Journal of Hard Tissue Biology 7[1] (1998) pp21-pp26

CODEN-JHTBFF, ISSN 1341-7649/98

ORIGINAL

Transchondroid Bone Formation Displayed in BMP-Induced Heterotopic Osteogenesis

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(Accepted for publication, January 20, 1998)

Abstract : It has been stated that BMP induces undifferentiated mesenchymal cells to become chondrocytes in the first stage of the BMP-induced heterotopic osteogenesis. The cartilage is replaced by bone in a manner similar to that in normal endochondral (indirect) ossification. It is suggested that the BMP induced-bone occurs through an endochondral-like ossification; however the cell differentiation patterns differ from those of the normal fetal endochondral ossification process. On the other hand, intramembranous (direct) ossification is observed in some cases. Therefore, we examined histopathologically the nature of the BMP induced heterotopic osteogenesis, that is, examining histological features that are more like bone than cartilage but whose cells were not distinguishable from chondrocytes. Round chondrocytes-like cells and smaller osteocyte-like cells coexisted in the chondroid bone matrix. Furthermore, there were some chondroid patterns that still remained in the maturing bone matrix showing mosaic patterns. These findings seems to be a third ossification pattern, "transchondroid bone formation", which was described by Yasui et al. (1997)[11)] in an experimentally distraction osteogenesis model in the rat. "Chondroid bone", a tissue intermediate between bone and cartilage, was formed mainly in BMP-induced heterotopic osteogenesis.

Key Words: transchondroid bone formation, endochondral ossification, intramembranous ossification, bone morphogenetic protein (BMP), heterotopic osteogenesis, chondroid bone

Introduction

Bone morphogenetic proteins (BMP) are factors in growth and differentiation and belong to the TGF-β superfamily. Typically, they induce immature mesenchymal cells to differentiate to form bone tissue when they are experimentally implanted in heterotopic sites. It has been stated that BMP induces undifferentiated mesenchymal cells to become chondrocytes in the first stage of the BMP induced heterotopic osteogenesis, which are subsequently replaced by bone in a similar process of normal endochondral ossification. On the other hand, it is known that BMP induces various patterns of osteogenesis according to the cell environment [1,2)]. Inoue et al. (1995[1)]; 1996[2)]) suggested that BMP induces bone formation through endochondral ossification but the cell differentiation patterns differs from those in the normal fetal endochondral ossification process. Furthermore, some researchers reported that BMP induces direct bone formation in implantation with some carrier (Sasano et al. 1993[3)]; Kuboki et al. 1995)[4)] . Kawakami et al. (1997)[5)] reported on the perichondral ossification pattern in heterotopic osteogenesis using squalane as a carrier. This perichondral ossification occurs in the periphery after chondrocyte differentiation.

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Materials and Methods

BMP used in the present investigation was prepared according to same method as in our previous examination (Kawakami et al. 1993)[6]. The epiphyseal ends of bovine long bones were cut away and the diaphyses were mechanically scraped clean of soft tissues and washed extensively in cold water. The washed bones were frozen in liquid N_2, ground to a particle size of 1-2mm^3, defatted in chloroform/methanol (1:1) and dried at room temperature. The bone particles were then demineralized in 0.6N HCl at 4°C for 72 hours and again washed extensively in water. The demineralized washed bone particles were chemically extracted first with 2M $CaCl_2$ for removal of proteoglycans (1 hour) and then with 0.5M EDTA for removal of soluble non-collagenous protein (1 hour). The obtained result was demineralized bone matrix gelatin. The BMP was extracted at room temperature for 24 hours from the bone matrix gelatin with an inorganic/organic solvent mixture of 0.5 M $CaCl_2$ in 4 M guanidine hydrochloride containing 1mM N-ethylmaleimide and 1mM benzamidine-HCl to protect BMP against endogenous degradative enzymes. The extract was filtrated and concentrated to limit the molecular weight of proteins to 10-100 KDa. Then, the supernatant solution was dialyzed against water at 4°C and allowed to stand overnight in a cold room for precipitate formation. The precipitate was collected by centrifugation at 40,000g for 60 minutes, washed in cold deionized water and lyophilized. The BMP-osteoinductive activity was performed in the muscle hindquarter assay in ddY mice, and then the precipitate was used as partially purified BMP.

Adult female ddY mice (Japan SLC Inc., Hamamatsu, Japan), weighing approximately 25 g each, were anesthetized with ether. The hindquarter skin was then disinfected. Gelatin capsules containing BMP (5 mg) were immediately implanted in femoral muscle pouches through a dorsal incision site. The animals were kept in plastic cages, and were euthanized at intervals of 3, 5, 7, 10, 14, and 21 days after the operation under general anesthesia with ether. The implant with surrounding tissue was removed and immediately fixed in 4% formaldehyde neutral buffer solution (pH 7) at room temperature. Radiographic examinations were made with Softex CMB equipment (Softex Co. Tokyo, Japan). Materials were then embedded in paraffin with or without demineralization by EDTA, and sections were stained with hematoxylin and eosin (HE), Toluidine blue (TB), and Mallory's azan (MA).

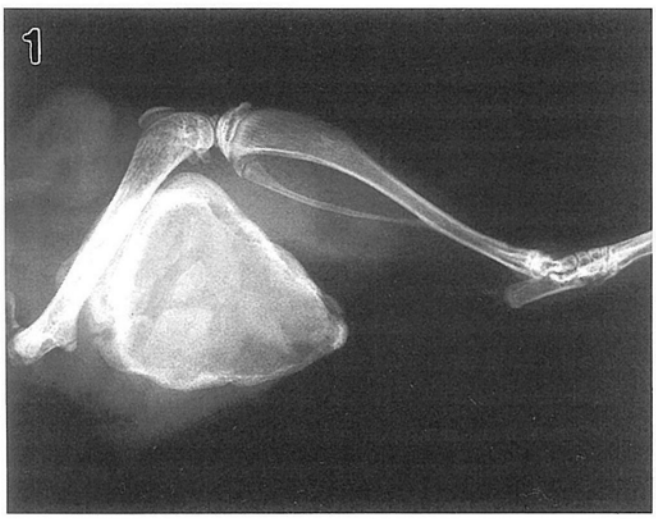

Fig. 1. Strong radiopaque region at the periphery suggesting direct perichondral bone formation (10-day specimen, × 2.5)

Results

Radiographic examination

There were no changes on the radiographs of 3, 5, and 7 day specimens. The radiographs of 10, 14, and 21 day specimens, however, showed bone-like radiopaque regions with comparatively clear demarcations (Fig. 1).

Histopathological examination

In 3-day specimens, the spindle shaped mesenchymal cells proliferated in the implanted site (Fig. 2). In 5 and 7 day specimens, the matrices were stained slightly by hematoxylin and eosin. Evidence of proliferation of undifferentiated cells having a light cytoplasm resembling poorly differentiated chondrocytes was seen at the periphery (Figs.3, 4). Within 10 days, perichondral ossification occurred, and the peripheral matrix of clusters of cartilage tissue changed to chondroid bone in connection with perichondral ossification sites (Fig. 5). The histopathological features were more like bone than cartilage, but the cells were not distinguishable from chondrocytes (Fig. 6) in 14-day specimens. Round chondrocytes-like cells and smaller osteocyte-like cells coexisted in forming the chondroid bone tissue. Furthermore, the phenomenon continually occurred within 14 days. In 21-day specimens, bone remodeling and colonization of the bone marrow were observed. However there were some chondroidal patterns (cartilage tissue with chondrocytes or chondroid tissues) that still remained in the trabecular bone tissue showing mosaic patterns (Fig. 7).

In histochemical examination, the perichondral ossification site was stained deeply blue by MA, although there were slightly stained blue areas in the chondral tissue region in 10 day specimens (Fig. 8). In 14 and 21 day specimens, mosaic patterns were observed in the chondro-osseous tissues. In general the periphery of the proliferated masses was stained

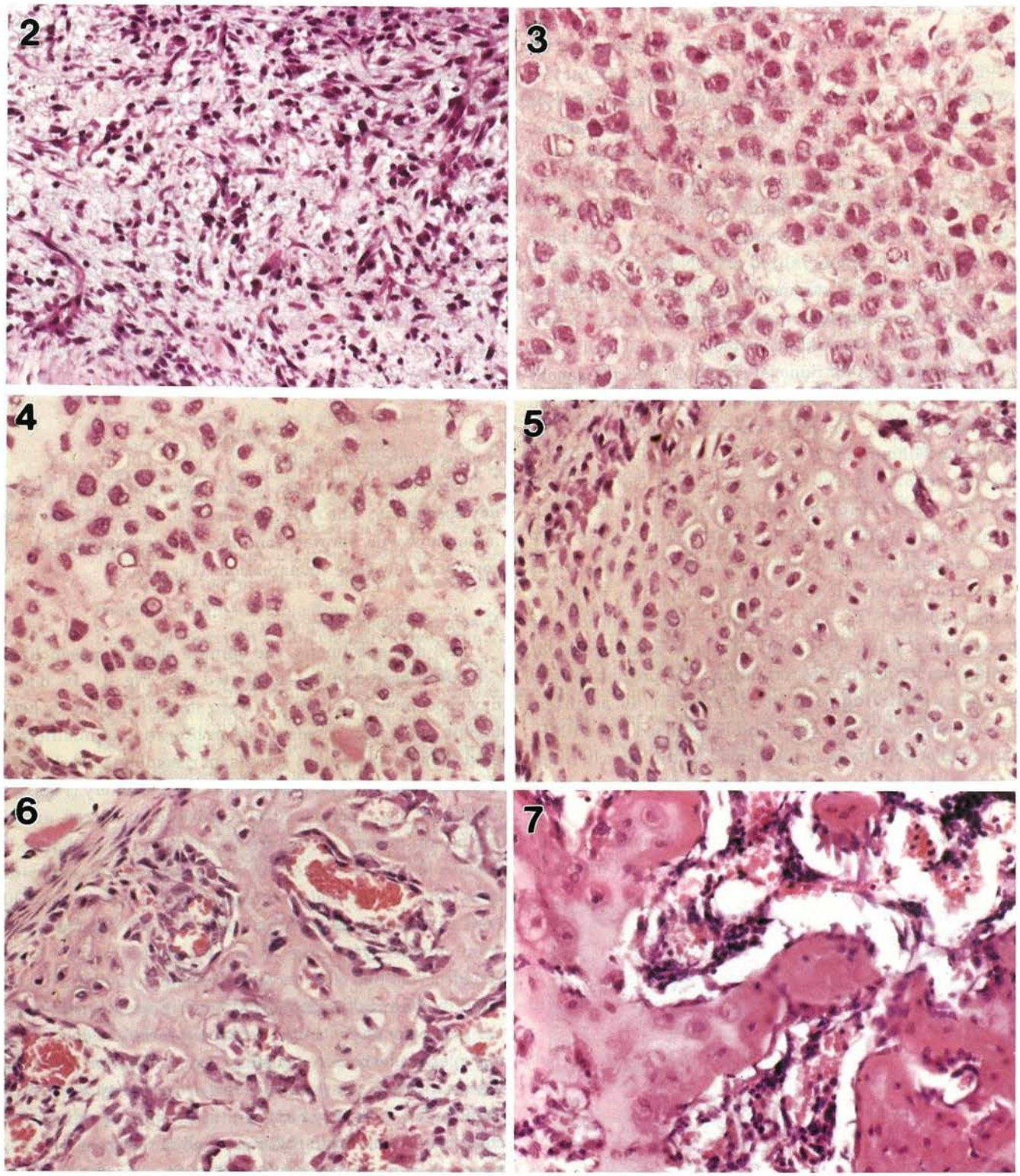

Fig. 2. Spindle shaped mesenchymal cell proliferation in the implanted site (3-day specimen, HE, × 250)

Fig. 3. Proliferation of poorly differentiated chondrocytes (5-day specimen, HE, × 250)

Fig. 4. Matrices were stained slightly among proliferated cells (7-day specimen, HE, × 250)

Fig. 5. Perichondral ossification and cluster of cartilage tissue (10-day specimen, HE, × 250).

Fig. 6. "Chondroid bone" containing round chondrocyte-like cells and osteocyte-like cells coexist (14-day specimen, HE, × 250)

Fig. 7. A chondroidal pattern still remained in the trabecular bone showing a mosaic pattern (21-day specimen, HE, × 250)

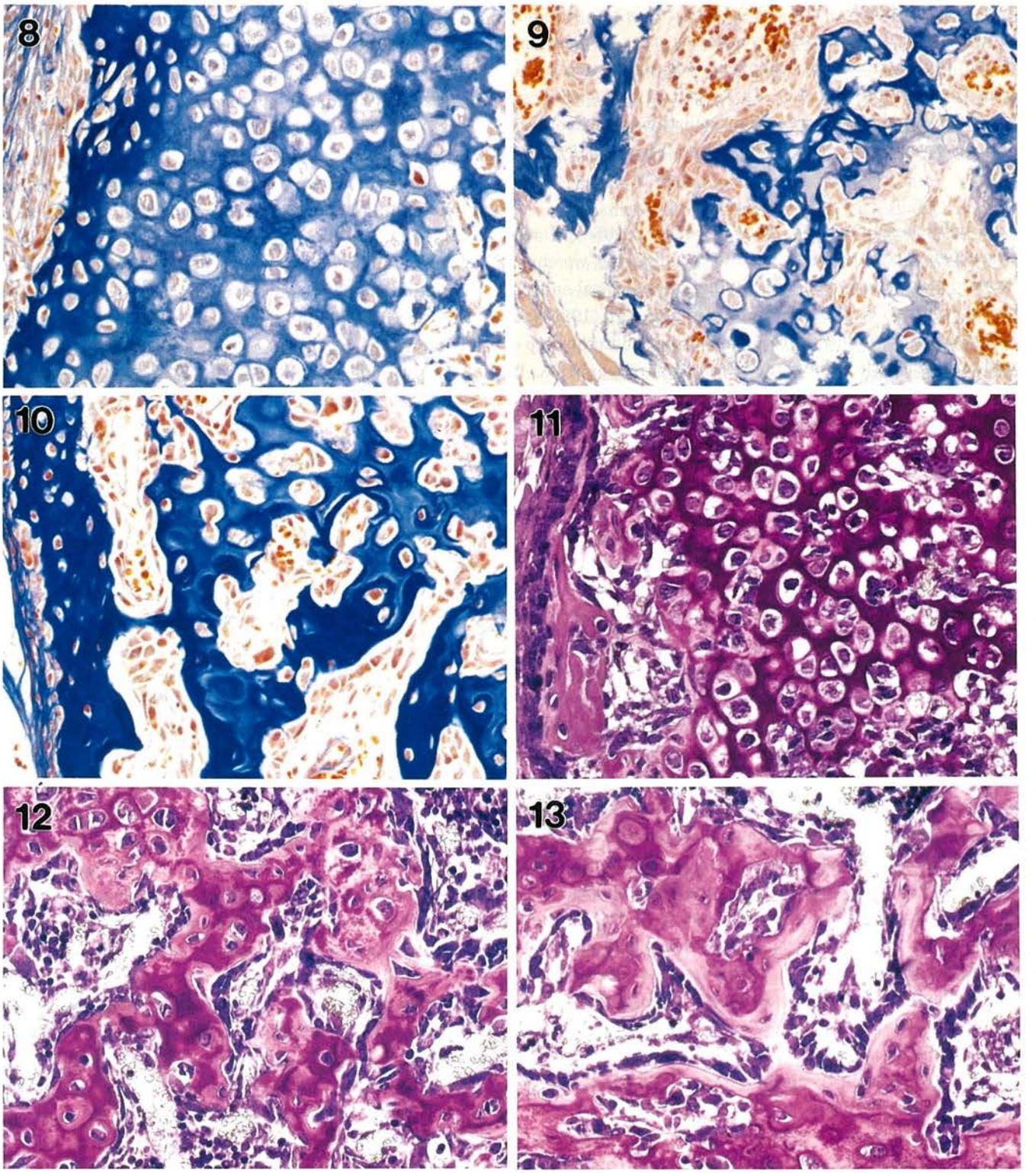

Fig. 8. The perichondral ossification site was stained deeply blue. (10-day specimen, MA, × 250)
Fig. 9. Mosaic patterns observed in the "chondroid bone". (14-day specimen, MA, × 250)
Fig. 10. The periphery of the proliferated mass was stained deeply blue. (21-day specimen, MA, × 250)
Fig. 11. Matrices of the chondroid tissue reacted to ortho-metachromasia. (10-day specimen, TB, × 250)
Fig. 12. Chondroid tissue reacted to ortho-metachromasia in the trabeculae. (14-day specimen, TB, × 250)
Fig. 13. "Chondroid bone" still remaining ortho-chromasia-reaction. (21-day specimen, TB, × 250)

deeply blue by MA (Figs. 9,10). According to specimens stained with TB, the matrices reacted to ortho-metachromasia, but the matrices of perichondral ossification sites did not show the reaction (Fig. 11). In 14 and 21 day specimens, some chondroid tissues reactions to ortho-metachromasia were observed in trabecular bone tissue (Figs. 12, 13).

Discussion

In published literature, BMP induces undifferentiated mesenchymal cells that develop into chondrocytes, which are replaced gradually by bone resembling physiological endochondral ossification. Furthermore, Kuboki et al. (1995)[4] reported that BMP induces direct bone formation in the case of implantation with porous particles of hydroxyapatite. In addition, we have stated that in heterotopic osteogenesis using squalane as a carrier in mice, perichondral ossification occurs after chondrocyte differentiation in the first phase (Kawakami et al. 1997)[5]. In this experiment, we considered that the clear demarcation of the newly formed bone tissue in the radiograph shows the occurrence of direct perichondral bone formation. As mentioned in the literature, it had been thought that there were two types of ossification modes: intramembranous (direct) and endochondral (indirect) in BMP-induced heterotopic osteogenesis.

Previously in the course of BMP-induced experimentally heterotopic osteogenesis, Nagai et al. (Nagai et al. 1995)[7]; Inoue et al. 1996[2]; Nosaka et al. 1996[8]) examined the expression of bone matrix proteins and their mRNA by immunohistochemical and in situ hybridization techniques, and considered that BMP induces chondro-osseous tissue through a process like that in the endochondral ossification mode, but the matrix components and cell differentiation patterns differ from those in the normal fetal endochondral ossification process. Moreover, Sasano et al. (1993)[3] have reported that BMP-induced cartilage which contained both types I and II collagen. Chondrocytes with large lacunae were randomly distributed in an irregularly calcified matrix. They could be classified as chondroid tissue rather than hyaline cartilage because of the immunohistochemical characteristics. "Chondroid tissue" is an intermediate tissue between cartilage and bone.

We have examined the expression of transforming growth factor beta 1 peptide and its mRNA in chondrocytes in the early phase of the BMP-induced heterotopic osteogenesis in mice. The expression patterns, both the peptide and mRNA, were different from those of normal chondrocytes in the endochondral ossification tissue (Kawakami et al. 1997[9]; Takei et al. 1997[10]). Therefore, we have also concluded that the BMP-induced heterotopic osteogenesis differs from normal endochondral osteogenesis, especially in the early phases.

Physiological ossification is usually classified into two types: intramembranous (direct) and endochondral (indirect). Intramembranous ossification is typically observed during embryonic development of the cranial vault, but at numerous other sites where there is no pre-existent cartilage model, new bone is directly formed by differentiated osteoblasts. Typical endochondral ossification is observed in the course of embryonic development of long bones. New bone formation may be regarded as endochondral when cartilage is formed first and later replaced by new bone. In the physical growth plate, for example, highly ordered structures of resting, proliferating, hypertrophic, and calcifying cartilage are first established by differentiating chondrocytes. The calcified cartilage matrix is then invaded by capillaries and new bone is formed by osteoblasts in the space previously occupied by hypertrophic chondrocytes. The fate of hypertrophic chondrocytes in calcifying cartilage remains controversial. Some authors believe that all the cells degenerate or are programmed to die, but others think that they survive to became osteoblasts under the influence of vascularization.

Yasui et al. (1997)[11] reported a "third" ossification mechanism producing"chondroid bone". This has not yet attracted much attention, although it was identified as an intermediate tissue between cartilage and bone some time ago. Previously some hypertrophic chondrocytes were described as undergo further differentiation into osteoblast-like cells and participate in initial bone formation[12, 13]. Furthermore, it was reported that chondrocyte-like cells and osteocyte-like cells coexisted in the chondroid bone with no clearly distinguishable boundary (Yasui et al. 1984)[14]. According to investigations of BMP-induced heterotopic bone tissue, Nagai et al. (1995)[7] suggested that chondrocyte-like cells demonstrate the two-phase function of chondrocytes and osteoblasts, and inductive cartilage tissues were classified as chondroid tissue rather than normal cartilage; chondroid tissue is an intermediate tissue between the cartilage and bone (Sasano et al. 1993)[2], which is named "chondroid bone" or "chondro-osseous tissue" (Inoue et al. 1996)[2].

Based upon the above discussion, the BMP-induced heterotopic osteogenesis was thought to be the third mode of ossification: "transchondroid bone formation" (Yasui et al. 1997)[11]. Our results and other published data indicated that the third ossification mode, "transchondroid bone formation", was chiefly displayed in the BMP-induced heterotopic osteogenesis. That is, the histopathological features of newly formed bone showed "chondroid bone" containing is-

lands of cartilage remnants and expression of both type I and II collagen in the same chondroid cells appearing in the early phase of immunohistochemical studies (Sasano et al. 1993[3]; Inoue et al. 1996[2]; Kimura et al. 1998[15]) and in situ hybridization (Nagai et al. 1995)[7] examinations.

Further histochemical, immunohistochemical and in situ hybridization evaluations of the "transchondroid bone formation" displayed experimentally in the BMP-induced heterotopic osteogenesis are in progress, and the results will be reported on in the near future.

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