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In vivo bone-forming capacity of human bone marrow-derived stromal cells is stimulated by recombinant human bone morphogenetic protein-2

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Abstract In the present study, we investigated whether the in vivo bone-forming capacity of human bone marrow-derived stromal cells (HMSCs) could be enhanced by recombinant human bone morphogenetic protein-2 (rhBMP-2). The HMSCs obtained from seven donors (5–54 years of age) were passaged three to six times. Passaged HMSCs exhibited the osteoblastic phenotype in vitro, including: (a) an increase in alkaline phosphatase (ALP) activity in response to dexamethasone, ascorbic acid, and β-glycerophosphate; and (b) mRNA expression for markers of osteoblastic lineage (ALP, osteopontin, osteocalcin, and parathyroid hormone-receptor) and BMP-2, -4, and -6 detected by reverse transcription–polymerase chain reaction. For the in vivo assay, transplants were subcutaneously implanted into nude mice as follows: group A (vehicle); group B (rhBMP-2); group C (HMSCs with vehicle); and group D (HMSCs with rhBMP-2). Transplants were obtained 2 and 4 weeks after implantation. Correlated radiographic findings, histological observations, and in situ hybridization using species-specific probes showed that the group B transplants contained bone tissue of mouse origin, which was observed at the periphery of the transplants. Four weeks after implantation, small amounts of HMSCs-derived bone tissue were detected at the periphery in two of seven transplants in group C. In contrast, five of seven group D transplants exhibited HMSCs-derived bone tissue, which was located at the center of the transplants and was surrounded by mouse bone tissue. Furthermore, HMSCs-derived chondrogenesis was detected in two of seven group D transplants. The results of the present study demonstrate that culture-expanded HMSCs preserve the osteoblastic phenotype, and the in vivo bone-forming capacity can be promoted by rhBMP-2.

Key words bone marrow stromal cells · human · bone formation · recombinant human BMP-2 · in situ hybridization

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Introduction

To manage bone defects, autogenous bone grafting harvested from intact sites has become a major therapeutic option. Over the past decade, the desire to incorporate the favorable properties of different materials into an effective bone graft compound has led to the manipulation and development of a number of new synthetic bone-grafting products. It is thought that the ideal bone graft substitutes should provide three elements: (1) an osteoconductive matrix as scaffolding; (2) osteoinductive factors; and (3) osteogenic cells [1]. Supplementation of bone-forming cells and osteoinductive growth factors, which could additively activate bone formation, are expected to be good bone graft substitutes, while combined implantation of bone-forming cells and osteoinductive growth factors has not been examined thoroughly.

It has been shown that bone marrow stroma contains stromal precursor cells that can differentiate into osteoblasts, chondrocytes, adipocytes, fibroblasts, and hematopoietic supporting cells [2–8]. Recent progress in elucidating the osteogenic potential of human bone marrow-derived stromal cells (HMSCs) has demonstrated that they maintain their osteogenic potential during extensive subcultivation in vitro [9,10] and can form bone in vivo [11,12]. Although HMSCs are considered to be a potent cell source for bone regenerative therapy, little is known about whether they can be accelerated to form bone in vivo with a combination of osteoinductive growth factors.

Bone morphogenetic proteins (BMPs) play an important role in bone formation in vivo. Recombinant human BMP-2 (rhBMP-2) can induce osteoblastic differentiation of mesenchymal cells in vitro [13–15] and stimulate bone formation in vivo [16–20]. In addition, rhBMP-2 can increase alkaline phosphatase (ALP) activity in HMSCs [21,22]. A recent study has shown that human bone cells can form bone with rhBMP-2 using

diffusion chambers [23]. These data suggest that this molecule could be a candidate growth factor for osteoinductive treatment. However, the effect of rhBMP-2 on the bone-forming capacity of cultured HMSCs in vivo is not adequately understood.

To address the hypothesis that the in vivo bone-forming capacity of HMSCs is stimulated by rhBMP-2 when they are simultaneously implanted, we examined the in vivo bone-forming capacity of transplanted HMSCs with or without rhBMP-2 in a xenograft model. To distinguish between HMSCs- and mouse-derived bone formation, especially in the presence of rhBMP-2, we analyzed the origin of the bone-forming cells in transplants by in situ hybridization with species-specific probes [24].

Materials and methods

Preparation of culture-expanded HMSCs

Bone marrow specimens were obtained from seven patients (age range from 5–54 years; mean age 18.9 years) without metabolic bone disorders during pelvic or femoral osteotomy. Informed consent was obtained prior to surgery. After 6 ml bone marrow aspirate was harvested with sodium heparin from the ilium under general anesthesia, the aspirate was added to ice-cold serum-free α-modified minimal essential medium (α-MEM; Gibco BRL, Gaithersburg, MD, USA) up to 20 ml. After pipetting repeatedly, bone dust and undispersed cell aggregates were removed by filtration through a 70 μm nylon mesh cell strainer (Falcon, Lincoln Park, NJ, USA). Mononuclear cells were isolated by centrifugation over a Ficoll–Paque density gradient (Pharmacia Biotech, Piscataway, NJ, USA) at 400 *g* for 30 min at 20°C. Cells from the interface were washed twice with phosphate-buffered saline (PBS) and counted using a hemocytometer with Trypan Blue. Cells were plated into 25 or 75 cm^2 culture flasks (Corning, Corning, NY, USA) at a density of 0.8–1.6 × 10^5 cells/cm^2 in α-MEM containing penicillin (100 U/ml), streptomycin (100 μg/mL), and 10% fetal bovine serum (FBS; Gibco BRL). Culture media were changed on the 5th day and twice weekly thereafter. Cells were maintained in a humidified atmosphere (95% air and 5% CO_2) at 37°C. In primary culture, cells grew to reach a semiconfluent monolayer on approximately day 14. After washing twice with PBS, adherent cells were treated with 0.05% trypsin and 0.02% EDTA in PBS for 5 min at 37°C. Then, they were expanded at a 1:3 split ratio per subculture. Culture-expanded cells were used for subsequent characterization (passage 4–6) and implantation (passage 3–5). Cell donors are described in Table 1.

Characterization of culture-expanded HMSCs in vitro

Alkaline phosphatase activity. The HMSCs were plated into 24-well plates (Falcon) at a density of 1 × 10^4 cells/cm^2 and cultured in α-MEM supplemented with 10% FBS with or without 10^{-7} mol/l dexamethasone (Dex; Sigma Chemical, St Louis, MO, USA), 50 μg/ml ascorbic acid phosphate (Asc; Wako Chemical, Osaka, Japan) and 10 mmol/l β-glycerophosphate (β-gly; Wako). The activity of ALP was measured biochemically on day 14 as described previously [25] using *p*-nitrophenyl phosphate as a substrate. Statistical analyses were performed using unpaired Student's *t*-test.

Table 1. Induction of altaline phosphatase activity in human bone marrow stromal cells after 2 weeks in culture

Donor no.	Age (years), gender	Passage	ALP activity (nmol/min/mg protein)		Fold stimulation (treated/control)
			Control	Treated[a]	
1	5, M	5th	19.0 ± 4.6	258.2 ± 26.2*	13.6
2	5, F	4th	4.0 ± 1.6	225.9 ± 57.0*	56.5
3	5, F	6th	39.2 ± 4.9	104.6 ± 7.3*	2.7
4	8, F	4th	120.5 ± 6.3	517.1 ± 20.5*	4.3
5	14, M	4th	22.2 ± 2.3	408.1 ± 25.3*	18.4
6	41, F	6th	51.1 ± 2.8	337.1 ± 16.8*	6.6
7	54, F	5th	19.7 ± 0.9	249.3 ± 33.9*	12.7

Data are the mean ± SD of quadruplicate cultures

* $P < 0.001$ compared with control cultures (Student's *t*-test). Alkaline phosphatase (ALP) activity was measured as described in Materials and methods

[a] Human bone marrow stromal cells were treated with 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate in α-modified minimal essential medium plus 10% fetal bovine serum for 14 days. Control cells were cultured with vehicle

Reverse transcription–polymerase chain reaction. HMSCs were cultured in 6-cm dishes (Corning) for 2 weeks. Total RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan). Complementary DNA was synthesized from 1 μg total RNA using M-MLV reverse transcriptase (RT; Gibco BRL) with the oligo (dT) priming method in a 20 μl reaction mixture. A 1 μl sample of single-stranded cDNA was amplified in 20 μl of polymerase chain reaction (PCR) reaction mixture containing 0.5 U Taq polymerase (Promega, Madison, WI USA). The PCR primers for the osteoblastic phenotype and BMPs were prepared as described previously [8,26]: ALP, osteopontin (OPN), osteocalcin (OSC), parathyroid hormone receptor (PTH-R), and BMP-2–7. Amplifications were performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) under the following conditions: denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s, except for β-actin (25 cycles) and PTH-R (35 cycles). The amplified products (5 μl) were electrophoresed in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and were visualized using a an ultraviolet (UV) transilluminator.

Bone formation assay in vivo

Preparation of HMSCs and implantation into nude mice. Culture-expanded HMSCs from each donor were prepared in 75-cm^2 flasks at a density of 1.8–5.3 $\times$ 10^5 cells/flask (mean 3.3 $\times$ 10^5). Cells were cultured in the presence of Dex, Asc, and β-gly from day 2 after inoculation. By day 14, cells had reached confluency and grew into a uniform sheet of cells. (In preliminary studies, the confluent cell layer in a 75-cm^2 flask contained approximately 2 $\times$ 10^6 cells.) Cells were washed twice with PBS and scraped with a rubber policeman. Then, cells from two flasks each (approximately 4 $\times$ 10^6 cells) were collected in a 50-ml centrifugation tube (Corning) and were centrifuged to form a cellular pellet. Each pellet was prepared as follows: group A vehicle; group B rhBMP-2; group C HMSCs pellet with vehicle; and group D HMSCs pellet with rhBMP-2. Serum-free medium (180 μl for groups A and B; 80 μl for groups C and D) was added to adjust the volume of the pellets equally. Either rhBMP-2 (20 μg; kindly provided by Yamanouchi Pharmaceutical, Tokyo, Japan) or rhBMP-2 dilution buffer were added. The total volume of each transplant was approximately 200 μl. To make the pellets clot-like and easy to implant without scaffolds, 20 μl fibrin glue (BOLHEAL; gift from Fujisawa Pharmaceutical, Osaka, Japan), at 10% of the final concentration, was added. Seven-week-old nude mice (BALB/c nu/nu; purchased from Nihon Clea, Tokyo, Japan) were anesthetized with ketamine hydrochloride (Sankyo, Tokyo, Japan) and xylazine hydrochloride (Bayer, Tokyo, Japan). A midlongitudinal skin incision was made along the back, and subcutaneous pockets were formed by blunt dissection. A set of four pellets was implanted into each pocket separately. The incision was closed with sutures. Specimens were obtained 2 and 4 weeks after implantation and were used in the following studies. These procedures were reviewed and approved by the Animal Care and Use Committee of Niigata University. The protocol for implantation is illustrated in Fig. 1.

Radiologic and histologic assessment of bone formation in transplants. After 24 h fixation with 4% paraformaldehyde in a 0.1 mol/l phosphate buffer, soft-X radiograms of specimens were taken under RNase-free conditions. Then, specimens were decalcified in 0.5 mol/l EDTA, dehydrated, and embedded in paraffin. Sections (4 μm) were mounted on 3-amino-propyltriethoxysilane-coated glass slides and stored at 4°C until subsequent analysis. For each sample, two different section levels and 15 sections for each level were analyzed using histology and in situ hybridization. Serial sections were stained with hematoxylin–eosin (HE) and Toluidine Blue-O.

Detection of bone-forming cells and identification of their origin by in situ hybridization. In the present study, bone formation was determined via the eosin-stained bone matrix with bone-forming cells strongly expressing type I collagen mRNA. The 0.37 kb of mouse α1 type I procollagen cDNA [24], which is highly homologous in humans (91% identical), was used. To identify the origin of bone-forming cells, human "Alu" and mouse "L1"-specific DNA sequences were used [24]. Digoxigenin-11-UTP (Boehringer Mannheim Biochemica, Mannheim, Germany)-labeled probes were prepared, and in situ hybridization was performed as described previously [24,27].

Results

Characteristics of cultured HMSCs in vitro

In all donors, the ALP activity of HMSCs treated with Dex, Asc, and β-gly on day 14 of culture was significantly higher (2.7–56.5-fold) compared with control (Table 1). Variability in the basal level of ALP activity and a fold increase in ALP activity were observed. These findings are consistent with previously reported ALP activity in human bone marrow stromal cell cultures [10,28].

Analysis with RT-PCR revealed that mRNA expression of markers for osteoblastic phenotypes, such as ALP, OPN, OSC, and PTH-R, was present in control

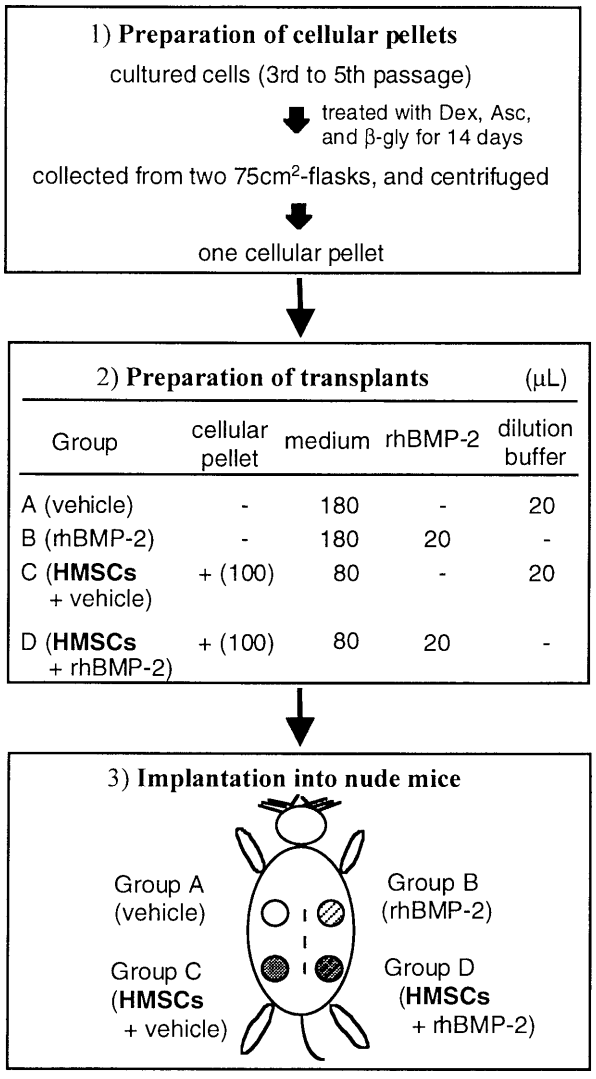

Fig. 1. Implantation protocol for human bone marrow stromal cells (*HMSCs*) in vivo. *1)* Culture-expanded HMSCs were treated with 10^{-7}mol/l dexamethasone (*Dex*), 50μg/ml ascorbic acid phosphate (*Asc*), and 10mmol/l β-glycerophosphate (β-*gly*) in α-modified minimum essential medium plus 10% fetal bovine serum (α-MEM + 10% FBS) for 14 days. *2)* The volume of the cellular pellets was approximately equal to 100μl. Serum-free medium was added to adjust the volume of the pellets. Then, 20μg recombinant human bone morphogenetic protein-2 (rhBMP-2) in 20μL dilution buffer was added. Transplants were prepared with fibrin glue (20μl), at approximately 10% of the final concentration, to make the pellets clot-like and easy to implant without scaffolds. *3)* A set of four pellets was subcutaneously implanted separately. Specimens were obtained after 2 and 4 weeks and used in subsequent studies

cells. There was an increase in ALP, OPN, and PTH-R mRNA and a decrease in OSC mRNA expression in treated cells. Expression of BMP-2, -4, and -6 mRNA was detected in control cells, and the expression of these BMPs increased in treated cells. Representative results are shown in Fig. 2. In RT-PCR analysis, no obvious

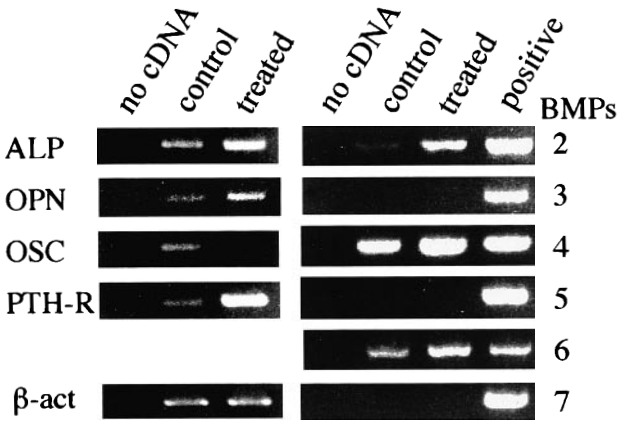

Fig. 2. Expression of markers for osteoblastic lineage and BMPs mRNA in culture-expanded HMSCs by reverse transcription–polymerase chain reaction (RT-PCR). The HMSCs were treated with or without 10^{-7}mol/l dexamethasone, 50μg/ml ascorbic acid phosphate, and 10mmol/l β-glycerophosphate in α-MEM + 10% FBS for 14 days. Total RNA was extracted, and cDNA was synthesized from 1μg total RNA using M-MLV RTase with oligo dT primer, followed by 30 cycles of PCR, except for β-actin (β-act; 25 cycles) and parathyroid hormone receptor (*PTH-R*; 35 cycles). The PCR products were electrophoresed in 1.5% agarose gels. Representative results are shown. *ALP*, alkaline phosphatase; *OPN*, osteopontin; *OSC*, osteocalcin. Human embryonic RNA was used as a positive control for BMPs

difference in mRNA expression was observed among the donors (data not shown).

Bone formation of HMSCs in vivo

Soft X-ray examination of transplants. Group A transplants (vehicle) were absorbed by day 5 after implantation. Therefore, no group A specimens were obtained at the time of death. In group B (rhBMP-2 only), opaque areas of mineralization with trabecular structure were clearly observed (Fig. 3a, b). In group C (HMSCs with vehicle), calcification without trabecular structure was observed (Fig. 3c, d). Group C specimens were smaller in size than those of groups B or D. In group D specimens (HMSCs with rhBMP-2), opaque areas of mineralization with calcified trabeculae were observed, and highly mineralized areas were detected in the central region of transplants after 4 weeks (Fig. 3e, f). There was no significant difference in size between groups B and D.

Histological findings of bone formation. In group B, bone tissue was formed mainly at the periphery of the transplants (Fig. 4a). Bone marrow was also generated, and the bone marrow area increased after 4 weeks. In group C, fibrous tissue was observed and no bone was

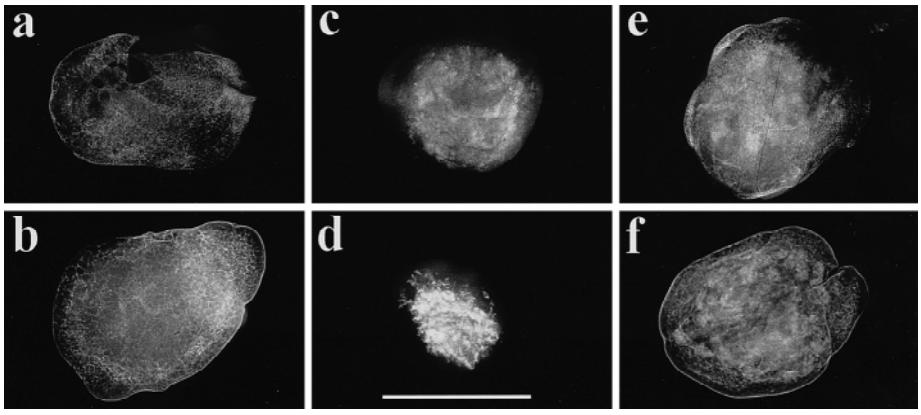

Fig. 3. Soft X-ray findings of transplants after 2 (**a,c,e**) and 4 (**b,d,f**) weeks. (**a,b**) Opaque areas of mineralization with trabecular structure are observed in group B (rhBMP-2). (**c,d**) The group C (HMSCs) specimen contains calcification, but does not have a trabecular structure. It is smaller in size than that of groups B and D. (**e,f**) Opaque areas of mineralization with calcified trabeculae are formed, and highly mineralized areas are detected in the central region of group D transplants (HMSCs + rhBMP-2). The results of donor 1 are shown. No specimen in group A (vehicle) was obtained because of absorption by day 5 after implantation. *Bar*, 1 cm

Table 2. HMSC-derived bone formation confirmed by DNA in situ hybridization

Donor no.	Age (years), gender	Passage	2 weeks		4 weeks	
			Group C	Group D	Group C	Group D
1	5, M	3rd	−	+	−	+
2	5, F	3rd	−	−	+	+
3	5, F	3rd	NE	NE	+	+
4	8, F	3rd	NE	NE	−	−
5	14, M	3rd	−	+	−	+
6	41, F	5th	−	−	−	−
7	54, F	4th	−	−	−	+
Total			0/5	2/5	2/7	5/7

Human bone marrow-derived stromal cells (HMSCs) were transplanted as described in Materials and methods. Bone formation that originated from HMSCs was confirmed according to positive signals for human-specific Alu (+) and negative signals for mouse L1. Group C, HMSCs with vehicle; group D, HMSCs with recombinant human bone morphogenetic protein-2; NE, not examined; (−), no bone tissue with positive signals for Alu

detected after 2 weeks. After 4 weeks, no bone tissue was detected in five transplants, but a small amount of immature bone tissue was found in two specimens (donors 2 and 3). This was located at the edge of the transplants (Fig. 4d). Bone marrow was not generated in any of the transplants in group C. In group D, bone was formed primarily at the periphery of the transplants, and bone marrow was also generated. In addition, bone tissue was formed at the center of the transplants (Fig. 4g).

Cell origin of bone formation: do transplanted HMSCs or recipient cells form bone tissue? In group B, bone-forming cells were positive for the mouse L1 probe (Fig. 4b), while they were negative for the human Alu probe (Fig. 4c). These findings indicate that mouse cells were successfully stimulated to form bone by rhBMP-2.

In group C, small amounts of immature bone tissue were detected only at the periphery of two transplants after 4 weeks, and bone-forming cells in the transplants were identified as being of human origin. In addition to bone tissue, there was fibrous tissue in the transplants, and this was mixed up with human and mouse cells. (Fig. 4e, f).

In group D, Alu-positive HMSCs-derived bone tissue was observed at the center of the transplants, but not at the periphery (Fig. 4i). Bone tissue at the periphery and bone marrow were identified as being of mouse origin (Fig. 4h). Bone formation by transplanted HMSCs was confirmed in five of seven transplants from different donors after 4 weeks (Table 2). The amount of bone

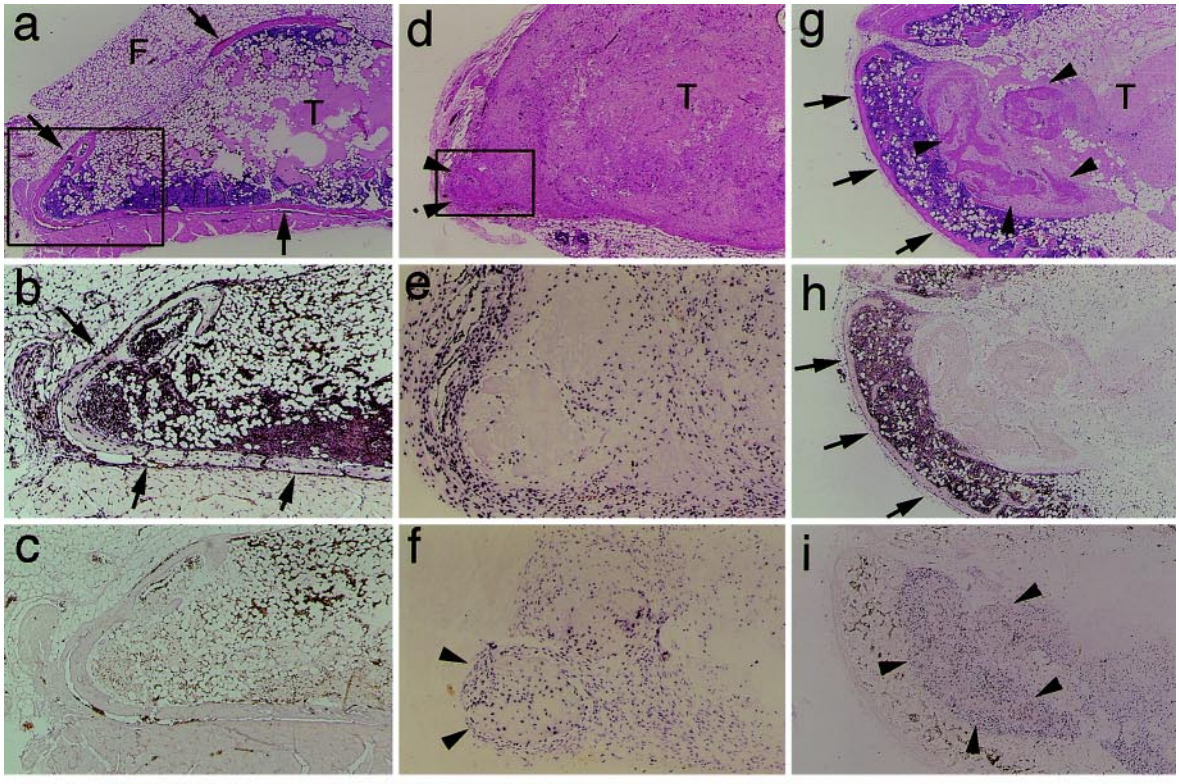

Fig. 4

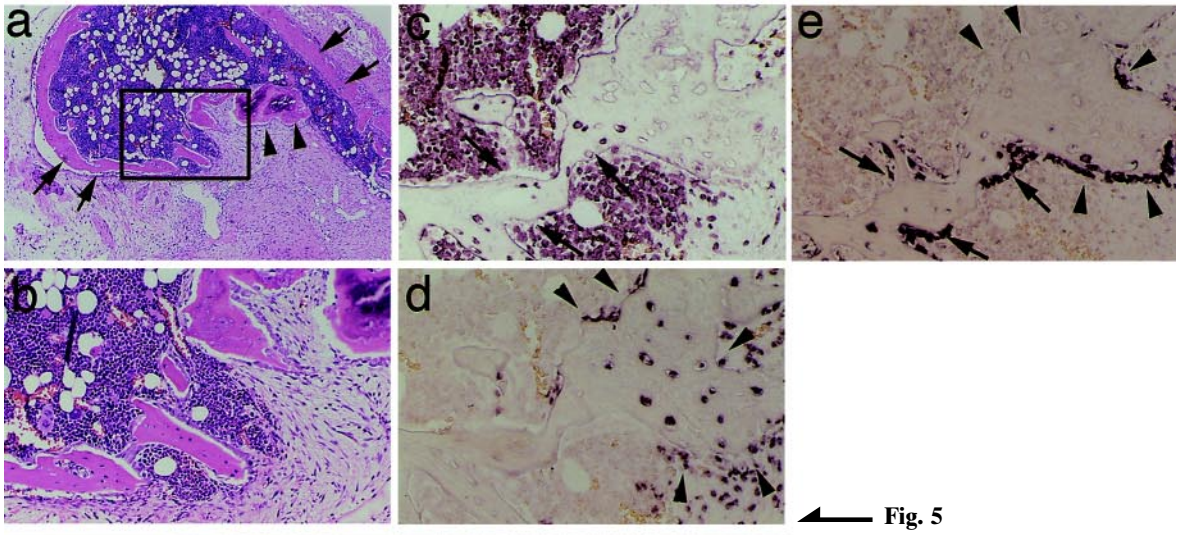

Fig. 5

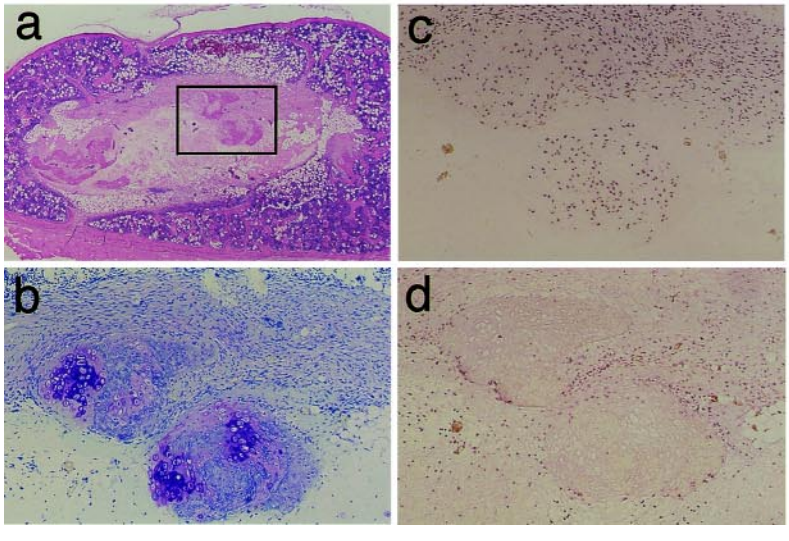

Fig. 6

tissue consisting of human cells was very scant after 2 weeks, but increased after 4 weeks.

In group D transplants, two remarkable findings were observed. First, "mixed" bone formation was observed in donors 1 and 2. Human and mouse bone-forming cells strongly expressing type I collagen mRNA coexisted in the same bone trabecula (Fig. 5). Second, chondrogenesis by HMSCs was observed in donors 2 and 3. Cartilage tissue was detected, as evidenced by the appearance of Toluidine Blue metachromasia (Fig. 6a, b). Chondrocytes were derived from HMSCs (Fig. 6c, d). Mouse-derived cartilage was not detected after 2 and 4 weeks, possibly because endochondral ossification was already complete, as suggested in a previous study [18].

Discussion

Although the bone-forming capacity of HMSCs has been elucidated [11,12], the factors stimulating bone-forming capacity in vivo have not been well characterized. The present in vitro studies revealed that the ALP activity of culture-expanded HMSCs was significantly enhanced in response to osteogenic supplements (Dex, Asc, and β-gly). RT-PCR analysis showed that mRNA expression of osteoblastic markers was detected in culture-expanded HMSCs, and an increase in ALP, OPN, and PTH-R mRNA expression, except for OSC, was observed in the presence of osteogenic supplements. These data indicate that HMSCs preserved the phenotype of osteoblastic lineage and the potency of differentiation into active osteoblastic cells in response to osteogenic supplements, even after being passaged several times. We simultaneously confirmed the bone-forming potency in vivo in the presence or absence of rhBMP-2. Our data demonstrate that bone formation in group D after 4 weeks was detected in donors with the relatively low control activity and high-fold stimulation of ALP activity. This raises the possibility that HMSCs, including more undifferentiated stromal stem cells, may have higher bone-forming capacity.

It has been shown that BMP-2, and -4–7 are bone-inductive molecules [29–31]. In the present study, the expression of BMP-2, -4, and -6 mRNA was detected in HMSCs in culture, suggesting that culture-expanded HMSCs have the potential to produce BMP molecules and act as osteogenic cells in vivo. Histological findings revealed that HMSCs without rhBMP-2 (group C) developed less bone tissue, and HMSCs in the presence of rhBMP-2 (group D) showed a higher incidence of bone formation than group C. These results indicate that the amount of endogenous BMPs in HMSCs transplants may be not enough to induce bone formation.

In group D transplants, rhBMP-2 strongly promoted HMSCs-induced bone formation. The role of rhBMP-2 in bone formation can be assumed to be as follows: (i) rhBMP-2 directly stimulates HMSCs to form bone and cartilage; and (ii) it provides an appropriate environment for HMSCs by inducing bone and bone marrow of mouse origin. It is well known that rhBMP-2 strongly induces bone formation, especially in rodents [18,19,30,31]. Histological findings have demonstrated that none of the mouse-derived bone cells was detected in group C, whereas mouse-derived bone and marrow cells surrounded the cell aggregates of HMSCs in group D. Human bone marrow-derived stromal cell-derived

Fig. 4. Origin of bone formation in group B transplants (rhBMP-2), group C HMSCs transplants, and group D (HMSCs + rhBMP-2) transplants after 4 weeks by DNA in situ hybridization. (**a**) In group B, new bone tissue is formed at the periphery of the transplants (*arrows*). (**b**) Bone-forming cells are positive for mouse L1 (*arrows*), and (**c**) no signal was detected using the human Alu probe. (**d**) In group C, a small amount of immature bone tissue is formed at the periphery (*arrowheads*), and it is negative for mouse L1 (**e**) but positive for human Alu (**f**) (*arrowheads*). (**g**) In group D, bone tissue is observed at the periphery (*arrows*) and center (*arrowheads*) of the transplants. (**h**) Bone tissue at the periphery (*arrows*) and bone marrow cells had positive signals for mouse L1. (**i**) Human Alu-positive bone tissue was observed at the center of the transplants (*arrowheads*). The results of donor 3 are shown. (**a,d,g**): Hematoxylin–eosin (HE) stain. (**b,c,e,f**) are magnifications of the indicated areas in (**a**) and (**d**), respectively. Original magnification: (**a,d,g–i**) ×10; (**b,c**) ×20; (**e,f**) ×25. *T*, center of transplants; *F*, fat tissue

Fig. 5. Mixed bone formation by HMSCs- and mouse-derived bone-forming cells in group D (HMSCs + rhBMP-2) transplants after 4 weeks. (**a**) Lower and (**b**) higher magnification of the transplants (HE stain). New bone is formed at the periphery (*arrows*) and the center (*arrowheads*) of the transplants. (**c**) Mouse cells have positive signals for L1 (*arrows*), and (**d**) human cells are positive for Alu (*arrowheads*). These mouse and human bone-forming cells coexist in the same bone trabecula. (**e**) Type I collagen mRNA is detected in the bone-forming osteoblastic cells of both origins. These findings were observed in two of seven transplants. (**b–e**) Higher magnifications of the area indicated in (**a**). All sections are sequential. Original magnification: (**a**) ×16; (**b–e**) ×80

Fig. 6. Formation of cartilage tissue in group D (HMSCs + rhBMP-2) transplants after 4 weeks. (**a**) Lower magnification of transplants (HE stain). (**b**) Cartilaginous tissue indicates metachromasia with Toluidine Blue-O stain. Chondrocytes are human Alu-positive HMSCs-derived cells (**c**) and negative for mouse L1 (**d**). These findings were observed in two of seven transplants. (**b–d**) Higher magnifications of the area indicated in (**a**). All sections are sequential. Original magnification: (**a**) ×6.6; (**b–d**) ×25

bone tissue existed in the center of group D transplants. These findings indicate that rhBMP-2 provides more suitable conditions, such as a biological capsule or barrier, for forming bone by stimulating mouse cells surrounding transplanted HMSCs. Furthermore, a cell–cell interaction between HMSCs and mouse cells (e.g. bone-forming and hematopoietic cells) may take place in the process of bone formation. These effects resulted in mixed bone formation derived from donor (human) and recipient (mouse) cells. This suggests that combined transplantation of bone-forming cells with growth factors will be beneficial in bone regenerative treatment.

Fibrin glue, which can act as a distributor of BMP [32], made it easy to transplant rhBMP-2, HMSCs, and HMSCs with rhBMP-2. However, it cannot become a supportive scaffold for cells. Kuznetsov et al. have demonstrated that human marrow stromal cells are able to form bone with supportive vehicles without growth factors [12]. The osteoconductive scaffolds for cells play an important role in supporting the bone-forming environment. As examined in group C transplants (HMSCs), subcutaneous implantation without osteoconductive scaffolds resulted in less bone formation. These data indicate that HMSCs could successfully form bone with a strong inducer or appropriate environment.

Mixed bone formation and chondrogenesis in HMSCs with rhBMP-2 was detected in 5-year-old donors. Although these data from a limited number of donors is not sufficient to make any definitive conclusions, it suggests the possibility that the bone-forming capacity of culture-expanded HMSCs is highly preserved in donors of a very young age. It has been reported that there is considerable difference in the osteogenic potential relating to age [33]. We have reported that the number of osteoprogenitor cells from iliac bone marrow decreases with advancing age [34]. The low number of osteoprogenitor cells existing in HMSCs of adult donors may result in a lower capacity for bone formation.

In conclusion, HMSCs harvested from iliac bone marrow have bone-forming potential in vivo. Bone formation of HMSCs could be stimulated by rhBMP-2. This study raises the possibility that a combination of HMSCs and rhBMP-2 is an effective composite as a bone graft substitute. Ex vivo-expanded HMSCs could be a source of osteogenic cells, and combined implantation with osteoinductive growth factors could be used as an alternative to autogenous or allogeneic bone grafts for bone regeneration in the future. Further combined application with cells, growth factors, and osteoconductive scaffolds will be evaluated.

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