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# Influence of the porosity of hydroxyapatite ceramics on *in vitro* and *in vivo* bone formation by cultured rat bone marrow stromal cells

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**Abstract** The *in vitro* and *in vivo* osteoblastic differentiation of rat bone marrow stromal cells (MSCs) was assessed on hydroxyapatite disks with 3 different porosities: 30%, 50%, and 70% (HA30, HA50, and HA70, respectively). MSCs obtained by 10-day culture of fresh bone marrow cells were subcultured for 2 weeks on 3 kinds of porous HA disks in the presence and absence of dexamethasone (Dex). After 2 weeks of subculture, alkaline phosphatase (ALP) activity and osteocalcin production of MSCs/HA composites with Dex were higher than those without, and increased with increasing porosity. The resultant bone tissue grafts "cultured-bone/HA constructs" were implanted subcutaneously into the backs of syngeneic rats, and harvested 1, 2, and 4 weeks after implantation. At 1 week, only cultured-bone/HA70 constructs exhibited expanded bone formation. At 2 and 4 weeks, active osteoblasts and progressive bone formation were observed morphologically in both cultured-bone/HA50 and HA70 constructs. At 4 weeks, bone tissue was observed even in cultured-bone/HA30 constructs. ALP activity and osteocalcin production also increased with increasing porosity and time after implantation. In this *in vivo* model, different scaffold porosity with similar crystal morphology of the apatite phase demonstrated marked differences in ability to support osteogenesis by implanted rat MSCs.

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## 1. Introduction

Autogenous cancellous bone is widely regarded as an ideal construct for graft procedures, supplying osteoinductive growth factors, osteogenic cells, and a structural scaffold. However, procurement morbidity and constraints on obtainable quantities limit its use. Allograft is the next best alternative at present; however, immunogenic rejection and risk of disease transmission are unresolved issues. Although synthetic grafting materials eliminate these risks, these materials do not transfer osteoinductive or osteogenic elements to the host site [1].

Aiming at regeneration of autogenous bone grafts, we have already established an experimental model to show consistent bone formation in the pores of hydroxyapatite (HA) ceramic combined with the culture-expanded bone marrow stromal cells (MSCs) from rat or human bone marrow [2–5]. Porous HA ceramic is an excellent scaffold for supporting the osteogenic differentiation of MSCs because of its osteoconductivity and good biocompatibility. Furthermore, it is possible to vary the porosity and pore size of the HA structure and to construct various shapes with a high degree of precision [6]. Evaluation of the relationship between pore dimension and tissue ingrowth to the HA suggested that a pore size greater than 150 $\mu$m is an essential requirement for osteoconduction

[7]. Tsuruga and colleagues reported that the optimal pore size for attachment, differentiation, and growth of osteoblasts and for vascularization is approximately 300–400 $\mu$m [8]. Kuboki and colleagues reported that the pore size of HA controlled phenotype expression in BMP-induced osteogenesis or chondrogenesis, and the commitment to osteogenic lineage occurred by increasing the pore size of porous HA ceramics [9]. Several studies have shown that another important parameter for osteoconduction is porosity [10–14]. However, there is little information about the biochemical and quantitative evaluation of the relationship between porosity and ectopic bone formation in the pore regions of HA. We reported that with culture techniques, active bone formation in the pore regions of HA disks can be fabricated *in vitro*, and that they are useful as a bone graft substitute for autogenous bone [15]. In this study, we compared the osteoblastic differentiation of rat MSCs on HA disks with different grades of porosity (30%, 50%, and 70%) during a 2-week culture and *in vivo* bone formation by implantation to verify if the porosity of HA can influence its osteoconductive property.

## 2. Materials and methods

### 2.1. Materials

The three different porosities of HA ceramics (CELLYARD$^{TM}$ HA scaffold PENTAX Co., Tokyo, Japan) used in this experiment were 30%, 50%, and 70%, termed HA30, HA50, and HA70, respectively and the average diameter of the pores in all porosities was 0.1–500 $\mu$m, which showed in peak abundance at 10 $\mu$m and 100 $\mu$m (Fig. 1). The pore components around 10 $\mu$m or 100 $\mu$m in diameter have been referred to as micopores or macropores, respectively. The solid and porous components of the microstructure were well interconnected,

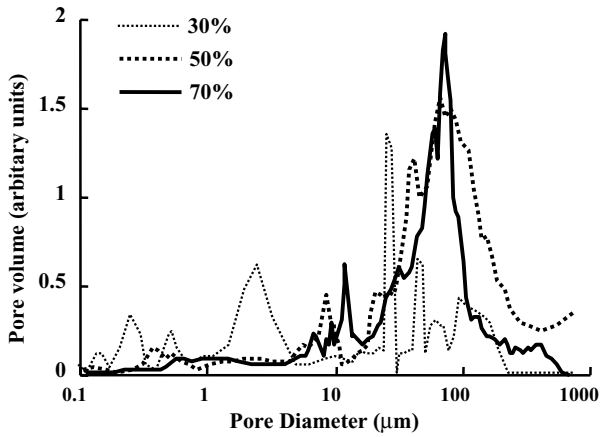


**Fig. 1** Pore size distribution of HA scaffold. The pore diameter in all porosities varied from 0.1 to 500 $\mu$m, which showed in peak abundance at 10 $\mu$m and 100 $\mu$m.

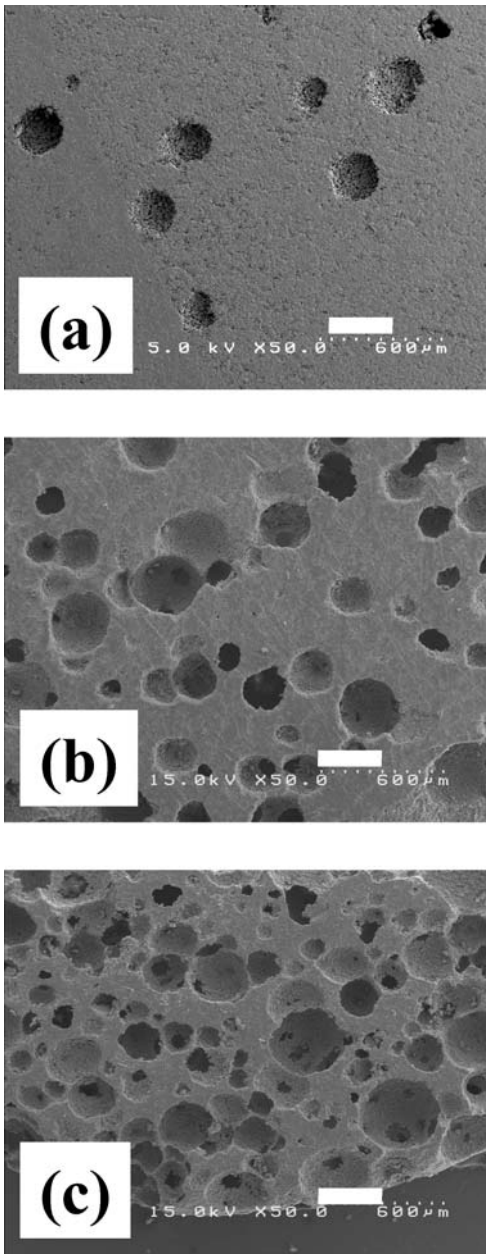


**Fig. 2** Scanning electron microscopy (SEM) image of HA ceramics. The open structure of the interconnected pores is evident, except for HA scaffold with 30% porosity. (a) HA scaffold with 30% porosity, (b) HA scaffold with 50% porosity, (c) HA scaffold with 70% porosity. bars: 300 $\mu$m.

except for HA30 (Fig. 2). Three porous HA ceramics were cut into a disk shape (5 mm in diameter and 2 mm in thickness), sterilized in a dry oven at 180 °C for 3 h, and stored in a dessicator until use. The standard culture medium was Earle's minimal essential medium (MEM; Nakalai Tesque, Inc., Kyoto, Japan) containing 15% fetal bovine serum (JRH Biosciences, Lenexa, KS) and a mixture of antibiotics (100 units/ml penicillin, 100 $\mu$g/ml streptomycin,

**Table 1** Number of rats and implants of experimental groups

| | Implants (for histology/for biochemical assey) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Rats | HA30 only | HA30[-] | HA30[+] | HA50 only | HA50[-] | HA50[+] | HA70 only | HA70[-] | HA70[+] | Harvest time after implantation (week) |
| 15 | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 1 |
| 15 | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 2 |
| 15 | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 10(4/6) | 4 |

and 0.25 $\mu$g/ml amphotericin B; Sigma Chemicals Co., St. Louis, MO).

### 2.2. Marrow mesenchymal stem cell preparation and culture

Using HA disks with three different porosities, we generated new bone constructs by subculturing composites consisting of culture-expanded MSCs and porous HA, as described previously [16].

As the first step, fresh bone marrow plugs were obtained from the femoral shafts of 7-week-old male Fischer 344 rats and flushed out using 10 ml of culture medium expelled gently from a syringe through a 21-gauge needle. The released cells were collected in a T-75 flask (Costar Co., Cambridge, MA) containing 15 ml of the standard medium described above. Cultures were maintained in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. The medium was changed after 24 h to remove nonadherent cells and subsequently renewed three times a week.

As the second step, after 10 days of primary culture, the adherent bone marrow MSCs were released with 0.25% trypsin, concentrated by centrifugation at 900 rpm for 5 min at room temperature, and resuspended to a density of $10^6$cells/ml in MEM. After the air bubbles in the MEM were aspirated, each sterilized HA ceramic (HA30, HA50, and HA70) was soaked in a cell suspension ($10^6$ cells/ml) for 16 h in a $CO_2$ incubator at 37 °C, resulting in MSCs combined with HA ceramics (MSCs/HA composites). HA50 and HA70 easily absorbed about 17 and 20 $\mu$l of the cell suspension and, therefore, approximately $1.7 \times 10^4$ cells and $2 \times 10^4$ cells were loaded on HA50 and HA70, respectively. In contrast, HA30 absorbed only about 6.0 $\mu$l cell suspension, resulting in $6 \times 10^3$ cells per one ceramic disk.

As the third step, each composite consisting of MSCs and porous HA ceramic was transferred into one well of a 24-well plate (Falcon, Franklin Lakes, NJ) and subcultured with 1 ml of the standard medium supplemented with 10 mM ß-glycerophosphate (Merck Japan Co., Tokyo, Japan) and 82 $\mu$g/ml ascorbic acid phosphate (Wako Pure Chemical Industries, Osaka, Japan), with or without 10 nM dexamethasone (Dex; Sigma Chemicals Co.) for 2 weeks. The culture medium was changed 3 times per week. During 2 weeks of culture, we had found previously that HA can be covered by a thin layer of bone tissue when incubated with MSCs [16]. Therefore, we designed the subcultured MSCs/HA composites as cultured-bone/HA constructs.

### 2.3. Implantation of HA ceramic

We prepared nine experimental groups of implants as follows: HA alone with 30%, 50%, and 70% porosity (HA30, HA50, and HA70), cultured-bone/HA constructs with Dex (cultured-bone/HA30[+], cultured-bone/HA50[+], and cultured-bone/HA70[+]) and without Dex (cultured-bone/HA30[-], cultured-bone/HA50[-], and cultured-bone/HA70[-]). Six cultured-bone/HA constructs with or without Dex were implanted subcutaneously on six sites of the back of each syngeneic 7-week-old male rat. The HA ceramics alone were soaked in MEM, then also implanted subcutaneously as control grafts. Six HA disks were implanted through separate incisions on the back of each rat by the procedure previously described [17, 18]. The implanted constructs were harvested at 1, 2, and 4 weeks after implantation and selected randomly from each experimental group for histological and biochemical analyses. The number of recipient rats and the number of implanted ceramics are described in Table 1. The total number of the recipient rats per time period was fifteen; consequently, each group received 10 implanted HA disks. Four implanted constructs from different rats for each group were used for histological examination, and the remaining six implanted constructs were used for biochemical analysis of ALP activity and osteocalcin content.

### 2.4. Biochemical analysis

Assays of ALP activity and osteocalcin content were carried out according to the method described in our previous report [19]. Briefly, each HA disk was crushed, homogenized in 500 $\mu$l of 0.2% Nonidet P-40/50 mM Tris-HCl buffer (pH 7.6) containing 1 mM $MgCl_2$, and centrifuged at $13,000 \times g$ for 15 min at 4 °C. An aliquot of supernatant was assayed for ALP activity using p-nitrophenyl phosphate as a substrate. Alkaline phosphatase activity was represented as $\mu$mol of

p-nitrophenol released per HA disk for 30 min at 37 °C. Osteocalcin in the HA constructs was extracted from the sediment of the Nonidet P-40 extract with 5 ml of 20% formic acid for 1 week at 4 °C. An aliquot of the formic acid extract was applied to a prepacked Sephadex G-25 column (NAP-25 column; Amersham Pharmacia Biotech AB, Uppsala, Sweden) and eluted with 10% formic acid. The protein fractions were pooled, lyophilized, and subjected to enzyme-linked immnoassay (Rat osteocalcin EIA kit; Biomedical Technologies Inc., Stoughton, MA, USA) for rat osteocalcin.

### 2.5. Histological examination

Four implanted specimens from each group were fixed for 3 days in 10% buffered formalin and decalcified with K-CX solution (Falma Co., Tokyo, Japan). After dehydration through a graded series of ethanol, the specimens were embedded in paraffin and cut into 8-$\mu$m sections at the middle of each specimen, parallel to the round surface of the HA disk, and stained with hematoxylin and eosin.

### 2.6. Statistics

The values for ALP activity and osteocalcin content represent the mean and standard error of the mean (SEM) per HA disk. Statistically significant differences in mean values among experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's PLSD test. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. *In vitro* bone formation of subcultured MSCs/HA composites

To quantify the osteogenic potential of cultured composites consisting of MSCs and porous HA with 30% to 70% porosity, biochemical parameters such as ALP activity and osteocalcin content were measured 2 weeks after subculture in the presence or absence of Dex.

After 2 weeks of culture, the ALP activity of the cultured-bone/HA constructs increased with increasing porosity of HA, regardless of the presence or absence of Dex. Significant differences in ALP activity were observed between cultured-bone/HA30[+] and cultured-bone/HA50[+] and between cultured-bone/HA30[+] and cultured-bone/HA70[+], but not between cultured-bone/HA50[+] and cultured-bone/HA70[+]. The activities of these cultured-bone/HA constructs with 50% and 70% porosity were also significantly higher ($p< 0.01$) than that of MSCs on culture dishes, in which a cell density of $2 \times 10^4$cells/24-well dish corresponded to the cell number per one HA disk (Fig. 3). Osteocalcin content also increased with increasing porosity. Significant differences were also observed between cultured-bone/HA30[+] and cultured-bone/HA50[+] and between cultured-bone/HA30[+] and cultured-bone/HA70[+], but not between cultured-bone/HA50[+] and cultured bone/HA70[+] (Fig. 4). As shown in Figs. 3 and 4, ALP activities and osteocalcin contents of the Dex-treated constructs were significantly higher than Dex-untreated constructs, regardless of porosity.

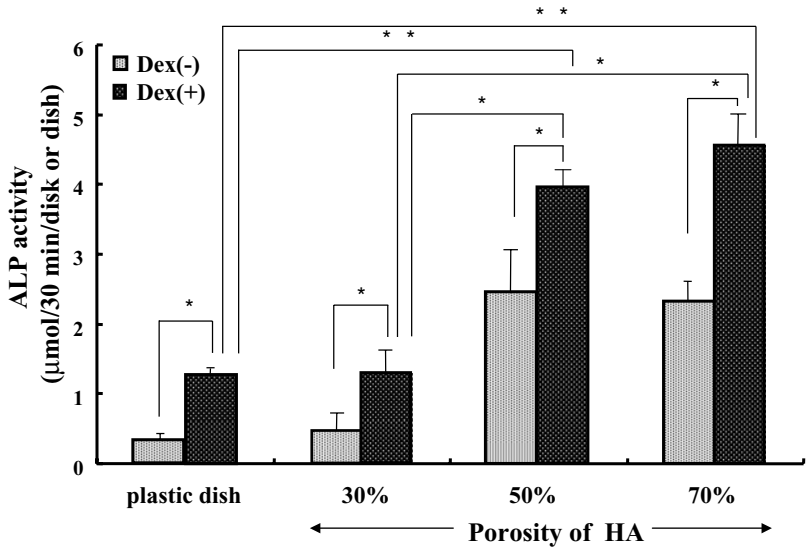


**Fig. 3** Alkaline phosphatase (ALP) activity of MSCs/HA (HA30%, 50%, and 70%) composites and MSCs on plastic culture dishes after 2 weeks of subculture in the presence or absence of Dex. The error bar indicates the standard error of the mean (SEM) of eight composites. The asterisks indicate significant differences (* $p< 0.05$, ** $p< 0.01$).

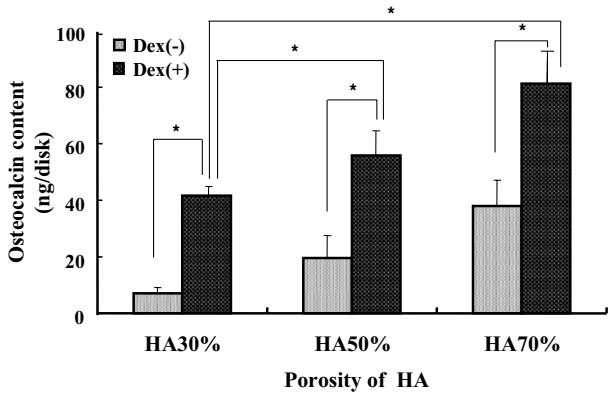


**Fig. 4** Osteocalcin content of MSCs/HA (HA30%, 50%, and 70%) composites after 2 weeks of subculture in the presence or absence of Dex. The error bar indicates the standard error of the mean (SEM) of eight composites. The asterisks indicate significant differences (*$p<$ 0.05).

### 3.2. *In vivo* bone formation of cultured-bone/HA grafts after implantation

Subsequent *in vivo* bone formation was examined by implantation of cultured-bone/HA constructs subcutaneously to syngeneic rats.

In the histological study, as early as 1 week after implantation, only cultured-bone/HA70[+] constructs exhibited expanded obvious bone formation in some pore areas (Fig. 5). At 2 weeks after implantation, both of the constructs with HA70[+] and HA50[+] exhibited obvious bone formation, together with active osteoblasts within many pores (Fig. 6). No bone formation was observed in the cultured-bone/HA30[+] constructs (data not shown). At 4 weeks after implantation, bone formation was observed in the cultured-bone/HA[+] constructs at all porosities associated with invasion of some vascular tissues (Fig. 7), and also in the cultured-bone/HA50[-] and cultured-bone/HA70[-] constructs, even though the amount of bone formation was less than that in the Dex-treated bone/HA50[+] or bone/HA70[+] constructs (data not shown). There were no differences between the cultured-bone constructs with HA50[+] and HA70[+], with regard to bone formation in many pores (Fig. 7). HA alone at any porosity did not induce bone formation at any time after implantation, and only fibrovascular tissue invasion into many pore areas was observed (Figs. 5–7).

ALP activity and the osteocalcin content of the implants were analyzed 1, 2, and 4 weeks after implantation. The ALP activity of these implants increased with increasing porosity, similar to *in vitro* bone formation in the subcultured MSCs/HA composites with three different porosities and with time after implantation. In particular, the cultured-bone/HA70[+] and /HA70[-] constructs showed 3–4.5-fold higher ALP activity during any implantation period than that before implantation (week 0) (Figs. 3 and 8). At 2 weeks after implantation, there was a significant level of osteocalcin in the cultured-bone/HA30, HA50, and HA70, regardless of the presence or absence of Dex, except for cultured-bone/HA30[-] constructs. At 4 weeks after implantation, the osteocalcin levels exponentially increased in the cultured-bone constructs with HA30, HA50, and HA70 ceramics (Fig. 9). The ALP activities and osteocalcin contents of the Dex-treated groups tended to be higher than those of Dex-untreated groups. Differences in biochemical parameters between Dex-treated and untreated constructs appeared to decrease with increasing porosity (Figs. 8 and 9).

## 4. Discussion

Scaffolds to support cell-based tissue engineering are critical determinants of clinical efforts to regenerate and repair bone. The porous HA ceramic exhibits a biocompatible and osteoconductive nature, and has been recognized, therefore,

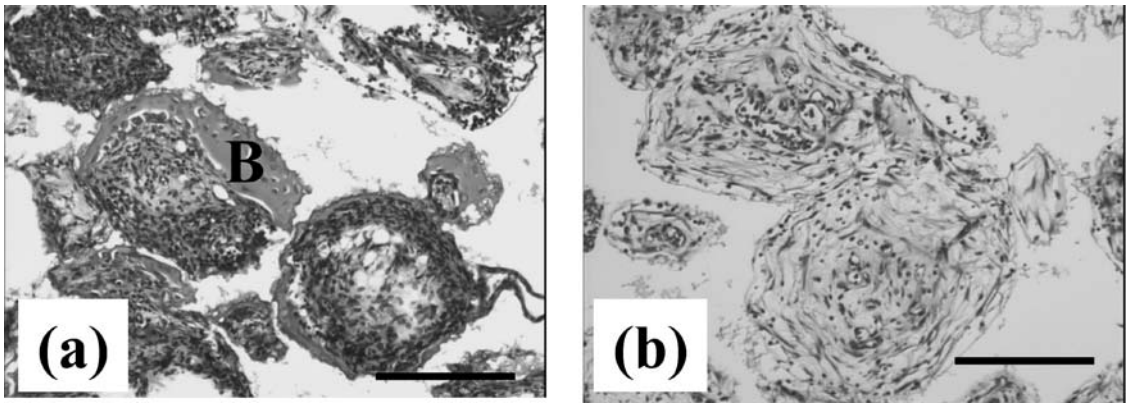


**Fig. 5** Photomicrographs of the histological section of an implant at 1 week after implantation. Early bone formation is detected in the pore areas of cultured bone/HA70[+] constructs (a). HA70 alone did not induce bone formation, and only fibrovascular tissue invasion into many pore areas was observed (b). The white area is the ghost of the HA ceramic produced by decalcification procedures. Hematoxylin and eosin stain; bars: 200 $\mu$m.

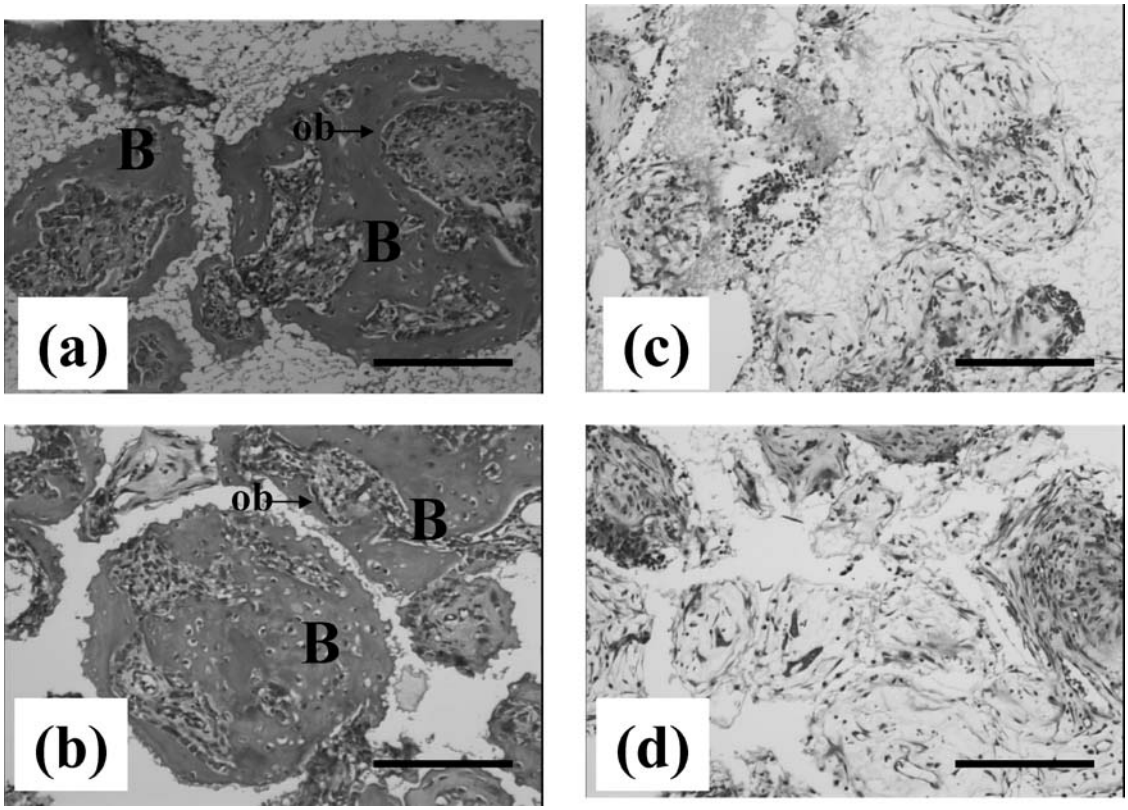


**Fig. 6** Photomicrographs of histological sections of implants at 2 weeks after implantation. Both of the constructs with HA70[+] (a) and HA50[+] (b) exhibited obvious bone formation (B), together with active osteoblasts (ob) within many pores. HA alone with any porosity did not induce *de novo* bone formation, and only fibrovascular tissue invasion into many pore areas was observed (c and d). The white area is the ghost of the HA ceramic produced by decalcification procedures. Hematoxylin and eosin stain; bars: 200 $\mu$m.

as an excellent scaffold for supporting the osteogenic differentiation of MSCs. We have proposed, using Dex-dependent subculture of MSCs in a porous HA framework, fabrication of transplantable artificial bone graft substitutes that can show continuous osteoblastic activity [14]. In this paper, we compared the *in vitro* and *in vivo* osteogenic differentiation of rat MSCs on porous HA with three different porosities (30%, 50%, and 70%).

When MSCs/porous HA composites were subcultured for 2 weeks, MSCs/HA70[+] showed the greatest osteogenic capacity, evidenced by high ALP activity and osteocalcin content. The ALP activity of MSCs/HA70[+] was 3.5-fold higher than that of MSCs on plastic culture dishes (Fig. 3), in which a cell density of $2 \times 10^4$ cells/24-well dish corresponded to the cell number per one HA disk, and increased with increasing porosity. We have described that in the absence of Dex, the composites with MSCs and HA (Interpore 200) showed only traces of ALP and osteocalcin at any time during culture and no mineralized collagenous matrix by scanning electron microscopic analysis [15]. We have also confirmed that Dex is required for osteoblastic differentiation of MSCs on culture dishes, followed by formation of a bone-like mineralized tissue outside cells [20]. Porous HA ceramics used in this study, however, showed a significant level of these proteins, even in the absence of Dex, though these protein expression levels with Dex were significantly higher than without Dex. We employed an aspirating step for easy removal of air bubbles in the pores of HA, which might allow for easy invasion of MSCs into pore areas. And the porous components of the microstructure of HA scaffold are distributed mainly around the HA disk (Fig. 2) and well interconnected but, unlike Interpore 200, which has a cancellous bone-like three-dimensional structure with a complete interpore connection through the disk. HA is also known as an excellent material to adsorb various protein molecules. Recently it was reported that serum contains a potent agent (a putative serum calcification factor) that participates in the re-calcification of bone [21] and the abundant protein, fetuin, which can form a fetuin-mineral complex and binds strongly to apatite [22]. Taken together, this subculture protocol with HA porosity as high as 50%–70% may allow serum proteins in the medium to adsorb and undifferentiated cells to adhere easily to the protein-coated pore surface of HA.

When the cultured-bone/HA constructs were implanted into syngeneic rats, the ALP activity of cultured-

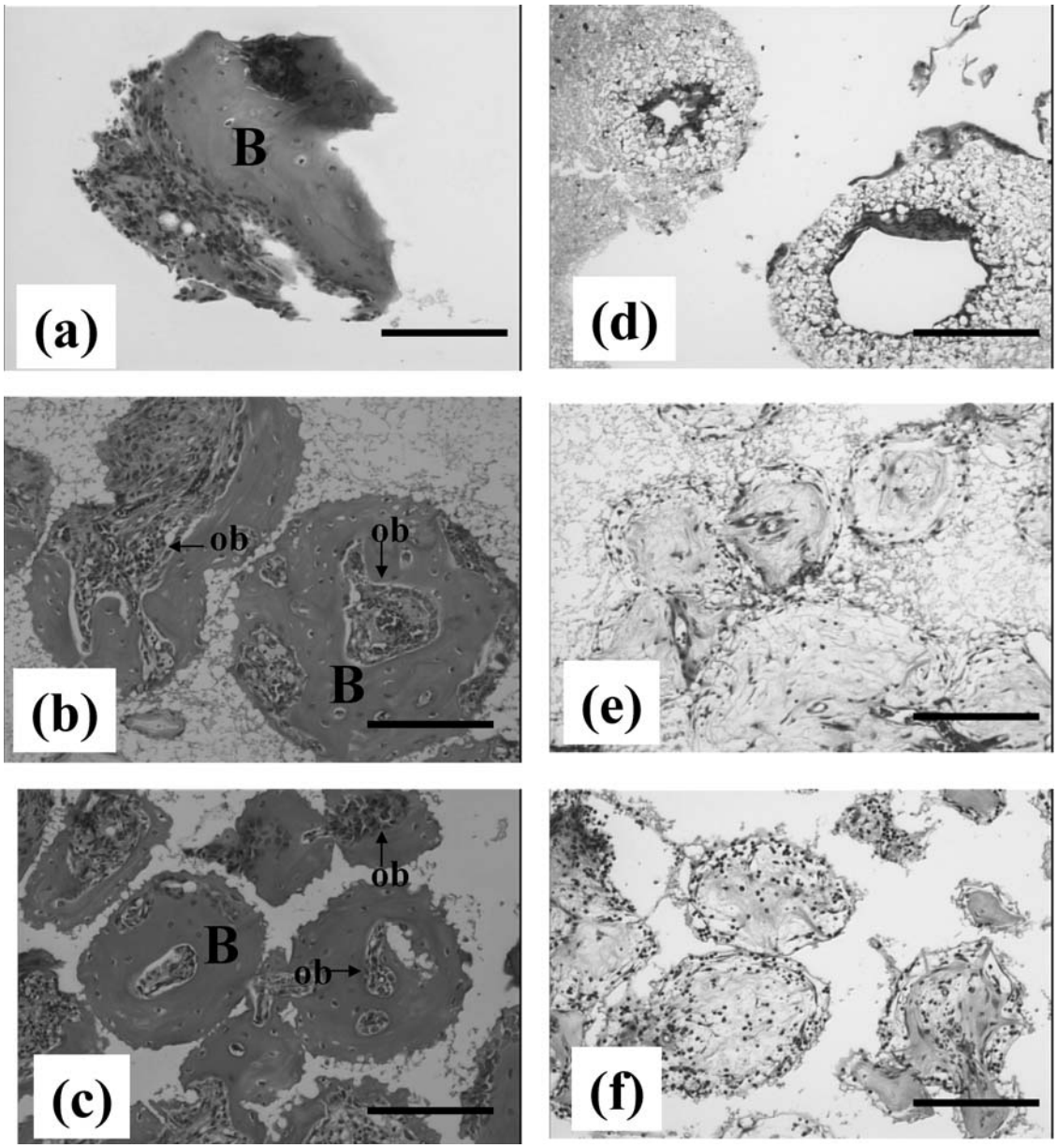


**Fig. 7** Photomicrographs of the histological section of an implant at 4 weeks after implantation. Bone formation (B) was observed in the cultured-bone/HA[+] constructs at all porosities associated with invasion of some vascular tissues (a–c). HA alone with any porosity did not induce *de novo* bone formation, and only fibrovascular tissue invasion into many pore areas was observed (d–f). The white area is the ghost of the HA ceramic produced by decalcification procedures. Hematoxylin and eosin stain; bars: 200 $\mu$m.

bone/HA50[+] and /HA70[+] constructs increased from 1 week onward, indicating a high level of osteoblastic activity. The ALP level increased with increasing porosity, and the high activity gradually rose, up to 4 weeks (Fig. 8). Osteocalcin also was detected before implantation and increased exponentially from 1 to 4 weeks after implantation. At 2 and 4 weeks after implantation, the ALP activity of cultured-bone/HA70[+] and /HA50[+] were higher than cultured-bone/HA70[-] and /HA50[-], respectively. However, significant differences were not observed between Dex-treated and Dex-untreated constructs. This difference between Dex-treated and -untreated constructs tended to decrease with increasing porosity.

Until now, to obtain consistent bone by grafting a porous HA disk mixed with primary cultured-expanded MSCs, a porous HA disk had to be soaked in a cell suspensions with an initial density of $10^7$ cells/ml [3, 19]. In contrast, with the present subculturing step, porous HA50 and HA70 ceramics that were soaked into cell suspensions at a density of $10^6$ cells/ml, resulting in initial cell densities of about 1.7 and 2.0 $\times$ $10^4$ cells/ceramic, respectively. These cell numbers were enough to show sufficient osteoblastic activity by

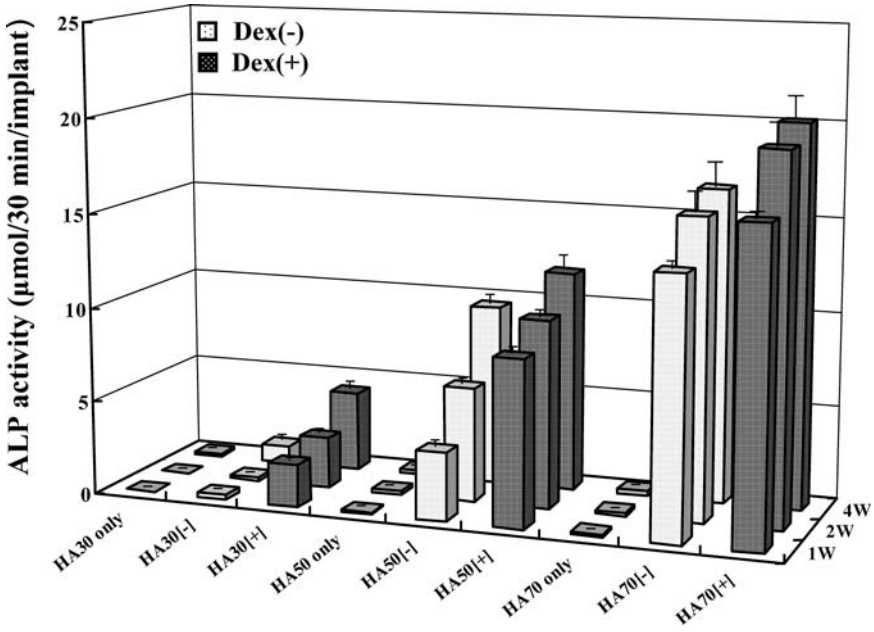


**Fig. 8** ALP activity of implants at 1, 2, and 4 weeks after implantation. Implants are HA (30%, 50%, and 70%) alone, and cultured-bone/HA (30%, 50%, and 70%) constructs after 2 weeks of subculture in the presence or absence of Dex. The error bar indicates the standard error of the mean (SEM) of six implants.

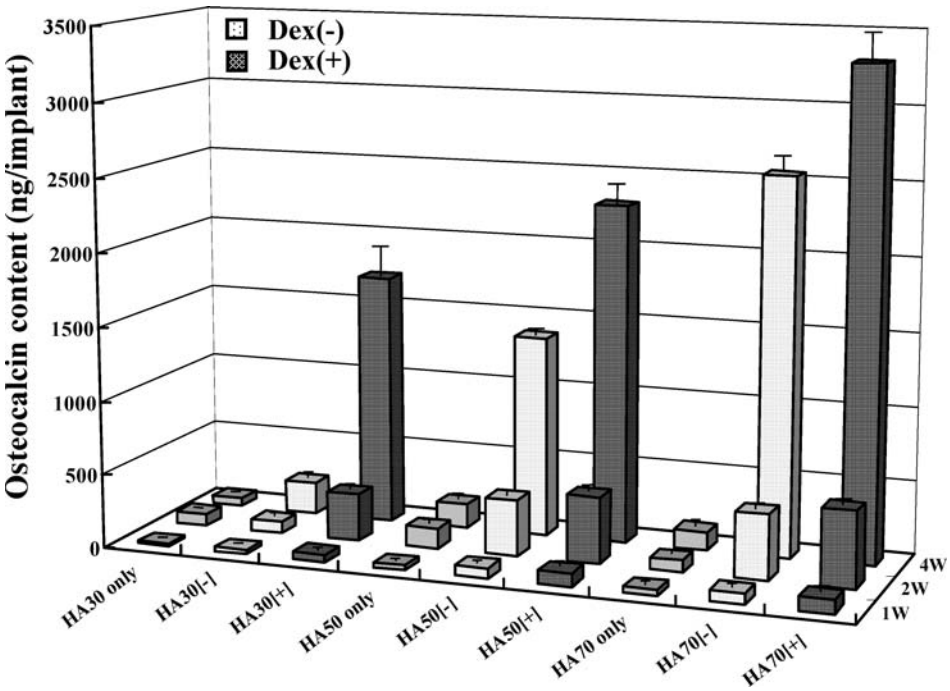


**Fig. 9** Osteocalcin content of implants at 1, 2, and 4 weeks after implantation. Implants are HA (30%, 50%, and 70%) alone, cultured-bone/HA (30%, 50%, and 70%) constructs after 2 weeks of subculture in the presence or absence of Dex. The error bar indicates the standard error of the mean (SEM) of six implants.

implantation. However, HA 30 shows less osteoblastic activity than HA 50 and 70, because of small number of cells loaded on HA30, and therefore the HA30 may be less valid. Thus, the cultured-bone/HA50[+] and /HA70[+] constructs could have a large bone-forming capacity, comparable to that of cancellous bone grafts.

The capacity of HA to enhance osteoblast differentiation is well known and is believed to be related to the pore size. The highest ALP activities and osteocalcin contents were both obtained with a pore size of 300–400 $\mu$m, a diameter that seems to favor the formation of osteon-like structures [7]. In comparison to the cultured-bone/HA (Interpore500) constructs incubated at a cell density of $10^6$ cells/ml in the reports of Yoshikawa and colleagues, at 4 weeks after implantation, the ALP activity was comparable to levels of the activity of cultured-bone/HA50 constructs. Furthermore, the ALP activity and osteocalcin content of cultured-bone/HA70 constructs were higher than HA (Interpore 500). Therefore, the HA ceramic, CELLYARD$^{TM}$ HA scaffold, with a porosity as large as 50%–70%, may prove to be efficient for intrapore

introduction of cells and to have more appropriate osteogenic ability compared with Interpore 500.

Subcutaneous grafting of the cultured-bone/HA constructs resulted in new bone formation without cartilage formation during any period of implantation, regardless of porosity. Kuboki and coworkers reported that the pore size of HA controlled phenotype expression in BMP-induced osteogenesis or chondrogenesis; smaller diameters (0.1 mm) induced cartilage followed by bone formation, while one with a larger diameter (0.35 mm) directly induced bone formation [9]. *In vivo* cartilage formation was not detected in porous HA (Interpore 200), in which the average pore size was 200 $\mu$m, even with the addition of cartilage-derived growth factor, GDF-5 [19]. Shimaoka and colleagues suggested that differentiation of MSCs is strongly affected by the characteristics of the surrounding microenvironment, such as oxygen tension, properties of biomaterials, cell densities, and so on [19]. The ceramics used in this study have pore diameters of 0.5–500 $\mu$m, which provide an open pore structure between micropores around 10 $\mu$m and macropores around 100 $\mu$m. Eggli and coworkers found that micropores with a diameter of less than 20 $\mu$m in HA helped to provide connections between macropores, which allow interstitial fluid circulation through them. These connections are believed to facilitate blood vessel development and tissue ingrowth into HA [23]. Scanning electron microscopy of HA scaffold (Fig. 2) shows no connections between macropores and micropores in HA with 30% porosity, but a high degree of interconnection in high porosity HA as well as 50% and 70% porosity HA. The pore distribution curve of HA with 30% porosity (Fig. 1) shows many small pores (less than 3 $\mu$m) and indicates the very poor pore interconnection. In addition, histological examination of implanted cultured-bone/HA50 and /HA70 constructs showed that satisfactory bone formation accompanied the vasculature in the pore areas, providing insights into important aspects of the vasculature-inducing geometry of the scaffold (Figs. 6 and 7).

According to an analysis of mechanical strength, as the porosity (0–70%) of HA increases, the breaking strength to both bending and compression strength decreases, and HA with 50%–60% porosity might be superior with regard to mechanical strength and osteoconductivity [11]. The substantial volume of 70% HA ceramics is small at only 30%, and therefore, the mechanical strength is very weak. The pores of cultured-bone/HA70[+] were filled with new bone tissue or bone matrices after implantation; therefore, the mechanical strength of cultured-bone/HA70[+] constructs could be stronger than HA70 alone. In this regard, osteocalcin is a bone-specific protein, and its contents paralleled the amount of bone matrix well. At 4 weeks after implantation, the osteocalcin content of cultured-bone/HA70[+] constructs was comparable to that of rat cancellous bone (0.2–2 mg/g bone). The data strongly suggest that plenty of bone matrices existed in the pore areas of the constructs.

In conclusion, the osteogenic ability of MSCs/HA composites increased with increasing porosity, not only *in vitro*, but also *in vivo*. Therefore, the most appropriate bone formation condition would consist of a primary culture of fresh bone marrow to expand the number of cells and subsequent subculture with Dex using HA of 70% porosity as the scaffold for cell growth. It might be anticipated that when MSC/HA70 composites are subcultured with Dex in this way, cultured-bone/HA70[+] constructs would be excellent bone grafts, with properties similar to those of autogenous bone.

## References


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