fullerton, CA). For preparation of autologous serum, whole blood was collected in a sterile tube and incubated at room temperature for a few hours. After the blood coagulated, the tube was centrifuged at 2380 *g* for 10 min, and the serum was collected from the upper layer. Considering the influence of the CPD solution in the plasma, serum was also mixed with the CPD solution at a 9:1 v/v ratio.

Preparation of BMSC/β-TCP constructs

Porous ceramic scaffolds. Porous β-TCP blocks (Osferion®; Olympus, Tokyo, Japan) were used in this study. The blocks were 5×5×5 mm in dimension and 75% porous, with a macropore size ranging from 200 to 400 μm and micropore size under 5 μm. The macropores were interconnected by paths with diameters of 100–200 μm.[5,34]

Experiment 1 (comparative study of BMSC/β-TCP, PPP/BMSC/β-TCP, and PRP/BMSC/β-TCP constructs). Cultured osteogenic BMSCs were suspended in the culture medium, autologous PPP, or PRP at a concentration of 2×10^6 cells/mL. The cell suspension using the culture medium was seeded into porous β-TCP cubes using the spontaneous penetration under the low-pressure method,[35] in which cell suspension is seeded into porous scaffolds under low pressure (50 mmHg) with a simple device and homogenously distributed into porous scaffolds. The procedure only requires a few minutes. Subsequently, the BMSC/β-TCP constructs using the culture medium (M group) were incubated for 3 h for cell attachment. The cell suspension prepared utilizing PPP or PRP was mixed with 2% calcium chloride at a 7:1 v/v ratio to initiate fibrin gel formation. It was immediately introduced into porous β-TCP cubes using the same technique as mentioned above. After gelation of each introduced cell suspension was completed, autologous transplantation of both PPP/BMSC/β-TCP (P group) and PRP/BMSC/β-TCP constructs (R group) was conducted with the M group and β-TCP without cells (control group) (Table 1A). Some constructs were used for analysis by scanning electron microscopy (SEM).

Experiment 2 (influence of BMSC concentration). To examine the influence of cell concentration on *in vivo* bone formation of the BMSC/β-TCP constructs, cultured osteogenic BMSCs were suspended in the culture medium or PPP at a series of concentrations: 2000×10^3 cells/mL (M2000 group or P2000 group), 200×10^3 cells/mL (M200 group or P200 group), and 20×10^3 cells/mL (M20 group or P20 group). Each construct was prepared via the same techniques as used in Experiment 1, and autologous transplantation was performed (Table 1B).

Experiment 3 (effect of fibrin and serum). The gel that formed in the implant prepared using autologous plasma is composed of fibrin networks, and serum (as basic nutrients) with low concentrations (PPP) or high concentrations (PRP) of growth factors derived from platelets. To elucidate the effects of fibrin and serum on bone formation, five groups of BMSC/β-TCP constructs were prepared and transplanted (Table 1C). Except for the 0S group, BMSCs were suspended at a concentration of 2×10^6 cells/mL in autologous serum or phosphate-buffered saline (PBS) containing the CPD solution at a 9:1 v/v ratio mixed with human fibrinogen powder (BOLHEAL®; Teijin Pharma, Tokyo, Japan) at each concentration (1, 4, or 16 mg/mL). Subsequently, the cell suspension was mixed with 7 units/mL thrombin (BOLHEAL) in 2% calcium chloride at a 7:1 v/v ratio to initiate fibrin gel formation and introduced into the scaffolds. To prepare the construct of the 0S group, BMSCs were suspended at a concentration of 2×10^6 cells/mL in autologous serum, seeded into the scaffold, and incubated for 3 h for cell attachment.

SEM examination

Extra implants from the M, P, and control groups were not implanted and were instead utilized for SEM analyses. The samples were fixed with 2.5% glutaraldehyde, divided into two halves, dehydrated through a graded series of ethanol, dried by a critical point dryer, mounted onto aluminum

TABLE 1. EXPERIMENTAL GROUPS

(A) Experiment 1

Groups	*Constructs*	*Cell concentration ($\times10^3$ cells/mL)*
M	BMSC/β-TCP	
P	PPP/BMSC/β-TCP	2000
R	PRP/BMSC/β-TCP	
Control	β-TCP	0

(B) Experiment 2

Groups	*Constructs*	*Cell concentration ($\times10^3$ cells/mL)*
M2000	BMSC/β-TCP	2000
M200		200
M20		20
P2000	PPP/BMSC/β-TCP	2000
P200		200
P20		20

(C) Experiment 3

Groups	*Constructs*	*Fibrinogen (mg/mL)*	*Cell concentration ($\times10^3$ cells/mL)*
4S	Fibrin/AS/BMSC/β-TCP	4	
4P	Fibrin/PBS/BMSC/β-TCP	4	
0S	AS/BMSC/β-TCP	0	2000
1S	Fibrin/AS/BMSC/β-TCP	1	
16S	Fibrin/AS/BMSC/β-TCP	16	

AS, autologous serum.

stubs, and sputter-coated with platinum. The samples were observed from the cut plane using a scanning electron microscope (S-4500; Hitachi, Tokyo, Japan).

Implantation

Three, three, and four male monkeys were used in Experiments 1, 2, and 3, respectively. Three implants were used for each group and each time point per monkey. The surgical procedures were performed under general anesthesia. After shaving and disinfecting the dorsal areas, a longitudinal skin incision was made to expose the muscle fascia. Separate fascia incisions were made, and using blunt dissection, intramuscular pockets were created that were filled with one of the constructs, according to a randomized scheme. After the constructs were implanted, the fascia and the skin were closed. After 1, 2, 3, and 6 weeks (Experiment 1), 5 weeks (Experiment 2), or 3 and 5 weeks (Experiment 3), implants were harvested under general anesthesia. The fascia and skin incision were closed, and the animals were not killed as a part of these experiments and continuously bred.

Histological and histomorphometric examination

The harvested implants were fixed in a 4% paraformaldehyde solution for 1 week, decalcified in a 4% EDTA solution, and embedded in paraffin blocks. Each block was sectioned at 5 μm thickness with five sections at equal intervals derived from one block. The sections were stained with hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP). For immunohistochemical analysis, after deparaffinized sections were treated in turn with 0.4 mg/mL proteinase K (DakoCytomation, Glostrup, Denmark), 3% hydrogen peroxide in methanol, and 5% skim milk, they were incubated with mouse anti-human CD31 monoclonal antibody (1:100 dilution; DakoCytomation) overnight at 4°C and reacted for 10 min with biotinylated pig anti-mouse/rabbit/goat immunoglobulin G (DakoCytomation). Immunostaining was detected with peroxidase-labeled streptavidin (DakoCytomation), followed by 3,3′-diaminobenzidine tetrahydrochloride staining; counter staining was performed with methyl green. For histomorphometric examination of newly formed bone, the area of bone formation was painted black using image-editing software (Photoshop®; Adobe Systems, San Jose, CA). The black area of each section was extracted using Photoshop and measured using image analysis software (Scion Image®; Scion Corporation, Frederick, MD). Modifying the report by Dennis *et al.*,[36] the bone formation area (BFA) was defined as the ratio of newly formed bone per whole sectional area including newly formed bone, pores, and β-TCP.[35] The BFA of a single implant was an average of five sections (Fig. 1).

Statistics

Average values were expressed as the mean ± standard deviation (SD). The Tukey–Kramer test (Experiments 1 and 3) and paired *t*-test (Experiment 2) were used to assess differences. Differences were considered to be statistically significant when the *p*-value was <0.05.

Results

Experiment 1 (comparison among the M, P, and R groups)

The constructs of the M, P, and control groups just before implantation were observed by SEM. Figure 2A demonstrates the porous structure of the control group (β-TCP only) with macropores ranging from 200 to 400 μm interconnected by paths with diameters of 100–200 μm and a micropore structure below 5 μm. In the M group, BMSCs were two-dimensionally attached to the pore surface of β-TCP (Fig. 2B, D). In contrast, BMSCs in the P group were three-dimensionally retained by the fibrin network formed in the pores (Fig. 2C, E). The number of platelets in whole blood, PPP, and PRP was 26.7 ± 6.9, 5.4 ± 0.9, and 152.3 ± 23.1 × 10^4/μL, respectively. The number of platelets of PRP was approximately 30-fold that of PPP in this study.

At 3 weeks after implantation, there was a large quantity of newly formed bone in the P and R groups in the peripheral area, while the M group had less bone formation in the partial peripheral area. Moreover, six of the nine M group implants (three implants per animal) showed no bone formation (details are shown in Table 2). The invasion of many blood vessels also appeared with thicker, newly formed bone in both the P and R groups, compared to the M group, which had few blood vessels (Fig. 3A). At 6 weeks, bone formation expanded into the central area, became thicker, and was homogenously distributed throughout the whole area in the

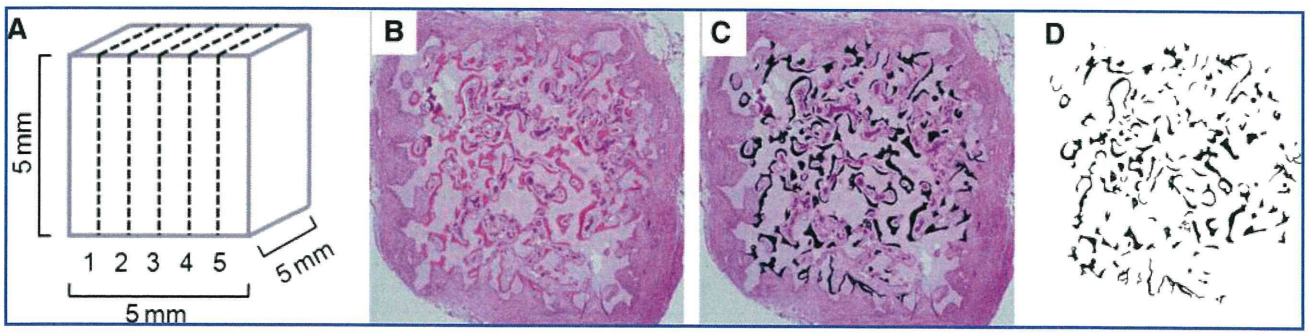


FIG. 1. Histomorphometric analysis of newly formed bone. (**A**) Five sections at equal intervals were made from one implant. (**B**) Each section was stained with H&E. (**C**) The area of bone formation was selected and painted black using Photoshop. (**D**) The area painted black in each section was extracted using Photoshop and measured using Scion Image. BFA was defined as the ratio of newly formed bone per whole sectional area including newly formed bone, pores, and β-TCP. The BFA of one implant was an average of five sections. Color image available online at www.liebertonline.com/ten.

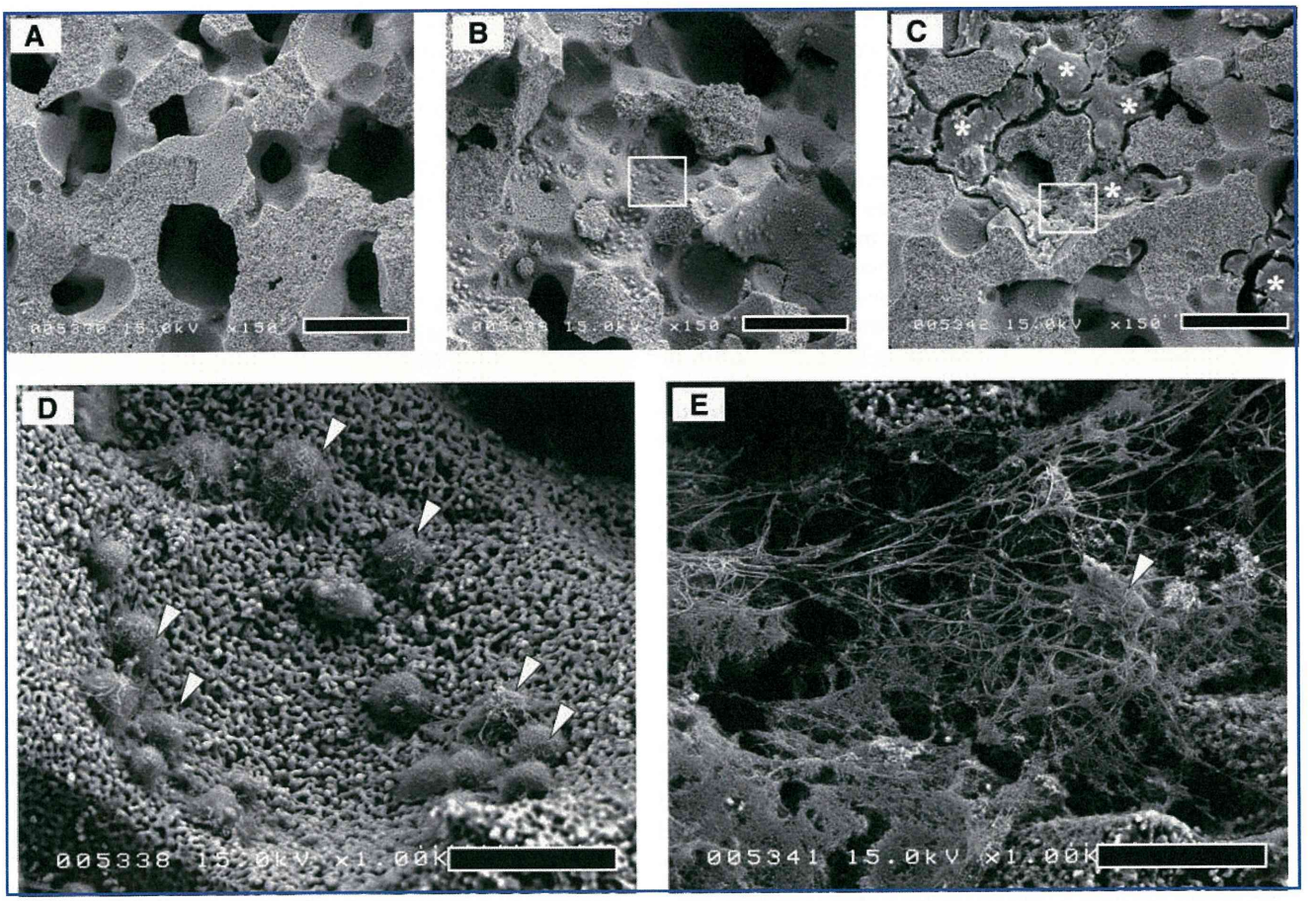


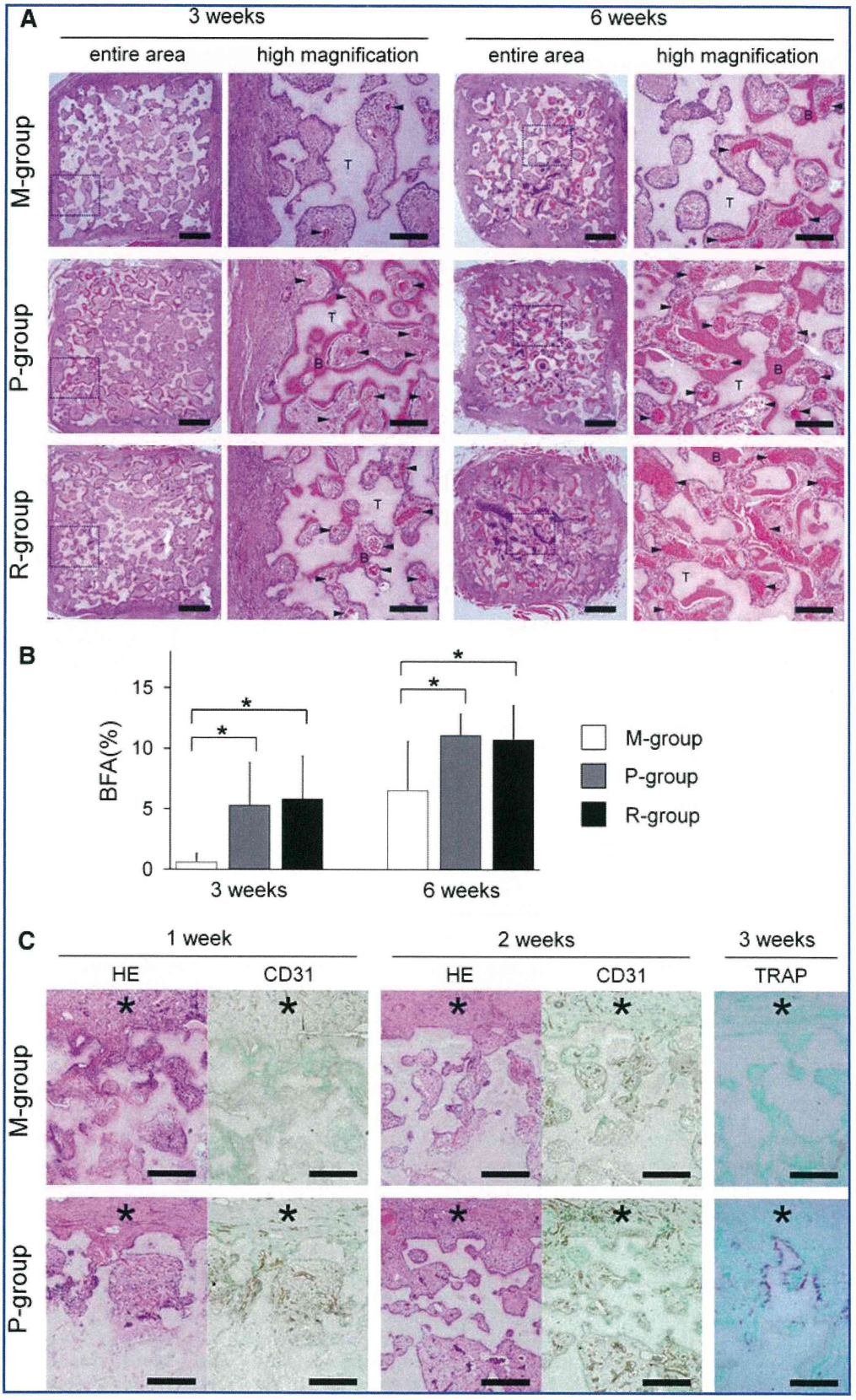
FIG. 2. SEM findings of the constructs before implantation. (**A**) Porous structure of the scaffold. The macropores and interconnecting pores are indicated. (**B, D**) The M group 3 h after seeding BMSCs. The BMSCs were attached to the pore surface. (**C, E**) The P group just after seeding BMSCs. Most of the macropores were filled with fibrin gel. The BMSCs were retained with a fibrin network formed in the pores. Arrowheads indicate seeded BMSCs; asterisks indicate fibrin gels. Scale bars: 200 μm (**A–C**) and 30 μm (**D, E**).

P and R groups; blood vessels had also developed. In the M group, there was less bone formation and invasion of blood vessels in the central area, and the distribution of newly formed bone was not uniform (Fig. 3A). In the control group, there was no bone formation at both 3 and 6 weeks (data not shown). In histomorphometric analysis of newly formed bone, the BFA in the P and R groups were significantly higher than the M group at both 3 and 6 weeks. Moreover, there was no significant difference between the P and R groups at these times (Fig. 3B). These results suggest that growth factors released from platelets had little effect on bone formation; therefore, we focused on PPP, which could be more abundantly obtained than PRP. Further, we examined the histological differences between the M and P groups in the early stages. At 1 week, in the M group invasion of more fibrous tissue into the implant was seen with few blood vessels stained with anti-CD31 antibody. On the other hand, comparatively many blood vessels appeared with less invasion of other tissues at the peripheral area in the P group (Fig. 3C). At 2 weeks, blood vessels extended and reached the central area in the P group, while invasion of blood vessels was delayed in the peripheral area in the M group (Fig. 3C). At 3 weeks, TRAP-positive cells appeared at the peripheral area in only the P group, according to the

TABLE 2. BONE FORMATION IN EXPERIMENT 1

		Case 1		*Case 2*		*Case 3*	
Groups	*Constructs*	*3 Weeks*	*6 Weeks*	*3 Weeks*	*6 Weeks*	*3 Weeks*	*6 Weeks*
M	BMSC/β-TCP	0/3	1/3	2/3	3/3	0/3	3/3
P	PPP/BMSC/β-TCP	3/3	3/3	3/3	3/3	3/3	3/3
R	PRP/BMSC/β-TCP	2/3	3/3	3/3	3/3	3/3	3/3
Control	β-TCP	0/3	0/3	0/3	0/3	0/3	0/3

Number of implants with bone formation/Total implants.


A
3 weeks
6 weeks
entire area
high magnification
entire area
high magnification
M-group
P-group
R-group
T
B
B
15
10
5
0
BFA(%)
*
*
*
*
3 weeks
6 weeks
M-group
P-group
R-group
C
1 week
2 weeks
3 weeks
HE
CD31
HE
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TRAP
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P-group
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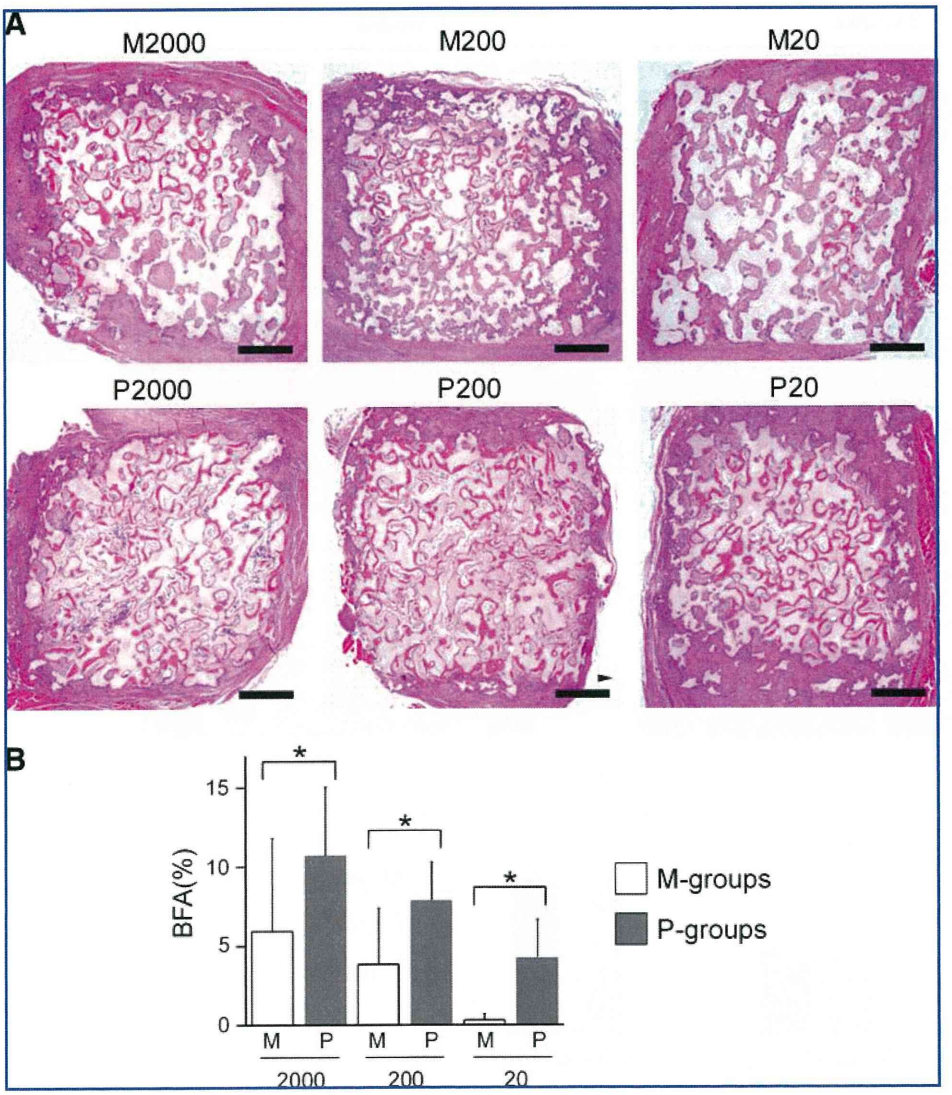

FIG. 4. Influences of BMSC concentration in the M and P groups (Experiment 2). (**A**) Representative histological sections at 5 weeks stained with H&E. Images taken from the central parts of each implant. The extent of bone formation in the M groups was reduced with a decrease in cell concentration. In contrast, the newly formed bone of both the P2000 and P200 groups distributed to the entire sectional area. Scale bar: 1 mm. (**B**) BFA at 5 weeks. BFAs of the P groups were significantly higher than those of the M groups at each concentration, especially at the concentration of 20×10^3 cells/mL. The data are expressed as mean ± SD ($^*p < 0.05$). Color image available online at www.liebertonline.com/ten.

progression of osteogenesis and angiogenesis (Fig. 3C). From these results, we confirmed that plasma enhanced osteogenesis and angiogenesis in the BMSC/β-TCP construct.

Experiment 2 (influence of BMSCs concentration in the M and P groups)

To investigate how cell concentration affects bone formation in the M and P groups, constructs were prepared with cell suspensions at three concentrations in each group. The extent of newly formed bone tended to be reduced in both groups according to the decrease in cell concentration. However, these influences were smaller in the P groups. The extent of bone formation of the P200 group was almost equal to that of the P2000 group; bone formation of the P20 group was distributed to nearly the same extent as in the M200 group. However, there was little or no bone formation in the implants of the M20 group (Fig. 4A and Table 3). The BFA of the P groups was higher than that of the M groups at each concentration. Though BFA was reduced with decreased cell concentration in both groups, the P group was less affected by the reduction of cell concentration. Further, the BFAs of

FIG. 3. Comparison among the M, P, and R groups (Experiment 1). (**A**) Representative histological sections at 3 and 6 weeks stained with H&E. Images taken from the central parts of each implant. At 3 weeks, there was much newly formed bone with many invaded blood vessels in the P and R groups in all the peripheral areas. At 6 weeks, bone formation invaded the central area, became thicker, and was homogenously distributed in the entire area in the P and R groups, differing from the M group. T, β-TCP; B, newly formed bone; arrowhead, blood vessels. Scale bars: 1 mm (whole area) and 250 μm (high magnification). (**B**) BFA at 3 and 6 weeks. The data are expressed as the mean ± SD ($^*p < 0.05$). (**C**) Representative histological sections of the peripheral area at early stages stained with H&E, TRAP, and immunohistochemistry for the anti-CD31 antibody. Blood vessels invaded and developed earlier in the P group than the M group. At 3 weeks, many TRAP-positive cells appeared only in the P group. Scale bars: 500 μm (H&E and CD31) and 200 μm (TRAP). Asterisks indicate outside area of implants. Color image available online at www.liebertonline.com/ten.

the constructs in the P groups (P200 and P20 groups) were almost equal to those of the M group constructs with, respectively, 10-fold higher cell concentrations (M2000 and M200 groups) (Fig. 4B).

Experiment 3 (effect of fibrin and serum)

Based on the results of Experiments 1 and 2, we hypothesized that the fibrin network and/or serum in plasma would be key factors promoting angiogenesis and osteogenesis. To clarify the effect of these components, we prepared and implanted constructs with five different compositions (Table 1C). To find the normal value of fibrinogen concentration in cynomolgus monkeys, the fibrinogen concentration in venous blood from monkeys was measured. The mean value of the fibrinogen concentration was 3.73 ± 1.27 mg/mL ($n = 10$). Therefore, we set 4 mg/mL as the physiological fibrinogen concentration in this experiment. At 3 and 5 weeks after implantation, constructs were extracted for histological examination; all of the extracted constructs showed bone formation. At 3 weeks, bone in the 4S and 1S groups was formed in the entire peripheral area, while there was less bone formation in the partial peripheral area in the 4P, 0S, and 16S groups (Fig. 5A). Many blood vessels were also seen in the peripheral area with newly formed bone in the 4S and 1S groups, as found in the P group (Fig. 5A). At 5 weeks, there was newly formed bone in almost the entire area of implants in all five groups. However, we observed less bone formation at the central area in the 4P, 0S, and 16S groups (Fig. 5A). In the quantitative analysis, the BFA of the 4S group was significantly higher than that of the 4P, 0S, or 16S group at 3 weeks (Fig. 5B). At 5 weeks, there was no significant difference among the five groups (Fig. 5C). These findings demonstrated that both fibrin networks from the physiological fibrinogen concentration (4 mg/mL) and serum played a crucial role for the enhancement of angiogenesis and osteogenesis, especially in the early stages.

Discussion

We showed here that the autologous plasma (PPP or PRP)/BMSC/β-TCP constructs have more potential for bone formation than the BMSC/β-TCP construct prepared using a culture medium in a monkey heterotopic bone formation model. We also showed that both fibrin networks formed from native fibrinogen concentration (4 mg/mL) and serum in plasma were essential elements for the enhancement of angiogenesis and osteogenesis in BMSC/β-TCP construct.

PRP has been widely used in the field of bone regeneration, due to the effect on bone formation of fibrin and various growth factors (transforming growth factor beta, platelet-derived growth factor, etc.) released from platelets;[25–27] therefore, we examined the bone formation capability of the PRP/BMSC/β-TCP construct using rats in previous study.[19] From this result, it was revealed that PPP, rather than PRP, had higher bone formation capability in the BMSC/β-TCP construct. This confirmed other reports that PRP had little effect on bone formation.[37,38] The function of PRP on bone formation is still controversial, and therefore, to clarify the potential effects of PPP and PRP/BMSC/β-TCP constructs, we conducted Experiment 1 using monkeys, which are most similar to humans.[33]

In contrast with our previous report using rats, there was no histological and histomorphometric difference between the P and R groups, although each of the two groups had a significantly higher BFA than the M group. The difference between our two studies suggests that PRP has diverse effects that depend on the difference in implantation sites, animal species, platelet concentration, reactivity to growth factors, and the like.[39] Considering that PPP is more easily and abundantly available than PRP and, therefore, is advantageous when applied to the repair of large bone defects, we focused on PPP and performed a detailed examination. Characteristically, enhanced angiogenesis and little other tissue invasion were found at early stages in the P group. These findings suggest that early nutrient supply by blood flow, without invasion of other tissues, could increase the potential of seeded BMSCs. Additionally, they might explain the reason why the extent of the bone formation in the P group was homogenous at 6 weeks, in contrast with the nonuniform results obtained in the M group. Based on the results of Experiment 1, we hypothesized that fibrin and serum component in plasma promoted angiogenesis and subsequent osteogenesis, even without growth factors from platelets.

In Experiment 2, the extent of bone formation and BFA were dramatically reduced in the M groups with a decrease in cell concentration, whereas these influences in the P groups were small both histologically and histomorphometrically. Additionally, it was demonstrated that the P groups required only one-tenth the number of BMSCs of the M groups. The potential of seeded BMSCs was possibly increased using PPP. Studies on bone formation that combine BMSCs and a porous scaffold generally utilize a cell suspension at concentrations of 1×10^6 to 3×10^7 cells/mL.[9–12] However, obtaining a large number of BMSCs is costly and requires long culture periods. Further, the osteogenic potential of BMSCs is known to decrease with proliferation.[40–43] Therefore, PPP, which could reduce the number of seeded BMSCs, is very beneficial in repairing massive bone defects.

Some studies reported the availability of plasma/BMSC/porous ceramic constructs.[17,19,31] However, these studies did not reveal which components in the plasma facilitated the *in vivo* bone formation capability. Therefore, we performed Experiment 3 to elucidate the mechanism of the augmentation by plasma. Because the BFAs of the P and R groups were almost equivalent in Experiment 1, we regarded effects of the growth factors released from platelets as negligible and used autologous serum from whole blood in Experiment 3. Considering that some combinations of purified fibrin/ceramic scaffolds were reported to have osteoinductivity in heterotopic sites[20] and that fibrin was known to induce angiogenesis,[22,23] the implants prepared with fibrin were predicted to obtain sufficient bone formation. Despite the prediction, the 4P and 16S groups showed statistically lower BFAs at 3 weeks, although the 0S group without fibrin showed lower BFA, as expected. In comparison with the 4S group, the result of the 4P group suggests that the fibrin network by itself is insufficient for early angiogenesis and osteogenesis; thus, serum was also necessary.

In the tissue engineering field, various porous materials have been used as scaffolds because the structure provides a large effective surface for cell expansion and tissue organization. However, the material structure was believed to have negative effects on nutrient diffusion surrounding tissues

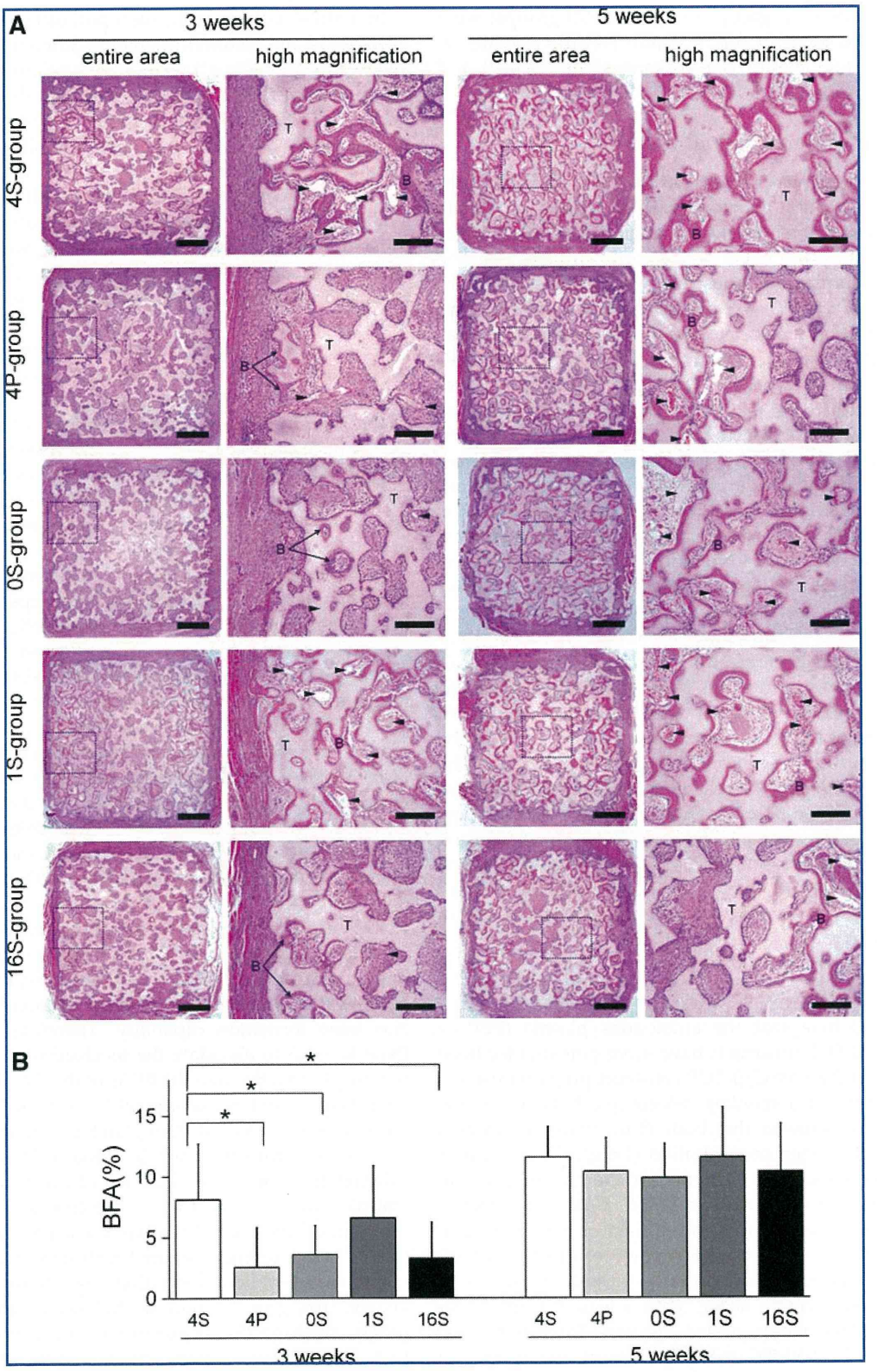

FIG. 5. Effects of fibrin and serum (Experiment 3). (**A**) Representative histological sections at 3 and 5 weeks stained with H&E. Images are from the central parts of each implant. By 3 weeks, a great quantity of bone and many blood vessels had formed in the entire peripheral area in both the 4S and 1S groups. At 5 weeks, in the 4S and 1S groups, thicker bone extended with many developed blood vessels to the central area, compared to the 4P, 0S, and 16S groups. T, β-TCP. Arrowheads indicate blood vessels. Scale bars: 1 mm (whole area) and 250 μm (high magnification). (**B**) BFA at 3 and 6 weeks. The data are expressed as mean ± SD (*$p < 0.05$). Color image available online at www.liebertonline.com/ten.

TABLE 3. BONE FORMATION IN EXPERIMENT 2

Groups	Constructs	5 Weeks		
		Case 1	*Case 2*	*Case 3*
M2000		3/3	3/3	0/3
M200	BMSC/β-TCP	3/3	3/3	0/3
M20		2/3	2/3	0/3
P2000		3/3	3/3	3/3
P200	PPP/BMSC/β-TCP	3/3	3/3	3/3
P20		3/3	3/3	3/3

Number of implants with bone formation/Total implants.

and resultant cell survival.[15–18] Therefore, as a mechanism of the augmentation of bone formation by plasma, the serum component contained in plasma was supposed to support BMSC survival after the implantation, especially in the early stages. Lower BFA in the 0S and 16S groups revealed that osteogenesis depends on fibrinogen concentration, and it is important that BMSCs are retained in the fibrin network formed from fibrinogen with the physiological concentration (the physiological fibrinogen concentration in cynomolgus monkeys is approximately 4 mg/mL). This result is consistent with the reports that fibrin from 5 mg/mL fibrinogen promoted osteogenic differentiation of BMSCs[19] and that a fibrin network formed from fibrinogen with a high concentration of inhibited cell growth and migration.[21] From these results, it was confirmed that both serum and fibrin networks that formed from the normal fibrinogen concentration, which was equal to normal plasma, are essential for enhanced angiogenesis and osteogenesis at the early stages of implantation. However, BFAs of the five groups were almost equivalent at 5 weeks, although there was a delay in bone formation in the central area in the 4P, 0S, and 16S groups. Considering the limitation of the available space for bone formation in the scaffolds, transplantation with decreased cell concentration might have revealed superior effects of the 4S group on bone formation also at 5 weeks.

This study, using nonhuman primates, indicates that utilization of the PPP/BMSCs/β-TCP construct, which can be easily prepared, will be a more advantageous strategy for clinical application of tissue-engineered bone regeneration.

Conclusion

The bone formation capability of the autologous plasma (PPP or PRP)/BMSC/β-TCP construct was enhanced in nonhuman primates compared to the BMSC/β-TCP construct prepared using a culture medium. The bone formation capability of PPP/BMSC/β-TCP construct was less influenced by the decrease in the number of BMSCs. Both fibrin networks, which formed from a native fibrinogen concentration, and serum were essential to the enhancement of angiogenesis and osteogenesis, especially in the early stages.

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Disclosure Statement

No competing financial interests exist.

References

1. Banwart, J.C., Asher, M.A., and Hassanein, R.S. Iliac crest bone graft harvest donor site morbidity. A statistical evaluation. Spine **20,** 1055–1060, 1995.
2. Arrington, E.D., Smith, W.J., Chambers, H.G., Bucknell, A.L., and Davino, N.A. Complications of iliac crest bone graft harvesting. Clin Orthop Relat Res **329,** 300–309, 1996.
3. LeGeros, R.Z. Properties of osteoconductive biomaterials: calcium phosphates. Clin Orthop Relat Res **395,** 81–98, 2002.
4. Yuan, H., van Blitterswijk, C.A., deGroot, K., and de Bruijn, J.D. Cross-species comparison of ectopic bone formation in biphasic calcium phosphate (BCP) and hydroxyapatite (HA) scaffolds. Tissue Eng **12,** 1607–1615, 2006.
5. Kondo, N., Ogose, A., Tokunaga, K., Umezu, H., Arai, K., Kudo, N., Hoshino, M., Inoue, H., Irie, H., Kuroda, K., Mera, H., and Endo, N. Osteoinduction with highly purified β-tricalcium phosphate in dog dorsal muscles and the proliferation of osteoclasts before heterotopic bone formation. Biomaterials **27,** 4419–4427, 2006.
6. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. Science **284,** 143–147, 1999.
7. Derubeis, A.R., and Cancedda, R. Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. Ann Biomed Eng **32,** 160–165, 2004.
8. Goshima, J., Goldberg, V.M., and Caplan, A.I. The osteogenic potential of cultured-expanded rat marrow mesenchymal cells assayed *in vivo* in calcium phosphate ceramic blocks. Clin Orthop **262,** 298–311, 1991.
9. Bruder, S.P., Kraus, K.H., Goldberg, V.M., and Kadiyala, S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am **80,** 985–996, 1998.
10. Petite, H., Viateau, V., Bensaid, W., Meunier, A., de Pollak, C., Bourquiqnon, M., Oudina, K., Sedel, L., and Guillemin, G. Tissue-engineered bone regeneration. Nat Biotechnol **18,** 959–963, 2000.
11. Nishikawa, M., Myoui, A., Ohgushi, H., Ikeuchi, M., Tamai, N., and Yoshikawa, H. Bone tissue engineering using novel interconnected porous hydroxyapatite ceramics combined with marrow mesenchymal cells: quantitative and three-dimensional image analysis. Cell Transplant **13,** 367–376, 2004.
12. Kruyt, M.C., Dhert, W.J., Yuan, H., Wilson, C.E., van Blitterswijk, C.A., Verbout, A.J., and de Bruijn, J.D. Bone tissue engineering in a critical size defect compared to ectopic implantations in the goat. J Orthop Res **22,** 544–551, 2004.
13. Kruyt, M.C., Persson, C., Johansson, G., Dhert, W.J., and de Bruijn, J.D. Towards injectable cell-based tissue-engineered bone: the effect of different calcium phosphate microparticles and pre-culturing. Tissue Eng **12,** 309–317, 2006.
14. Wang, J., Asou, Y., Sekiya, I., Sotome, S., Orii, H., and Shinomiya, K. Enhancement of tissue engineered bone formation by a low pressure system improving cell seeding and medium perfusion into a porous scaffold. Biomaterials **27,** 2738–2746, 2006.
15. Ishaug-Riley, S.L., Crane, G.M., Gurlek, A., Miller, M.J., Yasko, A.W., Yaszemski, M.J., and Mikos, A.G. Ectopic bone formation by marrow stromal osteoblast transplantation using poly(DL-lactic-co-glycolic acid) foams implanted into the rat mesentery. J Biomed Mater Res **36,** 1–8, 1997.

16. Muschler, G.F., Nakamoto, C., and Griffith, L.G. Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am **86,** 1541–1558, 2004.
17. Kruyt, M.C., Dhert, W.J., Oner, F.C., van Blitterswijk, C.A., Verbout, A.J., and de Bruijn, J.D. Analysis of ectopic and orthotopic bone formation in cell-based tissue-engineered constructs in goats. Biomaterials **28,** 1798–1805, 2007.
18. Viateau, V., Guillemin, G., Bousson, V., Oudina, K., Hannouche, D., Sedel, L., Logeart-Avramoglou, D., and Petite, H. Long-bone critical-size defects treated with tissue-engineered grafts: a study on sheep. J Orthop Res **25,** 741–749, 2007.
19. Tajima, N., Sotome, S., Marukawa, E., Omura, K., and Shinomiya, K. A three-dimensional cell-loading system using autologous plasma loaded into a porous beta-tricalciumphosphate block promotes bone formation at extraskeletal sites in rats. Mater Sci Eng C **27,** 625–632, 2007.
20. Abiraman, S., Varma, H.K., Umashankar, P.R., and John, A. Fibrin glue as an osteoinductive protein in a mouse model. Biomaterials **23,** 3023–3031, 2002.
21. Bensaid, W., Triffitt, J.T., Blanchat, C., Oudina, K., Sedel, L., and Petite, H. A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. Biomaterials **24,** 2497–2502, 2003.
22. Eyrich, D., Gopferich, A., and Blunk, T. Fibrin in tissue engineering. Adv Exp Med Biol **585,** 379–392, 2006.
23. Mosesson, M.W. Fibrinogen and fibrin structure and functions. J Thromb Haemost **3,** 1894–1904, 2005.
24. Fan, C.L., Gao, P.J., Gu, Y.J., Tang, X.F., Liu, J.J., Wei, J., Inoue, K., and Zhu, D.L. Therapeutic angiogenesis by intramuscular injection of fibrin particles into ischaemic hindlimbs. Clin Exp Pharmacol Physiol **33,** 617–622, 2006.
25. Marx, R.E., Carlson, E.R., Eichstaedt, R.M., Schimmele, S.R., Strauss, J.E., and Georgeff, K.R. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod **85,** 638–646, 1998.
26. Wiltfang, J., Schlegel, K.A., Schultze-Mosgau, S., Nkenke, E., Zimmermann, R., and Kessler, P. Sinus floor augmentation with beta-tricalciumphosphate (beta-TCP): does platelet-rich plasma promote its osseous integration and degradation? Clin Oral Implants Res **14,** 213–218, 2003.
27. Kitoh, H., Kitakoji, T., Tsuchiya, H., Mitsuyama, H., Nakamura, H., Katoh, M., and Ishiguro, N. Transplantation of marrow-derived mesenchymal stem cells and platelet-rich plasma during distraction osteogenesis—a preliminary result of three cases. Bone **35,** 892–898, 2004.
28. Mankani, M.H., Kuznetsov, S.A., Wolfe, R.M., Marshall, G.W., and Robey, P.G. *In vivo* bone formation by human bone marrow stromal cells: reconstruction of the mouse calvarium and mandible. Stem cells **24,** 2140–2149, 2006.
29. Minamide, A., Yoshida, M., Kawakami, M., Yamasaki, S., Kojima, H., Hashizume, H., and Boden, S.D. The use of cultured bone marrow cells in type I collagen gel and porous hydroxyapatite for posterolateral lumbar spine fusion. Spine **30,** 1134–1138, 2005.
30. Yuan, J., Cui, L., Zhang, W.J., Liu, W., and Cao, Y. Repair of canine mandibular bone defects with bone marrow stromal cells and porous beta-tricalcium phosphate. Biomaterials **28,** 1005–1013, 2007.
31. Kruyt, M.C., de Bruijn, J.D., Yuan, H., van Blitterswijk, C.A., Verbout, A.J., Oner, F.C., and Dhert, W.J. Optimization of bone tissue engineering in goats: a peroperative seeding method using cryopreserved cells and localized bone formation in calcium phosphate scaffolds. Transplantation **77,** 359–365, 2004.
32. Wang, T., Dang, G., Guo, Z., and Yang, M. Evaluation of autologous bone marrow mesenchymal stem cell-calcium phosphate ceramic composite for lumbar fusion in rhesus monkey interbody fusion model. Tissue Eng **11,** 1159–1167, 2005.
33. Izadopanah, R., Joswig, T., Tsien, F., Dofour, J., Kirijan, J.C., and Bunnell, B.A. Characterization of multipotent mesenchymal stem cells from the bone marrow of rhesus macaques. Stem Cells Dev **14,** 440–451, 2005.
34. Dong, J., Uemura, T., Shirasaki, Y., and Tateishi, T. Promotion of bone formation using highly pure porous beta-TCP combined with bone marrow-derived osteoprogenitor cells. Biomaterials **23,** 4493–4502, 2002.
35. Torigoe, I., Sotome, S., Tsuchiya, A., Yoshii, T., Takahashi, M., Kawabata, S., and Shinomiya, K. Novel cell seeding system into a porous scaffold using a modified low pressure method to enhance cell seeding efficiency and bone formation. Cell Transplant **16,** 729–739, 2007.
36. Dennis, J.E., Konstantakos, E.K., Arm, D., and Caplan, A.I. *In vivo* osteogenesis assay: a rapid method for quantitative analysis. Biomaterials **19,** 1323–1328, 1998.
37. Gruber, R., Karreth, F., Kandler, B., Fuerst, G., Rot, A., Fischer, M.B., and Watzek, G. Platelet-released supernatants increase migration and proliferation, and decrease osteogenic differentiation of bone marrow-derived mesenchymal progenitor cells under *in vitro* conditions. Platelets **15,** 29–35, 2004.
38. Sarkar, M.R., Augat, P., Shefelbine, S.J., Schorlemmer, S., Huber-Lang, M., Claes, L., Kinzl, L., and Ignatius, A. Bone formation in a long bone defect model using a platelet-rich plasma-loaded collagen scaffold. Biomaterials **27,** 1817–1823, 2006.
39. Grageda, E. Platelet-rich plasma and bone graft materials: a review and a standardized research protocol. Implant Dent **13,** 301–309, 2004.
40. Bruder, S.P., Jaiswal, N., and Haynesworth, S.E. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem **64,** 278–294, 1997.
41. Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., and Quatro, R. Proliferation kinetics and differentiation potential of *ex vivo* expanded human BMSCs: implications for their use in cell therapy. Exp Hematol **28,** 707–715, 2000.
42. Simonsen, J.L., Rosada, C., Serakinci, N., Justesen, J., Stenderup, K., Rattan, S.I., Jensen, T.G., and Kassem, M. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nat Biotechnol **20,** 592–996, 2002.
43. Vacanti, V., Kong, E., Suzuki, G., Sato, K., Canty, J.M., and Lee, T. Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. J Cell Physiol **205,** 194–201, 2005.

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