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Time-Dependent Expression of Bone Sialoprotein Fragments in Osteogenesis Induced by Bone Morphogenetic Protein[1]

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Bone sialoprotein is unique to bone and dentin, but its precise role in these tissues is still unknown, although several hypotheses have been presented. We chose ectopic chondro- and osteogenesis induced by bone morphogenetic protein (BMP) as a model system to examine the role of this protein. Partially purified bovine BMP obtained by a three-step chromatographic procedure contained all the active BMPs (natural BMP cocktail). It was combined with insoluble bone matrix and subcutaneously implanted into rats. Expression of bone sialoprotein (BSP) in the implants was followed by using a monoclonal antibody as previously reported. Immunostaining studies showed BSP in the osteoblasts lining the new bone surface at 5 weeks. Western blotting showed 53 and 30 kDa bands, instead of the 57 kDa band normally found in rat femur. These two fragments were metabolically labeled with [^{3}H]proline. The total amount of the fragments rapidly increased after 3 weeks, and at 5 weeks was 3 times as high as that at 2 weeks and still increasing. This time-dependent change was almost parallel to that of osteocalcin. The amount of bone estimated in terms of calcium content increased until 3 weeks and was remained at a plateau thereafter. Alkaline phosphatase activity was prominent only in the first 3 weeks. It was concluded that the 53 and 30 kDa BSP fragments might contribute to maintenance or remodeling in BMP-induced ectopic bone formation.

Key words: bone morphogenetic protein, bone remodeling, bone sialoprotein, fragmentation, immunolocalization.

Approximately 20% (w/w) of fresh bone tissue is composed of organic matrix, of which 90% is collagen and 10% is non-collagenous proteins. Bone sialoprotein (BSP), a major non-collagenous protein (*1-3*), has a molecular mass of 57 kDa as an extracted protein (*2*), and with 320 amino acids, based on the rat BSP cDNA sequence (*4*). Bovine and human BSP contains 46-50% carbohydrate and 12-13% sialic acid on a weight basis, and are characterized by the presence of an Arg-Gly-Asp (RGD) cell-attachment sequence (residues 289-291) and a poly glutamic acid sequence at the amino terminal (*4, 5*). In the carboxy-terminal segments, some tyrosine residues are sulfated. Several other proteins have been identified from bone matrix, including osteopontin, osteocalcin (*6*), and osteonectin (*7, 8*).

Although it is known that non-collagenous matrix proteins are expressed by bone cells at specific stages of development, the specific roles of these proteins are still unknown. Contrary to initial expectations, osteonectin and osteopontin are synthesized not only by osteoblasts, but also by a variety of other cell types (*9, 10*). As far as we know, only osteocalcin and BSP are localized almost exclusively in calcified tissues, and osteocalcin has been used as a marker of bone formation (*11, 12*). BSP or its mRNA has been detected in bone, dentin, mineralized cartilage and microcalcification foci of the placenta (*13-17*). BSP is expressed at the highest level by differentiated osteoblasts in *de novo* bone formation (*14, 18*). Thus, the functions of BSP should be related to bone formation or its metabolism and BSP provides a unique marker for differentiated osteoblasts and for *de novo* bone formation (*19*).

Bone morphogenetic proteins (BMP), growth and differentiation factors, and members of the TGF-β superfamily, stimulate immature mesenchymal cells and induce local cartilage and bone formation *in vivo* when they are subcutaneously implanted into rats with insoluble bone matrix (IBM) as a carrier (*12, 20-24*). The processes of cartilage and bone differentiation induced by BMP and IBM composites are similar to endochondral ossification (*25, 26*). These developmental cascades of BMP-induced bone formation consist of: (i) chondrogenesis at 1 week after implantation, (ii) osteogenesis at 2 weeks, and (iii) bone marrow forma-

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Abbreviations: ALP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolylphosphate; BMP, bone morphogenetic protein; BSP, bone sialoprotein; ELISA, enzyme-linked immunosorbent assay; IBM, insoluble bone matrix; OCPC, ortho-cresolphthalein-complexone; PBS, phosphate-buffered saline; RIA, radioimmunoassay; TBS, Tris-buffered saline; TTBS, Tris-buffered saline containing 0.05% Tween 20.

tion and bone remodeling at 4 weeks. This *in vivo* model is a superior system to examine the time-dependent changes in bone matrix proteins and to elucidate the functions of these proteins in the course of bone formation (*27, 28*). In this study we investigated the time-dependent changes of BSP in this model system of osteogenesis and found that this protein is uniquely expressed in the later stage of bone remodeling in smaller molecular sizes than that of the originally secreted form.

MATERIALS AND METHODS

Purification of BMP—Freshly obtained bovine metatarsal bones were powdered with a stainless steel mortar and pestle while being cooled under liquid nitrogen, then sieved to obtain particles smaller than 60 mesh. All the procedures thereafter were done at 4°C. The bone powder (1 kg per extraction) was washed with 1.0 M NaCl, 50 mM Tris-HCl, pH 7.4, containing protease inhibitors (50 mM aminocaproic acid, 5 mM benzamidine hydrochloride, and 1 mM benzylsulfonyl fluoride) and defatted with $CHCl_3/CH_3OH$ (1 : 1). The processed bone powder was then demineralized in diluted HCl, keeping the pH constant at 2.0 by adding 12 N HCl. The demineralized bone matrix was extracted with 4 M guanidine hydrochloride in 50 mM Tris-HCl, pH 7.4, containing the protease inhibitors described above. The extracts were centrifuged (20,000 × *g*, 30 min, 4°C) and the supernatants were filtered through a Nucleopore membrane (3 μm, Nucleopore, Pleasanton, CA, USA).

A detailed description of the procedure used for purification of the active fraction of the guanidine extract by an eight-step chromatographic procedure will be reported elsewhere (Kuboki *et al.*, in preparation). The final active preparation was shown by internal sequence analysis (residues 410–424) of the trypsin digest to contain a significant amount of BMP-3. In this study, a partially purified BMP fraction, obtained by the three-step chromatographic procedure described below, was used. A column (8.3 × 12 cm, 500 ml volume) packed with hydroxyapatite (FK-1, Apatite International, Tokyo) was equilibrated with 1 mM potassium phosphate buffer containing 6 M urea (pH 6.8) and the applied sample was eluted in a stepwise manner with 0.1 and 0.4 M phosphate in a potassium phosphate buffer containing 6 M urea (pH 6.8). The activity was recovered in the 0.4 M phosphate fraction, which was concentrated and replaced with the buffer used in the chromatography. The sample was applied to a heparin-Sepharose CL-6B column (Pharmacia LKB: 3 × 30 cm, 190 ml volume) equilibrated in 0.1 M NaCl, 50 mM Tris-HCl, 6 M urea, pH 7.0, and eluted with 0.1, 0.15, and 0.5 M NaCl in a stepwise manner. The activity was recognized in the 0.5 M NaCl eluate, which was concentrated, and the residue was taken up in the next solvent. Sephacryl S-300 HR (Pharmacia Uppsala: 2.2 × 141 cm, 500 ml volume) was equilibrated with 4 M guanidine-HCl/50 mM Tris-HCl, pH 7.4, and eluted with the same buffer. The BMP-containing fractions were pooled and designated S-300. The S-300 BMP preparation was pooled from at least 5 extractions to assure a uniform quality.

Preparation of BMP/IBM Composites—One-tenth milligram of the S-300 BMP fraction in 40 μl of 0.1% trifluoroacetic acid was absorbed on 20 mg of IBM. Preparations of BMP/IBM composites were lyophilized and then 40 μl of phosphate-buffered saline (PBS) was added.

Implantation of BMP/IBM Composites—BMP/IBM composites and control IBM alone were implanted subcutaneously into the back of male Wistar King rats 4 weeks old. Implants were harvested at 1, 2, 3, 4, 5, 6, 8, 10, and 20 weeks after implantation, and subjected to biological and histological analysis.

Biochemical Analysis—Implanted pellets were lyophilized and crushed. Samples were washed with PBS twice, homogenized in 1 ml of 0.2% Nonidet P-40, 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5, and homogenized. The homogenate was centrifuged at 12,000 rpm for 15 min and the alkaline phosphatase (ALP) activity of the supernatant was determined. ALP activity was measured by the Kind-King phenylphosphate method. Calcium content of the remaining samples was measured by the ortho-cresolphthalein-complexone (OCPC) method.

The residues of the homogenate were subjected to matrix protein extraction. They were decalcified with 40% formic acid for 12 h. During decalcification, non-collagenous proteins of bone matrix were extracted. The extracts were concentrated by ultrafiltration (Millipore, UFP1, LCC 24); PBS was added to neutralize the extract while filtration was under way. The extracts were used for analyses of phenotype expression.

Immunological Analyses—For analysis of osteocalcin in the pellet, a radioimmunoassay (RIA) was performed. Rat osteocalcin (Biomedical Technologies, USA) was used as a standard osteocalcin. Goat anti-rat osteocalcin (Biomedical Technologies) was used as a first antibody. Donkey anti-goat IgG (Biomedical Technologies) was used as a second antibody. Rat osteocalcin was labeled with ^{125}I by the lactoperoxidase method (ICN Pharmaceuticals, USA).

To determine the expression of BSP in the pellets, the extracts were subjected to Western blotting using Immobilon (Millipore, IPVH000 10) transfer membranes that were incubated with mouse anti-bovine BSP monoclonal antibodies in TBS (20 mM Tris-HCl, 0.15 M NaCl, pH 7.4)/2% bovine serum albumin (BSA). Membranes were incubated with a second antibody (Dako, peroxidase-conjugated rabbit anti-mouse immunoglobulins, Denmark) and developed with a diaminobenzidine substrate. As the positive control, bovine BSP and rat BSP-containing fractions were used. Bovine BSP was extracted and purified from bovine tibiae as described in a previous report (*29*). Rat BSP-containing fractions were partially purified from rat femoral bone. Demineralized rat bone was extracted with 4 M Gdn-HCl in the presence of protease inhibitors. The extracts were chromatographed on a DEAE-cellulose (DE-52) column eluted with 0.1 and 1.2 M sodium acetate in a stepwise manner in 7 M urea, 10 mM Tris-HCl, pH 6.0. Then 1.2 M sodium acetate fractions were collected as BSP-containing fractions. Mouse anti-bovine BSP monoclonal antibodies were kindly prepared by Dr. H. Matsuyama, Sapporo Medical University School of Medicine (Matsuyama *et al.*, in preparation).

We measured the contents of BSP in implanted pellets by enzyme-linked immunosorbent assay (ELISA) (*2*). Bovine BSP, dissolved at 1.17 μg/ml in carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8), was used to coat the wells of ELISA plates (Corning, Disposable Sterile ELISA plates) by incubation overnight. The plates were then rinsed throughly with TTBS (containing 0.05% Tween 20).

A dilution of purified bovine BSP and samples to be analyzed were preincubated for 9 h with mouse anti-bovine BSP monoclonal antibodies diluted at 1:1,200 with TBS/2% BSA. Measured samples of the antigen/antibody mixture were added to the wells coated with bovine BSP, and the plates were incubated for 1 h. After rinsing of the wells with TTBS, bound antibody was detected by incubation with a second antibody diluted at 1:1,000 with TBS/2% BSA. After incubation for 1 h, the wells were rinsed and enzyme activity was measured by incubation with an *o*-phenylenediamine substrate. All ELISA processes were performed at room temperature. The absorbance at the dual wavelengths of 490 nm/630 nm was measured with a plate analyzer (Toyo, ETY300).

Metabolic Labeling—We then attempted to elucidate whether BSP was synthesized in the pellets by metabolic labeling. Pellets were removed at 5 weeks after implantation, cut in pieces and incubated at 37°C for 24 h in serum-free medium containing 0.1 mCi [^{3}H]proline (Amersham, L-[5-^{3}H]Proline, USA). The medium was then withdrawn, and the free radioisotope in the medium was separated by ultrafiltration (Amicon, Centricon-3, Code 4202). Labeled proteins were immuno-precipitated with mouse anti-bovine BSP monoclonal antibodies and coupled to Pansorbin cells (Calbiochem, USA). The immuno-absorbed proteins were analyzed by SDS-PAGE on 12.5% gel. The gel was impregnated with Amplify (Amersham) and the radioactive bands were detected by fluorography.

Histological Analysis—Implanted pellets were fixed in 10% formaldehyde, decalcified in 10% formic acid, dehydrated through a graded series of ethanol, and embedded in paraffin. The sections were stained with hematoxylin and eosin.

For analysis of immunolocalization of BSP, pellets were fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.4, and then frozen and cut into thin sections. The sections were stained by the avidin-biotin alkaline-phosphatase complex technique. The sections were developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate and counterstained with methyl-green.

RESULTS

Histological Observations—In BMP/IBM composite implants, bone and cartilage formation was histologically observed at 2 weeks after implantation (Fig. 1A). The results were consistent with previous reports (*12, 20–23*). Cartilage was formed in the pellets, especially in spaces intervening between IBM particles at 1–2 weeks. Bone formation occurred at 2 weeks after implantation. At 5 weeks after implantation, cartilage had already disappeared and woven bone formation accompanied with differentiation of bone marrow could be recognized, along with formation of layers of mineralized bone matrix having appositional cement lines (*30, 31*) (Fig. 1B). IBM had already disappeared. Figure 1C shows that woven bone was replaced by lamellar bone at 20 weeks after implantation. These results suggested that the remodeling of bone had occurred after 5 weeks. In no case did any bone or cartilage

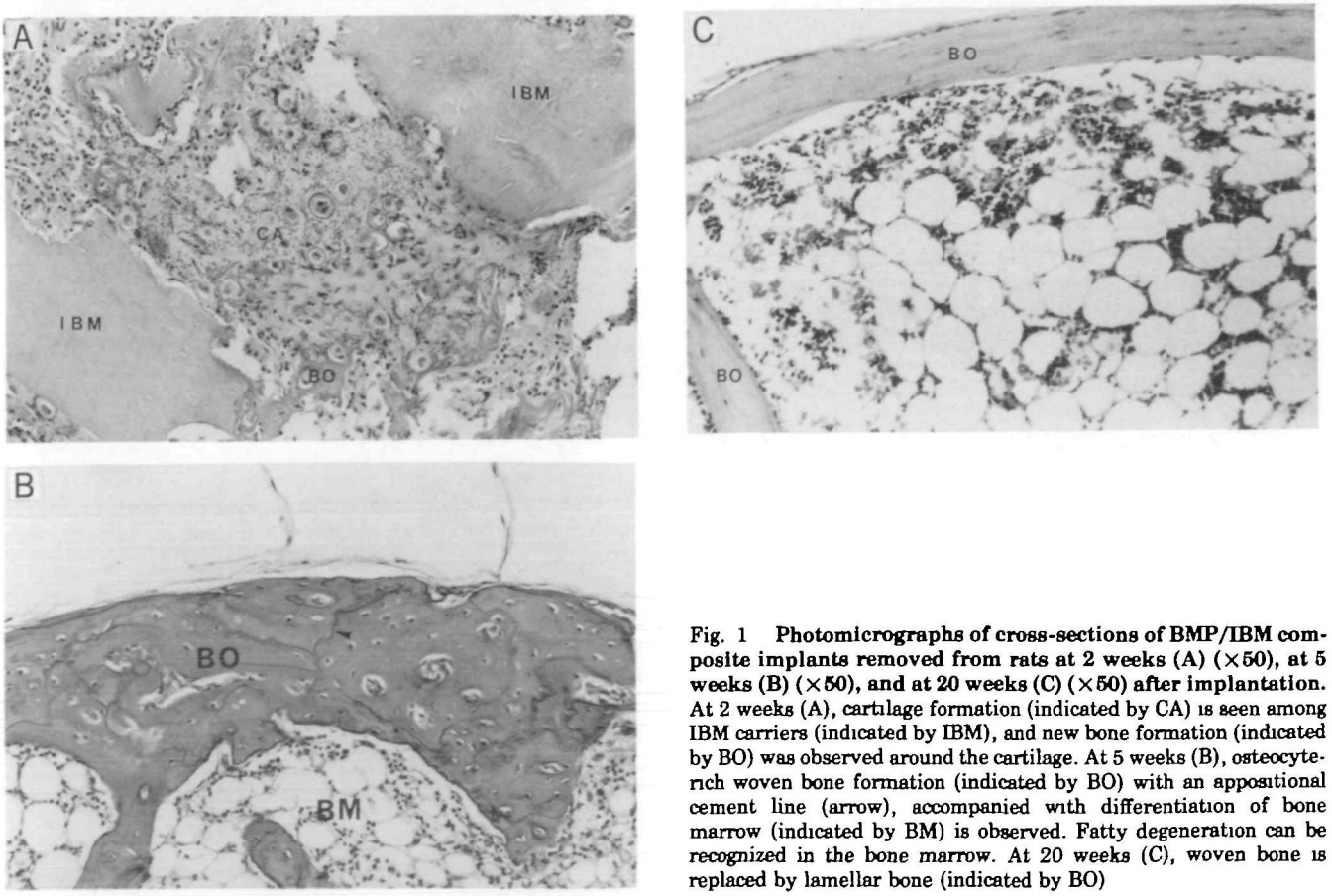
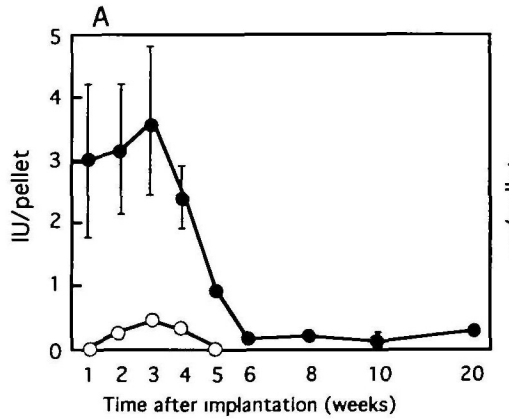


Fig. 1 **Photomicrographs of cross-sections of BMP/IBM composite implants removed from rats at 2 weeks (A) (×50), at 5 weeks (B) (×50), and at 20 weeks (C) (×50) after implantation.** At 2 weeks (A), cartilage formation (indicated by CA) is seen among IBM carriers (indicated by IBM), and new bone formation (indicated by BO) was observed around the cartilage. At 5 weeks (B), osteocyte-rich woven bone formation (indicated by BO) with an appositional cement line (arrow), accompanied with differentiation of bone marrow (indicated by BM) is observed. Fatty degeneration can be recognized in the bone marrow. At 20 weeks (C), woven bone is replaced by lamellar bone (indicated by BO)



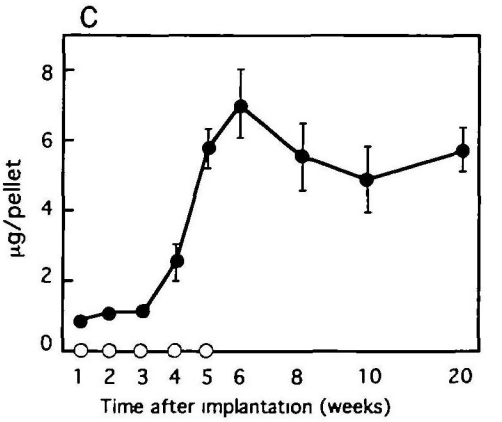
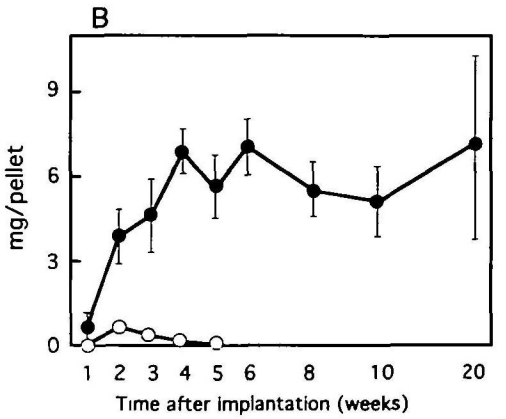


Fig. 2 **Time-dependent changes of alkaline phosphatase (ALP) activity (A), calcium content (B), and osteocalcin content (C) in the BMP/IBM composite implants.** In BMP/IBM composites (closed circles), high ALP activity was observed from 1 to 3 weeks after implantation followed by a decrease (A), and calcium content increased rapidly after 1 week, reaching a maximum at 4 weeks and remaining at an approximately steady level (B). In BMP/IBM composites (closed circles), osteocalcin increased rapidly at 5 weeks after implantation and was essentially stable after this (C). In implants of IBM alone (open circles), there was no significant change.



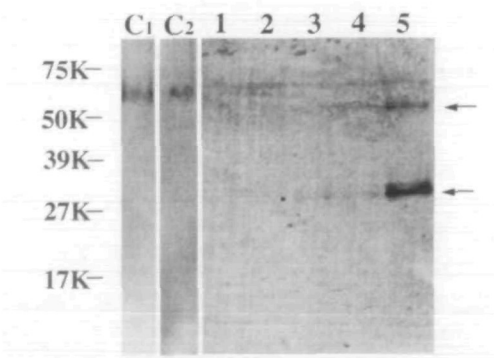


Fig. 3. **Immunoblotting analysis of BSP.** Bovine BSP (lane C1) and rat BSP-containing fractions (lane C2) were used as positive controls. At 1 and 2 weeks after implantation (lanes 1 and 2, respectively) there was no detectable reaction. After 3 weeks (lanes 3, 4, and 5), reactions to anti-BSP monoclonal antibody were observed at 53 and 30 kDa. The maximum reaction was at 5 weeks after implantation (lane 5). Reactions were observed at 60 kDa in lanes 1-5 These were considered non-specific because they remained after competition with bovine BSP.

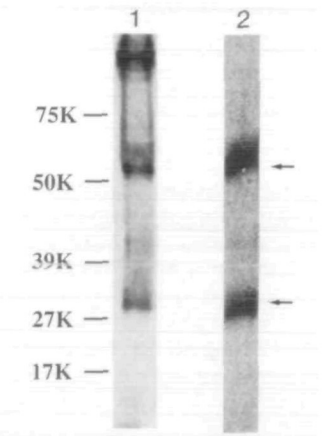


Fig 4 **SDS-PAGE analysis fluorogram of [^{3}H]proline-labeled proteins secreted in serum-free media.** Lane 1, incubated media; lane 2, immunoabsorbed proteins with anti-bovine BSP monoclonal antibodies.

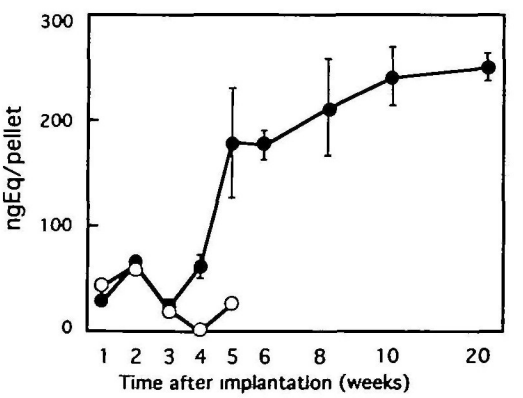


Fig. 5 **Time-dependent changes of the total amount of BSP fragments in the implanted pellets measured by ELISA.** In BMP/IBM composites (closed circles), the total amount of BSP fragments increased rapidly at 5 weeks after implantation. At 5 weeks the amount was about 3-fold greater than that at 2 weeks. After 5 weeks the total amount remained at a plateau level.

form with implants consisting of IBM alone. Moreover, such implants could not be recovered because of resorption after 5 weeks.

Biochemical Analyses—Time-dependent changes of ALP activities, calcium contents, and osteocalcin contents confirmed that BMP/IBM composites induced cartilage and bone formation (Fig. 2). In contrast, there was no significant change with implantation of IBM alone. High ALP activities were observed at 1 to 3 weeks after implantation (Fig. 2A), and decreased rapidly thereafter. Calcium content increased rapidly after 1 week, reaching a maximum at 4

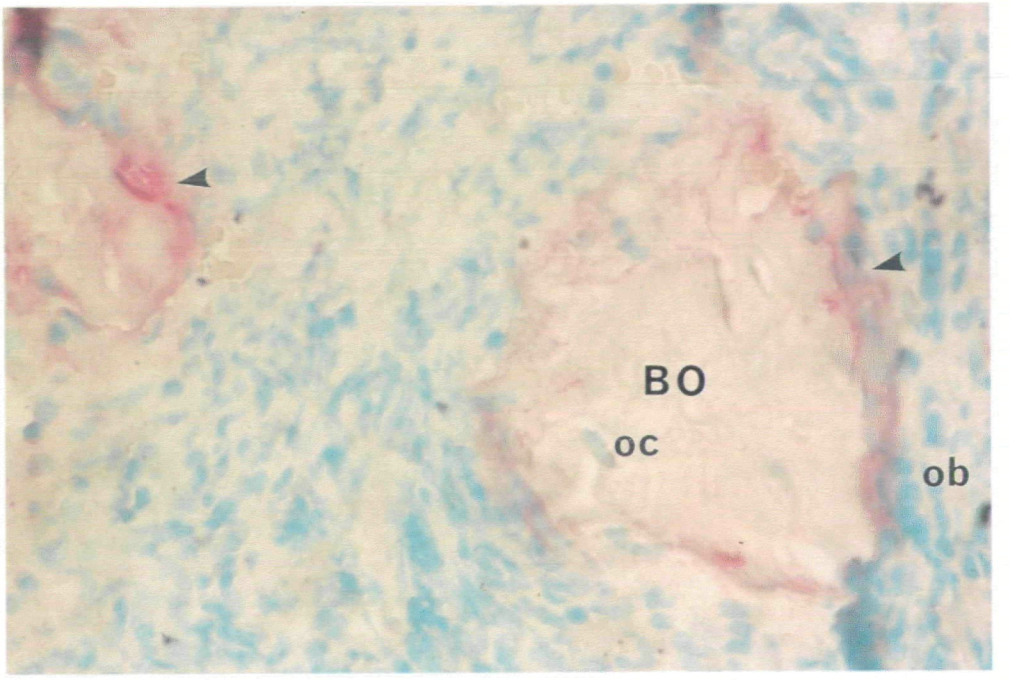


Fig. 6. Immunolocalization of 53 and 30 kDa BSP fragments in BMP/IBM composite implants at 5 weeks after implantation (×150). A reaction product (indicated by arrow) generated by the BCIP substrate was used to identify the presence of antigen; BO, bone; ob, osteoblast; oc, osteocyte. Strong reactivity to anti-BSP monoclonal antibodies is observed in osteoblasts lining the bone surface matrix

weeks and then became virtually constant (Fig. 2B). The results indicated that cartilage formed from 1 to 3 weeks and was replaced by bone up to 4 weeks, followed by bone remodeling. These data support the histological results. Osteocalcin is an established marker of bone formation in BMP-induced bone (*12*). The time-dependent changes of osteocalcin contents in implanted pellets are shown in Fig. 2C. Osteocalcin increased rapidly at 5 weeks after implantation, that is, at the onset of bone remodeling. From 5 weeks to 20 weeks after implantation, osteocalcin contents were essentially stable.

Characterization of BSP—Figure 3 shows the immunoblotting results with bovine BSP monoclonal antibodies in the bone matrix proteins of implants. After 3 weeks, reactions to anti-BSP monoclonal antibodies were observed at 53 and 30 kDa. Maximum reactions were at 5 weeks after implantation. These proteins had different molecular weights from the 57 kDa of intact bovine BSP and rat BSP, as shown in lane C_1 and lane C_2. We then performed a competition experiment with bovine BSP, and the 53 and 30 kDa protein bands disappeared (data not shown). This confirmed that the 53 and 30 kDa proteins were BSP-derived fragments.

To show that the 53 and 30 kDa fragments were synthesized in the pellets, implanted pellets were incubated with [^{3}H]proline in serum-free media. These media were withdrawn after 24 h and [^{3}H]proline-labeled proteins were analyzed by fluorography (Fig. 4). The 53 and 30 kDa proteins were observed as major synthesized proteins in the incubation medium (lane 1). After immunoprecipitation, these proteins were the only bands detected (lane 2). From these results, it was confirmed that the 53 and 30 kDa BSP fragments were synthesized products in the ectopic bone induced by BMP.

Figure 5 shows the time-dependent changes in the contents of both BSP fragments. Both fragments increased rapidly at 5 weeks after implantation, at the onset of remodeling, and then reached an approximately steady level. This pattern of change was coincident with that of osteocalcin content. The total amount of BSP fragments may be much more than shown, because rat BSP may react more weakly with the anti-BSP monoclonal antibodies than bovine BSP.

Immunolocalization—Figure 6 shows the immunolocalization of 53 and 30 kDa BSP fragments in the pellets. At 5 weeks after implantation, at the onset of remodeling, both fragments were localized in bone, especially in osteoblasts lining the bone surface matrix.

DISCUSSION

In this study, we have demonstrated the time-dependent change of phenotype expression in BMP-induced bone formation. We further found that 53 and 30 kDa BSP-derived fragments were localized in BMP-induced bone. Earlier studies have shown that BSP extracted from rat bone contains 23-33 kDa fragments (*1, 2, 32*). It was suspected that cleavage of BSP might be mediated by endogenous proteases released from osteoblasts or osteoclasts (*33*). In this study, we also demonstrated that low-molecular-weight fragments of BSP were secreted in the serum-free metabolic labeling medium within 24 h. It is possible that BSP is not bound to the matrix in the intact form, but only after degradation to a low-molecular-weight form. But low-molecular-weight fragments of BSP appeared rapidly and were afterwards taken into the matrix. These phenomena merit further study.

In previous studies, BSP has been suggested to have a close relation to mineralization (*34*), especially the initial stage of mineralization (*35*). In this study, however, fragments derived from 53 and 30 kDa BSP were clearly increased at 5 weeks after implantation, at the onset of bone remodeling, while calcium content reached the maximum level at 4 weeks after implantation. Thus, there

is a time-lag of 1 week between the increase of calcium content and that of BSP fragments. The developmental stage in which these fragments increased was identified as that of remodeling, based on the characteristic morphological changes. The morphological pattern observed at 5 weeks was characterized by a clear lamellar bone structure and secondary osteoid formation, in contrast to the fibrous bone formed at the early stage of bone development. The most striking finding concerning the stage-specific appearance of BSP was that in the immunohistological study, these fragments were only observed in bone later than 5 weeks after implantation, the period of bone remodeling, but not in mineralized cartilage or in the early stage of bone. Thus, it was concluded that, at least in BMP-induced osteogenesis, BSP fragments may be involved in bone remodeling, but not in initial mineralization.

BSP has also been shown to promote attachment and differentiation of osteoblast-like cells (*4, 29, 36*). In those studies, the localization of the fragments showed a high specificity for bone matrix, especially for the "lamina limitans" (*14, 30, 37, 38*), osteoblasts lining the bone surface matrix. Also, previous *in vitro* studies indicated that BSP has significant affinities for hydroxyapatite (*34, 35, 39, 40*) and collagen (*41, 42*). Combining these observations with our present results, we consider that BSP and its fragments may regulate osteoblasts and or osteoclasts through attachment between the cells and the mineral-containing collagenous matrix in bone.

It was observed that osteocalcin content increased and then became constant in the period of bone remodeling. Osteocalcin is necessary for the recruitment of osteoclast progenitors, as a chemotactic factor for osteoclasts and for promotion of differentiation to osteoclasts (*43-46*). It was also reported that BSP has binding ability to osteoclasts (*15, 46-51*). Ingram *et al.* (*52*) showed that BSP has multiple roles in bone remodeling. In accordance with these reports, we found that BSP-derived fragments were localized in the lamina limitans (cell-matrix interface). Further, the contents of BSP increased at the onset of bone remodeling and were remained at a plateau during bone remodeling. These previous studies and our present observations strongly suggest that 53 and 30 kDa BSP fragments are involved in bone remodeling rather than initiation of calcification, as previously suggested (*34, 35*).

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