Enhanced Osteoinduction by Intramuscular Grafting of BMP-\(\beta\)-TCP Compound Pellets into Murine Models

Chung-Hsien Wu¹, Kohji Hara¹ and Hidehiro Ozawa²

Department of Periodontology¹ and Department of Oral Anatomy², Niigata University School of Dentistry, Niigata, Japan

Received December 27, 1991

Summary. The osteoinductive effects of bone morphogenetic protein (BMP, derived from murine osteosarcoma) were studied with regard to its use combined with \(\beta\)-tricalcium phosphate (\(\beta\)-TCP). BMP and \(\beta\)-TCP were molded into pellets by the “pressure method”, originated by us and transplanted to ddY mice. Control mice received interdorsal muscular implantations of either the BMP or \(\beta\)-TCP pellets. The animals were sacrificed 1, 2 and 3 weeks after grafting, for radiological, histochemical, and ultrastructural observations. The BMP-\(\beta\)-TCP compound pellets induced faster cartilage and bone formation, whereas these activities were slower when pellets made solely of BMP were used. The \(\beta\)-TCP pellets demonstrated no osteoinductive properties. Observations revealed two types of \(\beta\)-TCP resorbing multinuclear giant cells. One was osteoclastic, expressing calcitonin receptors, having numerous mitochondria and ruffled border-like structures; the other was not osteoclastic in nature. In animals grafted with the compound pellets, a great number of osteoclastic cells gathered on the pellets, much earlier than those grafted with the pellets made of BMP alone. Then, osteoblastic bone formation over the cement lines followed an osteoclastic resorption of both \(\beta\)-TCP and newly formed bone. In contrast, BMP induced few osteoclastic cells, resulting in slower bone coupling. Furthermore, the faster bone formation induced by the compound pellets seemed to be associated with the presence of \(\beta\)-TCP. Porous by nature, \(\beta\)-TCP would entrap BMP within its micropores, and thus, the intrinsically diffusible BMP is retained and its action consequently prolonged. In addition, the compound pellet offered increased surface contact between BMP and mesenchymal cells. Therefore, BMP-\(\beta\)-TCP compound pellets induce cartilage and bone formation more rapidly than does BMP alone.

Bone grafting is a therapy often applied to bone defects of periodontal, traumatic, developmental or surgical origin. Most of the bone grafting materials currently available can be classified according to whether or not they are osteoinductive.

URIST (1965) reported the presence in decalcified bone of bone morphogenetic protein (BMP) which proved to be osteoinductive. Since then, the possible applications of BMP as a bone graft material have been the subject of extensive investigations. This protein is either extracted from dentin (YEOMANS and URIST, 1967; KAWAI and URIST, 1989) or osteosarcoma tissue samples, or obtained in several recombinant forms (WOZNEY et al., 1988; PARALKAR et al., 1991). However, BMP lacks plasticity and is, by itself, too diffusible to remain in the vicinity of the graft site. In practice, a combined use of the protein with other substances is required to overcome the above problems.

Several artificial bone graft materials have recently been developed, but none have proved to be osteoinductive. Despite their lack of osteoinductive property, however, some of them did, in fact, promote the filling of bone defects. One of the materials now considered for supplementing BMP is \(\beta\)-tricalcium phosphate (\(\beta\)-TCP) (HARA and SHIMIZU, 1985; SHIMIZU, 1986a, b; WADA et al., 1989a, b). This nonimmunogenic, biodegradable material possesses adequate strength for grafting, and is now a choice material for use in combination with BMP (URIST et al., 1984; MEIKI et al., 1989). Nevertheless, the interaction of BMP and \(\beta\)-TCP and the respective roles they play in bone formation during their combined use remains unknown.

In an attempt to clarify whether the combined use of BMP and \(\beta\)-TCP enhances BMP's osteoinductive activity or promotes ossification, we designed an in vivo morphological study on their osteoinductive activities.
MATERIALS AND METHODS

Graft pellets forming procedure
BMP used in this study was a gift from Dr. K. TAKAOKA, Department of Orthopedics, Osaka University School of Medicine. The protein was extracted from murine osteosarcoma and had a ca. 20,000 molecular weight (TAKAOKA et al., 1980, 1981, 1982). β-TCP was obtained from a commercial source (Synthograft, Johnson & Johnson, U.S.A.).

We designed a device for preparing three types of grafting pellets by compression (Fig. 1). The main system of this device is a stainless mold simulating experimental bone defects, i.e., those created in animal models to assess the effect of various grafts. Briefly, the procedures consisted of the following: 6 mg β-TCP, 6 mg BMP or 6 mg of both grafts were mixed well and placed in a polished cylindrical cavity drilled in a stainless block. The cavity was washed with sterilized distilled water, then sterilized in an autoclave prior to use. A stainless bar fitting the cavity was inserted, on which a gradually increasing compressive force of respectively 4 kg/mm², 4.8 kg/mm², then 5.6 kg/mm² was applied for 30 sec by a calibrated mechanical stress-loading press (Model 3763, Mori Testing Machine, Tokyo, Japan). The compression was completed after application of a 6.4 kg/mm² force for 1 min. The materials were thus formed into three types of solid pellets composed of BMP alone (BMP pellets), β-TCP alone (TCP pellets) or both BMP and β-TCP (compound pellets). This procedure is referred to as the "pressure method".

One pellet of each type was randomly selected from the lots to observe its surface, first with binoculars. They were then ion-coated (quick-coater, model IB-3, Eiko, Japan) and observed under a scanning electron microscope (SEM; model S-570, Hitachi, Japan).

Experimental animals and groups
Nineteen male Slc/ddY mice of 3 weeks of age and weighing from 10 to 15 g were divided into three groups A, B and C. Group A was composed of 7 mice, and groups B and C were composed of 6 mice respectively. The three groups received pellets composed of 6 mg β-TCP, 6 mg BMP and 6 mg of both grafts, respectively. All pellets were grafted into the right dorsal intermuscular space.

Experimental procedure
In group A, 6 out of the 7 mice were divided into three subgroups of two mice each. The mice of each subgroup were sacrificed 1, 2 and 3 weeks after grafting (week 1, 2, 3), respectively. All six mice were fixed by perfusion fixation with a solution containing 3% glutaraldehyde, 0.1% sucrose, 0.5% dextran, 0.05 M cacodylate buffer (pH 7.3) and 0.05% CaCl₂. Tissue blocks were sampled from the mice at the site of the graft. The blocks were then examined by soft X-ray (SRO-M50, Sofron, Japan) at 50 kV, 3 mA and 150 sec. Then, the blocks were randomly cut into three
pieces. One piece was decalcified with 5% Trichloroacetic acid, embedded in paraffin, sliced into 6 um serial sections and hematoxylin-eosin-stained for light microscopic observation. The second was decalcified with 4.13% ethylenediaminetetraacetic acid (EDTA), embedded in Technovit 7100 (Fa. Kulzer, Germany), and sliced into 2 um sections. The slices were incubated according to the method by Cole and Walters (1987). Briefly, the samples were incubated in a medium containing 50 mM L-(+)-tartaric acid with Naphthol AS-BI phosphate (Sigma, Germany) as substrate in 0.2 M acetate buffer (pH 5.2) for 40 min at 37°C, then stained with methylene blue for histochemical examination of tartrate-resistant acid phosphatase (TRACPase) activity. Control sections, incubated by Burstone's method (1958) in the same medium with the same substrate but without tartaric acid, were examined for acid phosphatase (ACPase) activity. The third block was also decalcified in 4.13% EDTA for 2 weeks, post-fixed with 1% osmium tetroxide, dehydrated in acetone and embedded in Epok-812. The blocks were then sectioned and stained with tannic acid, uranyl acetate and lead citrate for transmission electron microscopy (H-500, Hitachi).

In week 3, the last one of the seven mice of group A was anesthetized with diethyl ether. The tissue block was then sampled. The block was cut into two parts, with one part incubated in a modified BGJb medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mg/ml bovine serum albumin and 2.5 µCi/ml of 125I-labeled elcatonin (125I-eCT; courtesy, Toyo-Jozo Co., Japan) for 1 h at 37°C and 5% CO2. The other part was incubated in the same medium, but with 1 µg/ml non-labeled elcatonin 2.5 µCi/ml 125I-eCT (Toyo-Jozo Co., Japan). The samples were fixed by immersion with the same solution as used for the other 6 mice, decalcified with 4.13% EDTA for 2 weeks, post-fixed with 1% osmium tetroxide, dehy-
100 C.-H. Wu, K. Hara and H. Ozawa:

They were then sliced into 0.7 µm serial sections which were treated with autoradiographic emulsion (NR-M2, Konica, Japan) and stored in dry refrigeration. Five weeks later, they were developed using Konica-dol X (Konica, Co., Japan) and fixed using a fixation liquid (Fujifilm, Fujifoto Film Co., Japan). The samples were finally stained with 2% methylene blue and observed under the light microscope.

The six mice of group B were also divided into three subgroups of two mice each. They were sacrificed in weeks 1, 2 and 3 respectively, using the same perfusion fixation as for group A. Tissue blocks obtained from each mouse were examined by soft X-ray. The blocks from each subgroup were cut into three parts, with one embedded in paraffin and sliced. The sections were stained either with hematoxylin and eosin, or toluidine blue according to Ichikawa's method (1953), which uses McIlvain citric acid-phosphate buffer containing 0.05% toluidine blue (pH 4.5). The other was prepared for the detection of TRACPase activity as in group A. The third part was

Fig. 3. Photomicrographs of HE-stained tissue samples from β-TCP-transplanted mice. a. Many fibroblastic cells infiltrate the soft tissue surrounding the pellet, in week 1. A few multinuclear giant cells were observed (arrowheads). ×140. b shows tissue samples from week 2. The number of multinuclear giant cells has increased (arrowheads). T β-TCP. ×140

Fig. 4. Photomicrographs of the TRACPase activity of multinuclear giant cells surrounding β-TCP grafts. a. TRACPase-positive (△) and TRACPase-negative (▲) multinuclear giant cells are observed in week 1. ×350. b shows TRACPase activity in a β-TCP-transplanted mouse, in week 3. T β-TCP. ×560

3a

3b

4a

4b
Fig. 5. a. Electron micrograph of a β-TCP-resorbing multinuclear giant cell. Many vacuoles and mitochondria are observed in the cytoplasm. x5,200. b. A higher magnification of the area shown in the box in a, where the ruffled border-like structure is observed. x25,000. T: Ruffled border-like structure, TCP β-TCP, Cz clear zone, M mitochondria, V vacuoles.
processed for electron microscopy the same as for group A.

The six mice of group C were subdivided and examined by soft X-ray and light microscopy the same as the group B mice, and observed by TEM as for group A. The specimens obtained from this group also underwent detection of ACPase and TRACPase activities. Specimens from mice sacrificed on week 1 were submitted to a detection of ALPase and ACPase activities by a double-staining technique. After decalcification, the blocks were sliced into 60 μm tissue disks which were reacted overnight in 0.05 M Tris-maleate buffer (pH 7.4) (YOSHIKI et al., 1972), then incubated in the medium according to the method by MAYAHARA et al. (1967), using β-glycerophosphate as substrate and lead citrate as capturing agent at a pH 8.5 for 30 min at 37°C. After incubation, the sections were sulphurized with ammonium sulphide, and ALPase activity was detected. The tissue disks were then dehydrated with ethanol, embedded in Technovit 7100, and then thinly sectioned at 2 μm. The sections were incubated according to the method by Burstone as in group A prior to the detection of ACPase activity. Finally, the sections were sulphurized again, and stained with methylene blue to finalize the detection of ALPase activity.

RESULTS

Observation of pellet surfaces

The surface of the BMP and β-TCP pellets presented a homogeneous appearance, whereas the compound pellet showed areas with two distinct tones. The darker areas contained BMP, and the lighter areas, β-TCP (Fig. 2a). Under the scanning electron microscope, the surface of the β-TCP pellet presented more microparticles than unpelletized β-TCP. Observation of compound pellets disclosed BMP inserted into β-TCP particles (Fig. 2b). The BMP pellet surface appeared homogeneous.
Group A

In all samples, part of the β-TCP particles diffused and formed shadows observable by soft roentgenograph.

Light microscope observations in week 1 revealed a few multinuclear cells, many fibroblastic cells and capillary extensions surrounding β-TCP (Fig. 3a). In week 2, connective tissue inserted into the pellet and the number of multinuclear cells increased (Fig. 3b). In week 3, further invasion by connective tissue with a marked increase of multinuclear cells was observed. No cartilage or bone formation occurred.

Histochemical observation in week 1 showed some TRACPase-positive mono- and multi-nuclear cells. Most of the multinuclear giant cells (MNGCs) were TRACPase-negative (Fig. 4a). The number of TRACPase-positive MNGCs increased in week 2. In week 3, not only TRACPase-positive MNGCs but also TRACPase-negative MNGCs increased. TRACPase-positive, ruffled border-like structures were observed between TRACPase-positive MNGCs and β-TCP particles. The border layer between β-TCP and adjacent tissues was intensely stained by methylene blue (Fig. 4b).

Under the electron microscope, the cytoplasm of some MNGCs surrounding β-TCP contained many mitochondria and vacuoles. These cells also had yet undeveloped, ruffled border-like structures (Fig. 5). Some osteoblast-like cells surrounded β-TCP. However, no feature indicating bone formation was recognizable (Fig. 6).

In week 3, 125I-eCT autoradiography showed developing silver grains on many MNGCs and mononuclear cells surrounding β-TCP (Fig. 7). On the other hand, no silver grains developed in the sample incubated in the medium containing unlabeled elcatonin.

Group B

Roentgenograph of the area where the BMP pellets were grafted revealed no radiopaque shadow in week 1. However, shadows increased during weeks 2 and 3.

In week 1, light microscopy showed many mesenchymal cells gathering on the surface of the BMP pellet. Part of the cells infiltrated into the graft.
material. Metachromatic matrix surrounded some of the chondrocytes. In week 2, the area of cartilage formation increased. New bone formation occurred at the periphery of the cartilage. In week 3, bone tissue with bone marrow had replaced most of the cartilage.

Histochemical observation in week 1 showed a few TRACPase-positive cells. They were mostly mononuclear cells (Fig. 8a). In week 2, the TRACPase-positive mononuclear cells increased, and, furthermore, TRACPase-positive MNGCs were also found (Fig. 8b). In week 3, the TRACPase-positive MNGCs markedly increased. Bone remodeling occurred in the newly formed bone (Fig. 8c).

Group C
Throughout the experimental period, the area of shadow on the roentgenograph was greater in this group than in group B.

Light microscope observation in week 1 disclosed more cartilage formation than in group B during the same week. Many mesenchymal cells invaded the superficial portion of BMP-β-TCP pellets, especially the interface between BMP and β-TCP particles (Fig. 9a). Meanwhile, only a few cells infiltrated into the deeper portion of the pellet. No vascular extension was noticed in the inner portion of the pellet. In week 2, a large quantity of cartilage was recognized as surrounded by bone tissue with small bone marrow (Fig. 9b). There was much more newly formed cartilage and bone than in groups A or B in the same week. In week 3, extensive new bone formation, as

---

Fig. 8. Photomicrographs of the TRACPase activity of tissues from BMP-transplanted mice. a. In week 1, the TRACPase-positive cells are mostly mononuclear (MO). b shows TRACPase activity in week 2. While TRACPase-positive mononuclear cells increase, some TRACPase-positive multinuclear giant cells (MNGC) are also observed. Endochondral calcification (EC) occurs in newly formed cartilage. c. In week 3, the number of TRACPase-positive multinuclear giant cells (MNGC) is much greater than that observed during the previous week. Cement lines (CL) can be observed in the newly formed bone. Bm bone marrow. ×250
well as the presence of bone tissue with well-developed bone marrow was observed (Fig. 9d). 

TCP particles were embedded in the newly formed bone (Fig. 9d).

In week 1, histochemical observation disclosed many ALPase-positive cells and granules. Important populations of ACPase-positive mononuclear cells and MNGCs gathered around the \( \beta \)-TCP particles located on the pellet surface (Fig. 10a). These ACPase-positive cells were, for the most part, TRACPase-positive as well (Fig. 10b). The populations of TRACPase- and ACPase-positive cells were much larger than in groups A or B during the same week. In the inner portion of the pellet, only a few TRACPase-positive mononuclear cells and MNGCs gathered around the \( \beta \)-TCP particles. In week 2, ACPase- and TRACPase-positive MNGCs were located on \( \beta \)-TCP and new bone, and their spatial configuration appeared continuous to the cement lines over which osteoblastic bone formation had occurred (Fig. 11). In week 3, TRACPase-positive MNGCs continued to increase. Bone remodeling was observed.

Electron microscope observation in week 3 revealed newly formed bone both laying over and in direct contact with the \( \beta \)-TCP surfaces (Fig. 12a). Osteoclastic MNGCs resorbed both \( \beta \)-TCP (Fig. 12b) and bone (Fig. 12c). Osteoblasts aligned over the cement lines, and new bone formation was recognized.

**DISCUSSION**

Conventional methods for combining BMP with other substances include lyophilization (URIST et al., 1984; KAWAMURA et al., 1987; GLASS et al., 1989) and
dialysis (KAWAMURA et al., 1987). These techniques contain, however, one intrinsic problem: they carry the potential risk of either decreasing or altering the activity of BMP. Reported methods for the delivery of BMP in combination with other substances have consisted of capsule envelopment (URIST et al., 1984) and muscle pouches (MEIKI et al., 1989). Unfortunately, these methods provide only poor contact between grafts and surrounding tissues. Also, BMP and combined substances are easily dissociated by physical muscular movements, i.e., stretching and contraction.

On the other hand, our "pressure method", being purely mechanical, serves to avoid the degradation in BMP activity when compared with conventional methods. Another advantage of our method is that it provides well-mixed, solid graft pellets, which play a decisive role in maintaining the spatial relationship between BMP and β-TCP. Our technique can also provide a means of bringing novel insights into osteoinductive mechanisms by the grafting of the BMP-β-TCP compound. However, the pressure method is only a startpoint; a technique could eventually be developed for forming grafts which precisely fit the configuration of given bone defects.

In group A (β-TCP grafted alone), two types of β-TCP-resorbing MNGCs were observed. One type was TRACPase-positive, expressing calcitonin receptors and having many mitochondria and ruffled-border-like structures, all these characteristics indicating the osteoclastic nature of the cells (LUCHT, 1971; WARSHAWSKY, 1980; RAO et al., 1981; MINKIN, 1982; BARON et al., 1986a; NICHOLSON et al., 1986).

Fig. 10. Photomicrographs of ALPase, ACPase (a) and TRACPase (b) activity in tissue samples from compound pellet-grafted mice in week 1. a. ALPase-positive chondrocytes (*) and intercellular matrices (MX) are observed. ACPase-positive cells (arrowheads) are gathered around a β-TCP (T) particle. ×150. b shows TRACPase activity in the series section of a. Most of the ACPase-positive cells are also TRACPase-positive. T β-TCP. ×370
The other type presented morphological characteristics of MNGCs but lacked the features attributed to osteoclastic cells. Most of the \( \beta \)-TCP-resorbing osteoclastic MNGCs can be assumed to derive from TRACPase-positive mononuclear cells (BARON et al., 1986a) which express calcitonin receptors (RAO et al., 1981; NICHOLSON et al., 1986; HATTERSLEY and CHAMBERS, 1989). Although osteoclastic MNGCs resorbed \( \beta \)-TCP, we observed no bone formation in group A. This would be due to the \( \beta \)-TCP's lack of osteoinductive properties. Though \( \beta \)-TCP was resorbed, it did not induce recruitment of osteoblasts, thus resulting in the absence of any transition between \( \beta \)-TCP resorption and bone formation.

Our results demonstrated that the combined use of BMP and \( \beta \)-TCP induced cartilage and bone formation more rapidly than did BMP alone. Since \( \beta \)-TCP alone is not osteoinductive, the enhanced cartilage/bone induction should necessarily be related to the combined use of the two substances, especially the influence of \( \beta \)-TCP on BMP's osteoinduction capacity. One reason for the enhanced osteoinduction would be that porous \( \beta \)-TCP retained intrinsically diffusible (AMITANI and NAKATA, 1975; URIST et al., 1977) BMP within its micropores. In this case, the pressure method would be directly responsible for the enhanced bone formation. The retention of BMP by \( \beta \)-TCP would therefore contribute to prolonging the action of BMP. Our study also demonstrated that many mesenchymal cells invaded the BMP-\( \beta \)-TCP interface of the compound pellets. Furthermore, not only BMP, but also extensions of mesenchymal cells are suspected of inserting themselves into micropores of \( \beta \)-TCP, so that the contact area between BMP and surrounding tissues is therefore increased. Literature on the subject informs that BMP is osteoinductive when it enters into direct contact with undifferentiated mesenchymal cells (URIST, 1989). This increased contact area further enhances the inductive properties and results in faster cartilage and bone formation. Therefore, the porous nature of \( \beta \)-TCP would partly explain the faster cartilage/bone forma-

Fig. 11. A photomicrograph of ACPase activity in the tissue sample, from a compound pellet-grafted mouse in week 2. New bone (NB) formation occurs over the cement lines (CL) located on the surface of \( \beta \)-TCP (TCP). Osteoblasts (OB) align on the new bone (NB). Osteoclasts (OC) are observed on the continuum of the cement lines (CL). BM bone marrow. x 530
tion induced by the use of BMP-β-TCP combined pellets compared with that observed with pellets composed of BMP alone. Our results from group A clearly showed that some of the β-TCP resorbing MNGCs were osteoclastic. The β-TCP-resorbing osteoclastic cells were thought to play important roles in enhancing the osteoinductive properties of the compound pellets.

As our results indicate, the use of BMP alone first induced the formation of cartilage from undifferentiated mesenchymal cells. Following this, new bone formation via endochondral ossification occurred. In group B, the number of osteoclastic cells increased gradually through the process of ossification. In this case, the differentiation of osteoclastic cells would be associated with the mineral phase of bone formation (Glowacki et al., 1981; Krukowski et al., 1982). On the other hand, the grafting of compound pellets induced, in group C mice, a large population of osteoclastic cells on β-TCP, either before or at the initial stage of ossification. Though not osteoinductive, β-TCP alone seems to attract osteoclastic cells; our study demonstrates that BMP combined with β-TCP increases the number of osteoclastic cells gathering on the surface of the graft. This implies that an enhanced osteoclastic resorption would have taken place in group C, and even at a very early stage of ossification.

Assuming that β-TCP-resorbing osteoclastic cells release acid (Gay and Mueller, 1974; Baron et al., 1985; Baron, 1989; Blair et al., 1989) and dissolve β-TCP, the process would result in an uptake of β-TCP’s calcium ion by these osteoclastic cells, which in turn would lead to a Ca⁺⁺ release, as described by Baron et al. (1986b, 1988). These released Ca⁺⁺ would then accumulate around β-TCP and promote calcification. This explains why BMP-induced cartilage ossified more rapidly when combined with β-TCP than when BMP alone was used.

The process described above indicates that the

Fig. 12. Electron micrographs of tissue samples from compound pellet-grafted mice, in week 3. a. New bone (Bo) formed by osteoblasts (OB) is observed in direct contact with β-TCP (TCP). ×4,300. a and c. Osteoclastic cells (Oc) resorbed β-TCP (TCP) and newly formed bone (Bo). Osteoblasts (OB) are observed aligned on the cement line (CL). b: ×5,200, c: ×3,700
combined use of \( \beta \)-TCP and BMP provides an environmental priming for ossification, which also allows the coupling phenomenon.

The “coupling phenomenon” is suggested to be a transition from osteoclastic bone resorption to osteoblastic bone formation (Baylink and Liu, 1979; Howard et al., 1981; Baylink et al., 1982). During osteoclastic bone resorption, acid (Gay and Mueller, 1974; Baron et al., 1985; Baron, 1989) and lysosome enzymes (Minkin, 1982; Baron et al., 1986a, 1988; Baron, 1989) released by osteoclasts excavate and activate BMP (Yashikawa et al., 1986), TGF-\( \beta \) (Yashikawa et al., 1988; Oreffo et al., 1989) or other growth factors (Mohan and Baylink, 1991) present in the bone matrix. These factors then induce the differentiation of neighboring undifferentiated mesenchymal cells into chondrocytes or osteoblasts. Osteoclasts move, resorb bone, and form resorption surfaces which later become cement lines. The surface layer of resorption lacunae are thought to contain lysosome enzymes and/or activated growth factors, otherwise suspected to act as coupling factors inducing osteoblast recruitment and new bone formation (Baron et al., 1984; Miller, 1985; Huffer, 1988; Oguro and Ozawa, 1988, 1989; Bonewald and Mundy, 1990; Goldring and Goldring, 1990). Therefore, osteoclastic resorption is the initial cellular event in the coupling phenomenon, and results in osteoblastic bone formation.

In group B of our study, the population of osteoclastic cells increased only slowly and concomitantly with the progress of ossification of BMP-induced cartilage. However, in group C, many osteoclastic cells gathered on \( \beta \)-TCP either before or in the initiation phase of ossification. Also noteworthy was the great number of osteoclastic cells observed much earlier in group C than in group B. Our study also indicated that osteoclastic cells resorbed \( \beta \)-TCP as well as newly formed bone, resulting in the formation of cement lines over which osteoblastic bone formation occurred. Our morphological findings showed that osteoclastic cells which resorbed \( \beta \)-TCP and newly-formed bone took part in a phenomenon resembling bone coupling. Acid and/or lysosome enzymes released by the \( \beta \)-TCP-resorbing osteoclastic cells likely dissolve \( \beta \)-TCP and release calcium ions. These substances released by the osteoclastic cells could also excavate and enhance the activity of BMP retained in the \( \beta \)-TCP particles' micropores. The enhanced BMP's osteoinductive activity would, in its turn, enhance cartilage and new bone formation. Therefore, BMP-\( \beta \)-TCP compound pellets induced earlier and faster cartilage and bone formation that did pellets made of BMP alone.

Furthermore, in group C, the early appearance of osteoclastic cells followed by osteoclastic resorption results in early bone coupling in newly formed bone. The above could also result in a further recruitment of osteoblastic new bone formation. This, then, is why the coupling phenomenon and the following new bone formation occurs much more speedily in group C than in group B. This seems also to explain the enhanced bone formation by the use of the combined pellets as the graft.

Acknowledgments. Thanks are due to Dr. Kunio Takaoka, Department of Orthopaedics, Osaka University School of Medicine, for kindly supplying us with BMP. The authors wish to thank Dr. Seigo Okawa, Department of Dental Materials and Technology for his assistance. We are also grateful to Dr. Sadakazu Ejiri, Department of Oral Anatomy, and Dr. Takashi Suzuki, Department of Periodontology for their helpful discussion in the preparation of the manuscript.

REFERENCES


———: Cytochemical studies of the cellular events sequence in bone remodeling: cytological evidence for a coupling mechanism. J. Bone Mineral Metab. 7: 30-36 (1989).


Dr. Kohji Hara
Department of Periodontology
Niigata University School of Dentistry
Gakko-cho-2-5274, Niigata
951 Japan

原 耕 二
951 新潟市学校町通2番町5274
新潟大学歯学部
第二保存学教室