Architecture of Implanted Bone Matrix Gelatin Influences Heterotopic Calcification and New Bone Formation (43241)

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Abstract. **Heterotopic bone formation in skeletal muscle induced by compacted demineralized bone matrix gelatin (BMG) was studied histologically and biochemically. BMG was obtained by dehydrating diaphyseal shafts of femora and tibiae of 4-week-old male Sprague-Dawley rats, cutting the bone into chips, and demineralizing and extracting the chips with various solutions. The BMG was treated with 4 *M* guanidine-HCl, and compacted BMG was prepared by centrifugation. The compacted BMG was implanted into the rectus abdominis muscle of 5-week-old male Sprague-Dawley rats. The resulting specimens were examined histologically, and their alkaline phosphatase activity and the calcium content of the tissues were measured 3, 5, 7, 10, and 15 days after implantation. The BMG (separated BMG) with 75- to 500-μm particle sizes were implanted into control rats. The results showed that calcification, alkaline phosphatase activity, and bone formation were suppressed by implantation of the compacted BMG and that scarcely any vascularization occurred. Calcification, vascularization, and alkaline phosphatase activity were related and were indispensable for bone formation. In the control group, bone formation was observed at sites of high activity of alkaline phosphatase and well-developed vascularization. These results suggested that compacting of BMG suppressed vascularization, decreased calcification, and consequently reduced the induction of bone formation.** [P.S.E.B.M. 1991, Vol 197]

Intramuscular or subcutaneous implantation of demineralized bone matrix is known to induce new bone formation by endochondral ossification (1). Recently, a bone-inducing factor (bone morphogenetic protein) was purified from bone matrix and its cDNA was obtained (2–4), but its mechanism of bone induction is still unknown. In particular, very little is known about the calcification that occurs before new bone is formed. Reddi and co-workers (5, 6) reported that coarse powders (74–420 μm) of demineralized bone induced new bone, but that fine powders (44–74 μm) induce no bone, neither *in vitro* nor *in vivo*. Syftestad and Urist (7) reported that the yield of new bone from implants of pulverized, demineralized, whole matrix and bone matrix gelatin (BMG) declined as the particle size decreased in diameter below 125 μm. We thought their results probably depended on whether the structure of the implant had affinity for cells and provided a suitable architecture for vascularization out of the particle size. In the present study, we suppressed vascularization by compacting BMG into a pellet using the guanidine-ethanol precipitation method (8) and examined the significance of calcification of the BMG and the extent of vascularization in the mechanism of bone formation.

Materials and Methods

Preparation of Demineralized Bone Matrix Gelatin. Demineralized bone matrix gelatin was prepared as described by Urist *et al.* (9). Diaphyseal shafts of femora and tibiae from 4-week-old male Sprague-Dawley rats were cut into chips. Liquid nitrogen was used to freeze the bone shafts after their removal and while they were being cut into chips to avoid possible denaturation of proteins. The bone chips were extracted

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with chloroform/methanol (1:1) to remove lipid, demineralized in 0.6 N HCl, and extracted successively with solutions of 2.0 M $CaCl_2$, 0.5 M EDTA, and 8.0 M LiCl and then with water to remove soluble proteins (9). The bone chips were then pulverized with a Sample Chamber Heiko and sifted. Particles of 75–500 μm were collected by the testing sieve, lyophilized, and implanted as control group.

Preparation of Compacted BMG. BMG was compacted by the guanidine-ethanol precipitation method (7). For this, 2.0 mg of BMG was treated with 0.15 ml of 4 M guanidine-HCl, and then 0.85 ml of 100% ethanol was immediately added at 4°C. The mixture was centrifuged (10,000g for 15 min at 4°C) and the supernatant was discarded. The compact precipitate of BMG was washed three times with 85% ethanol in water and lyophilized. The compacted BMG was offered to the experimental group.

Implantation. As an experimental group, 32 5-week-old male rats were anesthetized by subcutaneous injection of pentobarbital sodium (Abbott Laboratories, North Chicago, IL), and 2.0 mg of compacted BMG were implanted into the right rectus abdominis muscle under sterile conditions. In the control group of 47 male rats, 2.0 mg of lyophilized BMG were implanted in the same way.

Histological Preparations. Muscle tissues containing implants were removed from five rats of the control group and three rats of the experimental group on Days 3, 5, 7, 10, and 15 after implantation. On each day, three specimens from the control group and two specimens from the experimental group were fixed overnight in 10% neutral buffered Formalin, washed, and dehydrated in an ethanol series. Selected regions of tissues with implants were removed, embedded in paraffin, and sectioned at 5-μm thickness. The two other specimens from the control group and one from the experimental group, on each day after implantation, were frozen in liquid nitrogen. Cryostat sections were cut at 5-μm thickness, mounted on glass slides, and air-dried for 15 min. The sections were then stained with hematoxylin and eosin, von Kossa stain, alizarin red S or, alkaline phosphatase (Sigma Kit No. 86-c). In addition, India ink was injected into the left ventricle of two rats in each group on Day 10 after implantation, and muscle tissues containing implants were removed and fixed overnight in 10% neutral buffered formalin. The specimens were then examined histologically in the same way as described above.

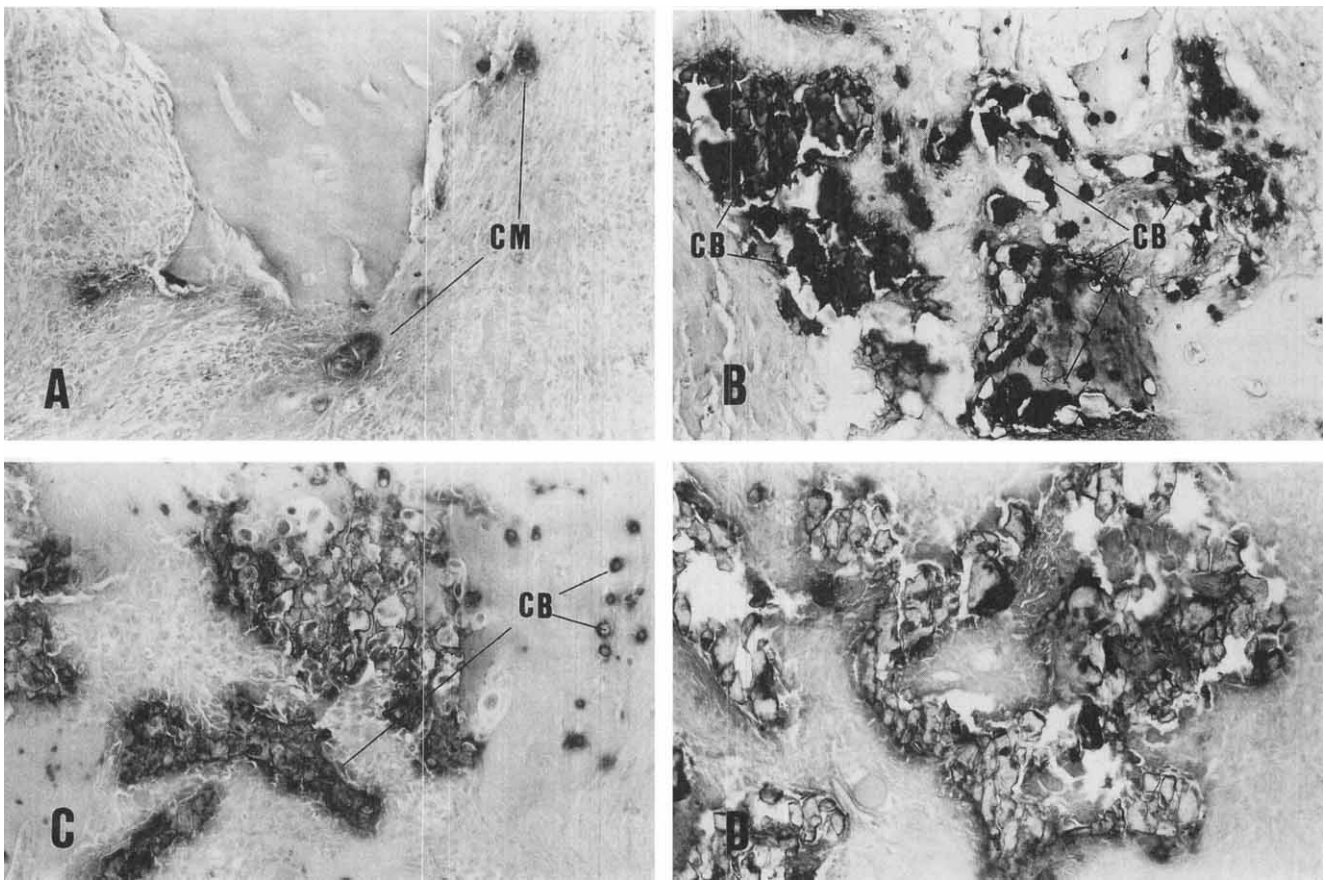

Figure 1. Alizarin red S staining of calcification of the BMG in the control group. Tissues were obtained on Day 5 (A), Day 7 (B), Day 10 (C), and Day 15 (D) (original magnification ×240). Calcification of muscle tissue (CM) was observed on Day 5 after implantation. Calcification of the BMG (CB) was observed as deposits of calcium on Day 7 and increased up to Day 15.

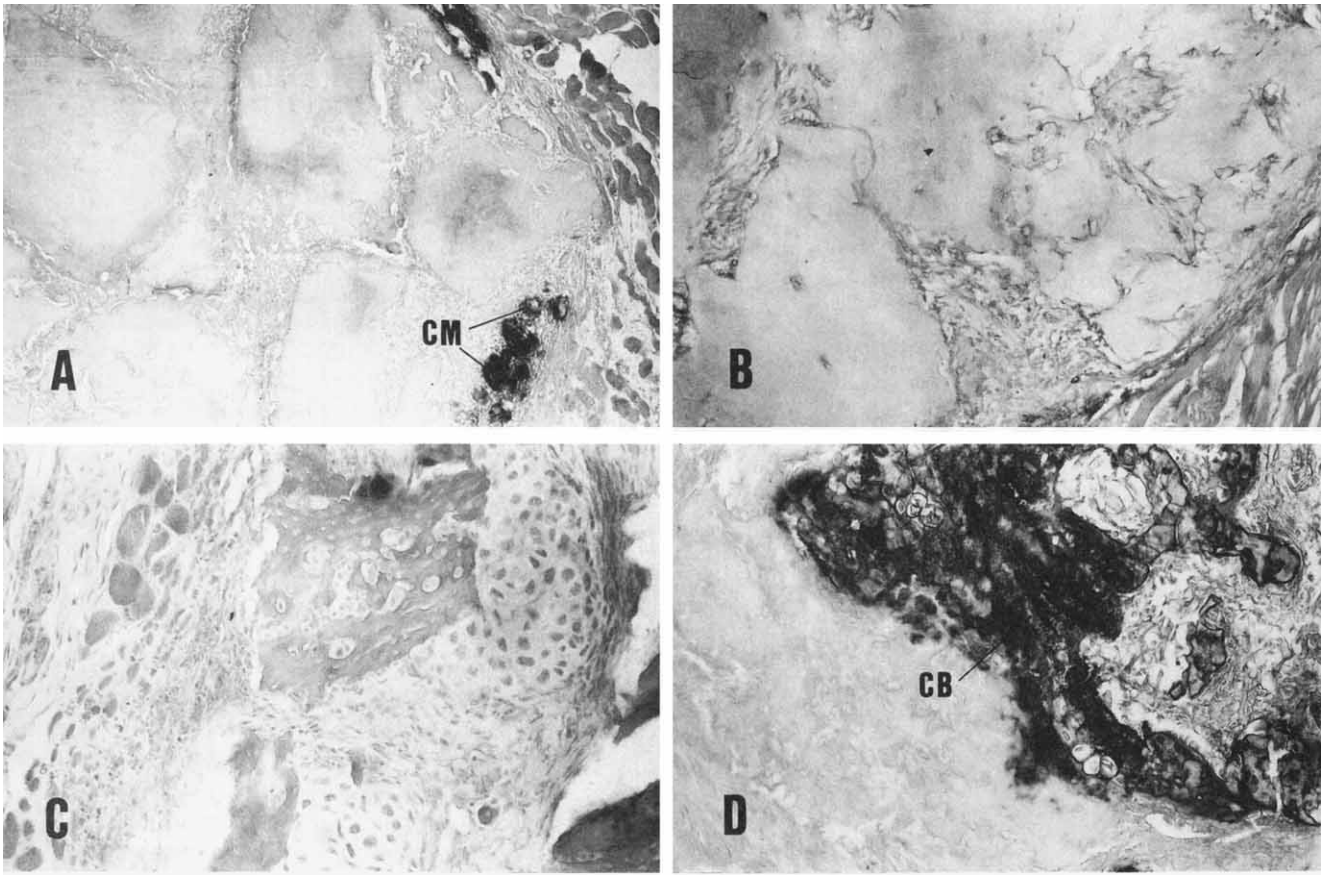

Figure 2. Alizarin red S staining in the compacted BMG group. Tissues were obtained on Day 5 (A), Day 7 (B), Day 10 (C), and Day 15 (D) (original magnification ×240). Calcification in muscle fibers (CM) was observed on Day 5. Cartilage formation was prominent on Day 10 (C). Calcification of the BMG was first seen on Day 15.

Enzyme Assays. Muscle tissues containing implants were removed from four rats of the control group and three rats of the experimental group on Days 3, 5, 7, 10, and 15 after implantation. The tissues were homogenized in 2 ml of ice-cold 0.25 *M* sucrose-3 m*M* $NaHCO_3$ in a glass-glass homogenizer for three 10-sec bursts at the maximum setting. The homogenates were centrifuged at 12,000*g* for 15 min at 2°C, and the supernatants were collected for enzyme assays. One unit of alkaline phosphatase (EC 3.1.3.1) activity was defined as the enzyme activity that liberated 1 μmol of *p*-nitrophenol per 30 min under the test conditions (10).

Quantitative Analysis of Calcium. The precipitates obtained by centrifugation of homogenates were ashed in a concentration of HNO_3, 30% H_2O_2, and 6 *M* $HClO_4$ on a hot plate and extracted with 4 ml of 25 m*M* $HClO_4$ for 48 hr. The acid-soluble extracts of the sediment were diluted with 6 ml of water for determination of calcium by ion-exchange chromatography (Dionex 20201). The calcium content was expressed as micromoles of calcium per implant.

Results

Calcification in BMG. The histological appearances of calcification of the BMG in the control group on Days 5, 7, 10, and 15 after implantation are shown in Figure 1. The intensity and distribution of calcified sites were clearly seen by staining with alizarin red S. On Day 5 after implantation, no calcified sites were seen, except in two rats (Fig. 1A). Many small calcified areas were seen in the adjacent muscle tissue. On Day 7 after implantation, many calcified deposits were seen on the surface of the BMG and several deposits were visible inside the BMG (Fig. 1B). On Day 10 after implantation, calcifying cartilage, and newly formed bone were seen with some small calcified deposits (Fig. 1C). On Day 15 after implantation, most of the BMG was calcified and extensive new bone formation was observed (Fig. 1D).

The histological appearance of specimens from the compacted BMG group are shown in Figure 2. On Day 5 after implantation, some parts of the adjacent muscle tissues had already calcified (Fig. 2A). On the other hand, scarcely any calcification of the compacted BMG or bone formation was observed on Day 5, 7, or 10 after implantation (Fig. 2A–C). However, on Day 15 after implantation, new calcifying cartilage and bone formation were observed (Fig. 2D).

Localization of Alkaline Phosphatase Activity

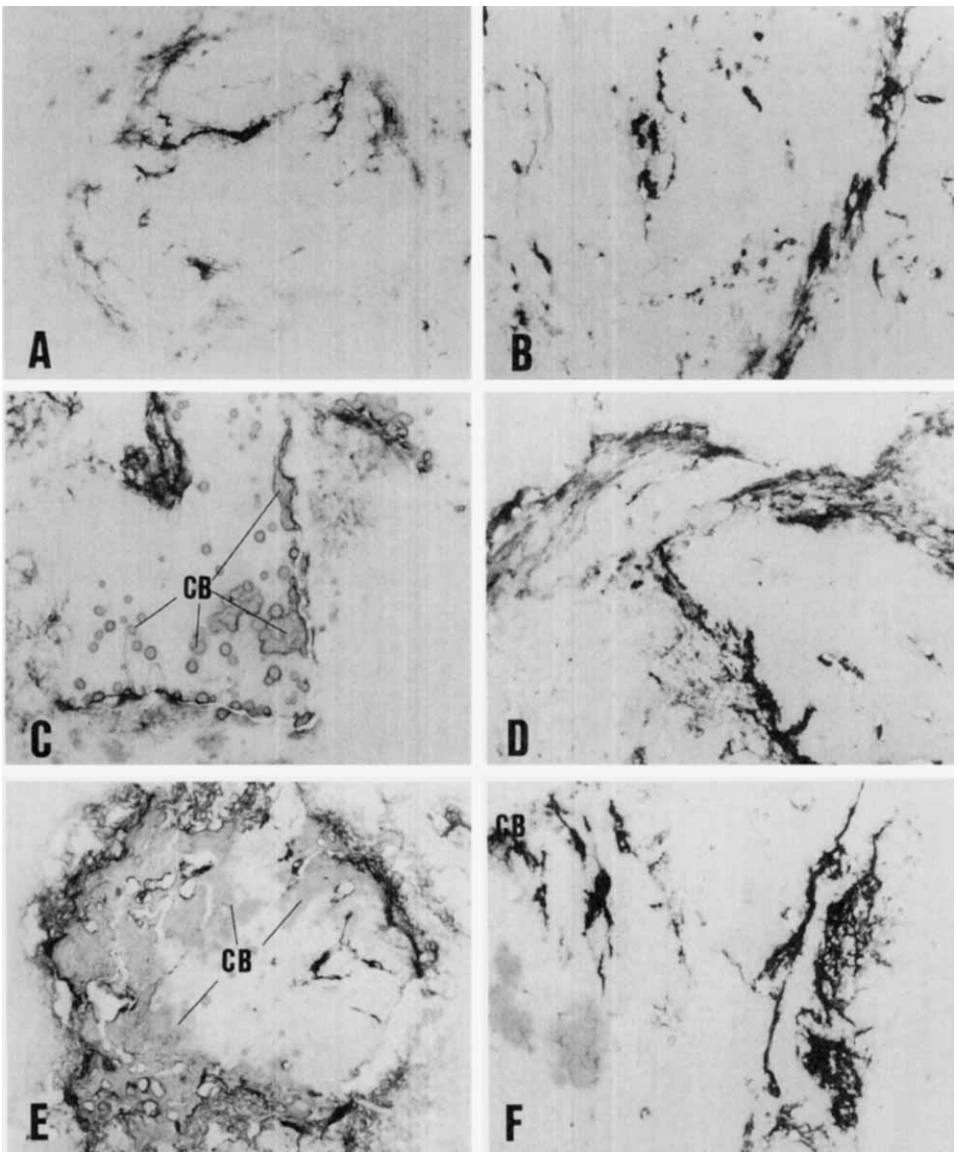

Figure 3. Localization of alkaline phosphatase activity. (A, C, and E) Tissues from the control group. (B, D, and F) Tissues from the compacted BMG group. Tissues were obtained on Day 5 (A and B), Day 7 (C and D), and Day 10 (E and F) (original magnification ×150) (alkaline phosphatase and alizarin red S staining). Alkaline phosphatase activity increased with time in both groups, but it was more extensive in the control group. The distribution of calcified sites of the BMG (CB) (alizarin red S positive areas) was different from that of high alkaline phosphatase activity (C).

and Enzyme Assay. The histological appearance of calcification and the localization of alkaline phosphatase activity in the two groups on Days 5, 7, and 10 after implantation are shown in Figure 3. Alkaline phosphatase activity was more marked in the control group (Fig. 3A, C, and E) than in the compacted BMG group (Fig. 3B, D, and F) in all cases. Alkaline phosphatase activity was first detected in crevices of the BMG on Day 5 after implantation and increased with time. The activity extended to the surface of the BMG, and then high activity was seen on the newly formed bones. Alizarin red S staining was also greater in the control group from Day 7. The distribution of alkaline phosphatase activity did not always correspond with calcification sites of the BMG.

The changes in alkaline phosphatase activity with time in the two groups are shown in Figure 4. On Days 7, 10, and 15, the total activity was markedly less in the experimental group than in the control group (Fig. 4B). In the control group, the total alkaline phosphatase activity increased rapidly from Day 7 to a peak on Day 10, and then decreased rapidly; the specific activity of alkaline phosphatase was highest on Day 10. In the compacted BMG group, the specific activity increased more slowly, but steadily, until at least Day 15 after implantation.

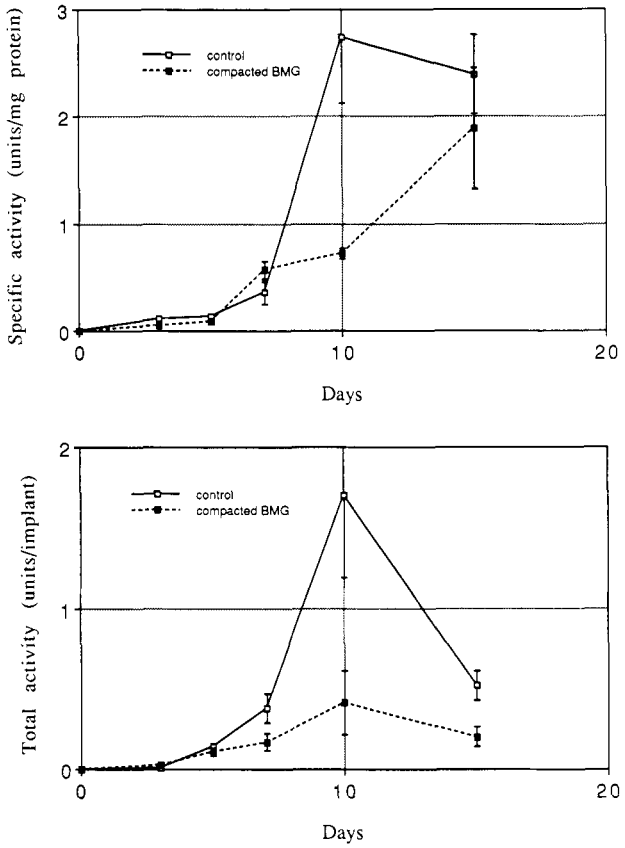

Figure 4. Changes in alkaline phosphatase activity in the control and compacted BMG groups. The top graph shows specific activity; the bottom graph shows total activity. Points and bars represent means and SD for three determinations in the compacted BMG group and four in the control group. The sp act increased until Day 15 after implantation in the compacted BMG group, but reached a peak on Day 10 in the control group. The total activity was much less on Days 7, 10, and 15 after implantation in the compacted BMG group than in the control group.

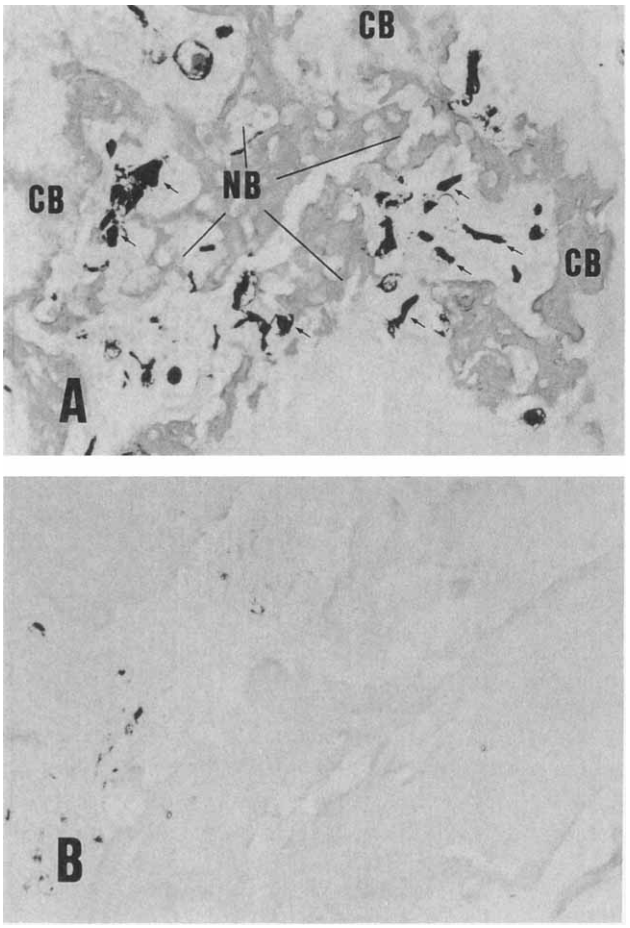

Figure 5. Localization of blood vessels demonstrated by injection of India ink. Tissues were obtained from the control group (A) and the compacted BMG group (B) on Day 10 after implantation (original magnification ×240) (alizarin red S staining). (A) New vascularization (arrows) is observed near the sites of calcified BMG (CB) and new bone formation (NB). (B) Scarcely any new vascularization is observed near the BMG.

Calcium Contents. In the compacted BMG group, there was little change in the calcium content, although the content was slightly increased on Day 5 (Day 3, 0.266 ± 0.025; Day 5, 0.481 ± 0.061; Day 7, 0.126 ± 0.020; Day 10, 0.486 ± 0.141; Day 15, 0.325 ± 0.094). In the control group, however, the calcium content increased remarkably from Day 7 (Day 3, 0.140 ± 0.016; Day 5, 0.179 ± 0.038; Day 7, 1.074 ± 0.182; Day 10, 1.564 ± 0.026; Day 15, 4.406 ± 0.068). A similar change of phosphate was also observed (data not shown).

Vascularization. The histological appearance of tissues on Day 10 after injection of India ink and staining with alizarin red S is shown in Figure 5. In the control group, vascularization was extensive near calcified sites of BMG and in the region of newly formed bones (Fig. 5A). On the other hand, in the compacted BMG group, scarcely any vascularization was observed in crevices of implanted BMG (Fig. 5B).

Discussion

No attention has been paid to the fact that calcification of the demineralized bone and the BMG precedes bone formation induced by its implantation (11, 12). Linden (13) mentioned that calcification of the decalcified bone and dentin was an important precondition for bone formation, but he did not explain details of this phenomenon. Urist *et al.* (14) reported that calcification initiator protein was extracted from bone matrix. But, the recalcification of BMG was not observed and the details of initial calcification were still unknown. In the present investigation, we examined the role of calcification before bone formation by BMG. We found that calcification of the BMG was first detectable as small deposits in the periphery of the BMG on Day 7 after implantation, and then extended into the BMG by fusion of the small deposits with one another (Fig. 1B and 3C–E). Calcification of the BMG began coincidentally with rapid increase of alkaline phosphatase activity, but the calcified sites did not

always coincide with the areas of intense alkaline phosphatase activity (Fig. 3C). These results imply that the cells adjacent to the calcified sites are not osteoblasts, because osteoblasts generally have high alkaline phosphatase activity. Therefore, the calcification may not depend on osteogenic cells, or any particular cells. In the present study, the most striking change after implantation of the BMG was the marked vascularization in the control group. It was observed that the calcification site of BMG corresponded with the area of new vascularization. Thus, the supply and concentration of calcium and phosphate for calcification may depend on the marked vascularization around the BMG (15, 16).

Reddi and co-workers (5, 6) discussed the problem of "anchorage dependence," which was the relation between cells and a solid substratum, and they concluded that the bone-inducing capacity of the demineralized bone powder depended on their particle sizes. They also found that subcutaneous implantation of coarse particles (74–420 μm) of demineralized bone resulted in local differentiation of endochondral bone, but that implantation of a particle of 44–74 μm did not induce bone. Syftestad and Urist (7) reported that the particle sizes of 125–1000 μm in demineralized whole matrix and 44–1000 μm in BMG were suitable for bone induction. Therefore, we used BMG with a particle size of 75–500 μm to induce bone formation. Judging from their findings, the particle size of the implant may be related not only to the surface architecture, but also to the inside architecture providing crevices for vascularization. We prepared the compacted BMG with smaller spaces and crevices inside the BMG by the guanidine-ethanol precipitation method usually used for reconstitution of bone morphogenetic protein and bone residue. We found, as expected, that in the compacted BMG group, no calcification of the BMG was detectable on Day 5, 7, or 10 after implantation, and that no bone formation occurred before Day 15, though much cartilage was seen. This group also showed considerably lower alkaline phosphatase activity and vascularization than did the control group. The difference between the two groups might be influenced not only by whether osteoblasts can attach to the compacted BMG as well as to coarse particles of BMG, but also by the circumstance for which many blood vessels were formed in the crevices of the coarse particles of BMG in the control group. Consequently, we found that by compaction of the particles, vascularization was retarded and, secondarily, both recalcification and bone development were delayed. It is said that vascularization was indispensable for new bone formation (15, 16).

The present results suggest that new vascularization is indispensable for calcification of the BMG and subsequent differentiation of osteoblasts. Hereafter, the relation between the calcification of the BMG and differentiation of chondroblast or osteoblast should be clarified.

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Erratum

In the article Absence of Estrogenic Activity in a Diet that Promotes Estrous Cyclicity in C57BL/6J Mice by Karelus and Nelson, Vol. 196, 321–324, there is an error in Table II. The probability value in the top row of the body of the table which corresponds with body weight (g) should read: <0.001 and *not* NS.

We regret any confusion this error may have caused.