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REVIEW

Immunohistochemistry of BMP-Induced Heterotopic Osteogenesis

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Abstract: Previously, our research group reported that the third ossification mode, "transchondroid bone formation", was chiefly displayed in the BMP-induced heterotopic bone formation model[1-3]. In this present paper, I describe the following: the histopathological and immunohistochemical features of BMP-induced heterotopic osteogenesis model in mice in the early phase of osteogenesis; and the localization of typical matrix proteins of cartilage and bone - type I and type II collagen, osteocalcin, and osteopontin - especially in the "chondroid bone matrix". Furthermore, I describe the expression of TGF-β peptide and its mRNA.

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

Introduction

Practical applications, such as for use in oral and maxillofacial surgery, are anricipated for some BMPs. In fact, successful repair of maxillofacial and other ossous defects has already been accomplished using BMP. One day, BMP may be synthesized in quantities large enough for regular clinical use.

In general, it has been stated that BMP, when implanted in heterotopic sites, induces undifferentiated mesenchymal cells to become chondrocytes in the first stage. These cells are replaced by bone in a manner similar to that of physiological endochondral (indirect) ossification. Although it has been suggested that BMP-induced bone occurs through endochondral-like ossification, the cell differentiation patterns differ from those in the physiological normal endochondral ossification process[5,6]. On the other hand, intramembranous (direct) ossification has also been observed in some cases[7,8]. We have previously described the occurrence of perichondral ossification, or, direct bone formation, observed histologically[9]. In those experiments squalane was used as a carrier. Perichondral ossification occurred in the periphery after chondrocyte differentiation.

There are two types of ossification modes in BMP-induced heterotopic osteogenesis: intramembranous (direct) and endochondral (indirect). However, the nature of heterotopic osteogenesis has not been clearly detailed. Therefore, I will discuss the nature of BMP-induced heterotopic osteogenesis in light of histopathological and immunohistochemical examinations.

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Examination Model

BMP used in the present manuscript was prepared according to same method as in our previous examination (Kawakami et al. 1993)[10]. Adult male 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 partially purified BMP 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 examined using histopathological (hematoxylin-eosin: HE), histochemical (Toluidine blue: TB, Mallory's azan: MA), immuno- histochemical (collagen type I, II, osteocalcin, osteopontin, TGF-β), and *in situ* hybridization (TGF-β mRNA) techniques.

Histopathology and Histochemistry

Histopathologically, in 3-day specimens, spindle-shaped mesenchymal cells proliferated in the implantation site. In 5- and 7-day specimens (Fig. 1), the matrices were stained slightly by HE. Evidence of proliferation of undifferentiated cells having

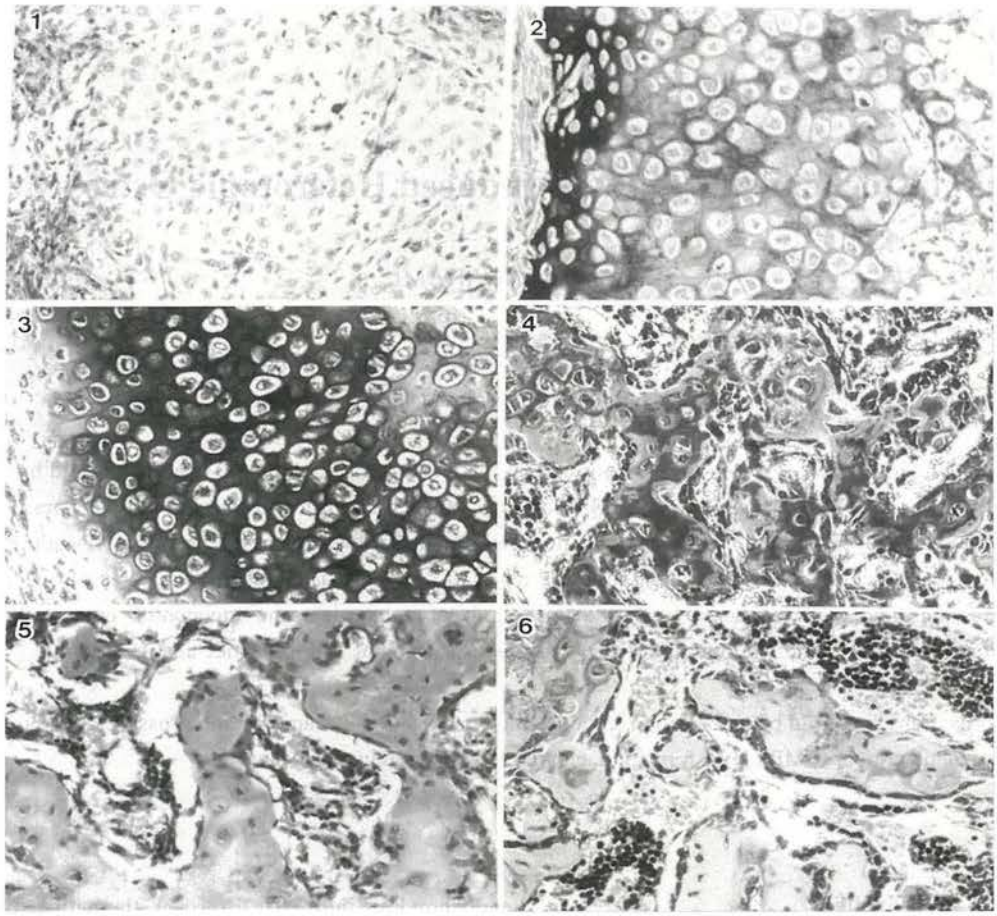

Fig. 1. Matrices were stained slightly among poorly differentiated proliferation cells. (7-day specimen, HE, ×250)
Fig. 2.The perichondral ossification site is stained deeply blue. (10-day specimen, MA, ×250)
Fig. 3. Peripheral matrices do not demonstrate ortho-metachromasia. (10-day specimen, TB, ×250)
Fig. 4.Chondroid tissue reacted to ortho-metachromasia in the trabeculae. (14-day specimen, TB, ×250)
Fig. 5. A chondroidal pattern still remained in the trabecular bone showing a mosaic pattern. (21-day specimen, HE, ×250).
Fig. 6. Ortho-metachromasia-reaction still remaining in "chondroid bone". (21-day specimen, TB, ×250)

light cytoplasm resembling poorly differentiated chondrocytes can be seen at the periphery.

Within 10 days, perichondral ossification had occurred, and the peripheral matrix of the cluster of cartilage tissue had changed to chondroid bone in connection with the perichondral ossification sites. In the histochemical examination, the perichondral ossification site was deeply stained blue by MA, although there were slightly stained blue areas in the chondral tissue region in 10-day specimens (Fig. 2). A 10-day specimen, stained with TB, shows matrix staining that demonstrates ortho-metachromasia, but the matrices of perichondral ossification sites do not show this (Fig. 3). In 2- and 3-week specimens, some chondroid tissues that displayed ortho-metachromasia were observed in the trabecular bone tissue. The Figure 4 shows a 2-week specimen.

The histopathological features are more like those of bone than those of cartilage, but the cells were not distinguishable from chondrocytes in 2-week specimens. Round chondrocyte-like cells and smaller osteocyte-like cells coexisted in the forming chondroid bone tissue. Furthermore, within 2 weeks, the phenomenon continually occurred.

In 3-week specimens, bone remodeling and colonization of the bone marrow were observed. However, there was a chondroidal pattern (cartilage tissue with chondrocytes or chondroid tissues) that still remained in the trabecular bone tissue, and it occurred in a mosaic fashion (Fig. 5), which was clearly observed by TB (Fig. 6).

Immunohistochemistry

Previously, in studying BMP-induced experimental heterotopic osteogenesis, Nagai et al.[11] examined the expression of bone matrix proteins and their mRNA by immunohistochemical and *in situ* hybridization techniques, and stated that BMP induces the chondro-osseous tissue through a process like that occurring in the endochondral ossification mode. However, they indicated that the matrix components and cell differentiation patterns differed from those found in the normal fetal endochondral ossification process.

Therefore, I will present some immunohistochemical results. Figure 7 show immunostaining for type-I collagen of a 7-day

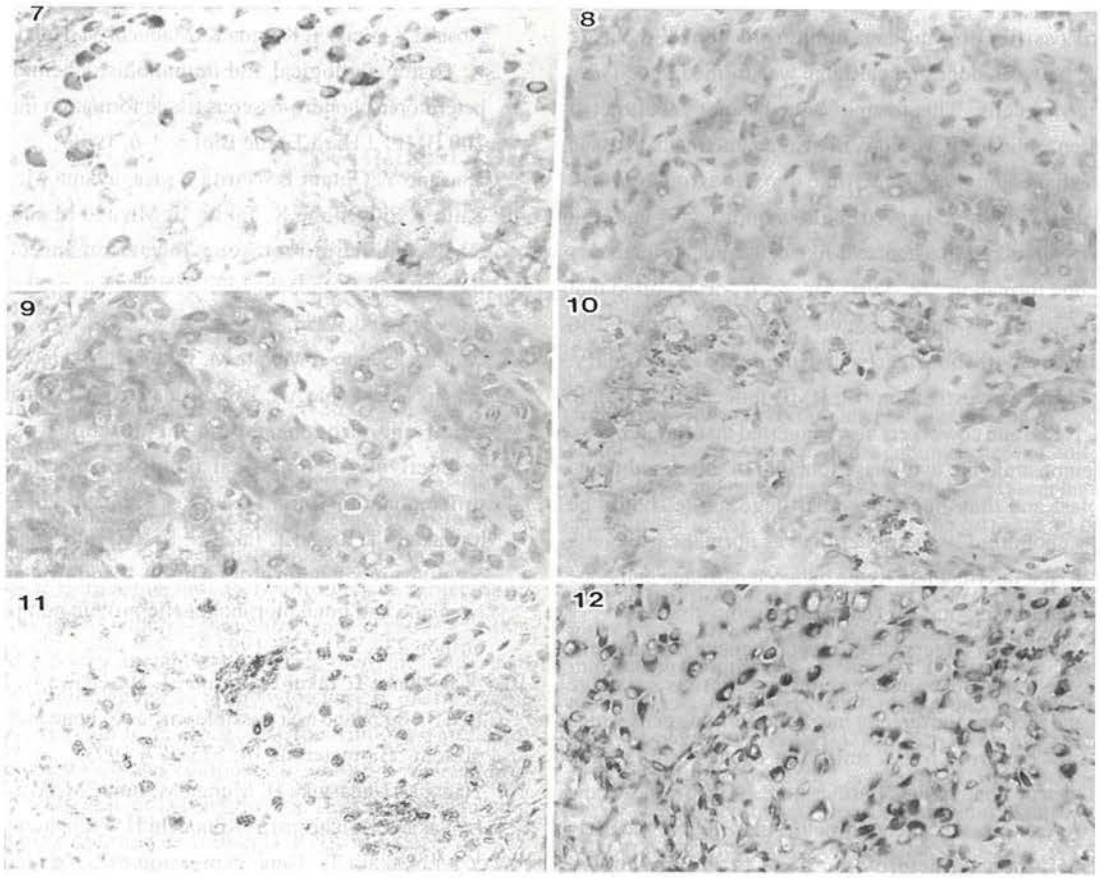

Fig. 7. Products of staining reaction are observed in the cells. (7-day specimen, Type I collagen, ×250)
Fig. 8. Positive staining is distributed in the matrices. (7-day specimen, Type II collagen, ×250)
Fig. 9. Immunostaining reactions are present in the matrices. (7-day specimen, Osteocalcine, ×250)
Fig. 10. Positive reactions are detected weakly in the matrices. (10-day specimen, Osteopontine, ×250)
Fig. 11. TGF-β peptide detected in some chondrocyte-like cells. (7-day specimen, TGF-β, ×250)
Fig. 12. The mRNA expression was apparent in some chondrocyte-like cells. (7-day specimen, TGF-β mRNA, ×250)

specimen. Type-I collagen is one of the bone matrix proteins. The products of the staining reaction are seen in the chondral cells. Type-II collagen, which is a typical matrix protein of cartilage tissue, is present in the chondral tissue of both the 7-day specimen (Fig. 8). The positive staining is distributed in the bone matrix. The results thus show that BMP induced cartilage that contained both type I and type II collagens. Chondrocytes with large lacunae were randomly distributed in an irregularly calcified matrix. This tissue could be classified as "chondroid bone" rather than hyaline cartilage because of its characteristics. "Chondroid bone" is an intermediate tissue between cartilage and bone.

Osteocalcin is another typical bone matrix protein. These specimens were immunostained for osteocalcin. A 7-day specimen is shown in Figure 9. Positive staining was observed. In particular, especially osteocalcin-positive proliferating cartilage cells were clearly observed. Therefore, once again, the tissue would appear to be "Chondroid bone", an intermediate tissue between cartilage and bone, with the expression patterns of osteopontin which are different from those of normal tissues (Fig. 10).

Next, I examined the immunohistochemical localization of TGF-β. This is one of the best known growth factors, and it is closely related to bone formation. In general, in the course of endochondral ossification, TGF-β appears in the stage of final differentiation to osteoblasts. It is detectable in chondrocytes and matrices in this 7-day specimen (Fig. 11). The 2-week specimen also shows its appearance in osteoblasts and matrices. So it is detected in various cells proliferating during the course of bone formation. Figure 12 shows TGF-β-mRNA in chondrocytes in a 7-day specimen, by *in situ* hybridization. The expression patterns of both the peptide and mRNA were different from those of normal chondrocytes in the endochondral ossification tissue. Therefore, we can conclude that BMP-induced heterotopic osteogenesis differs from normal endochondral osteogenesis in terms of TGF-β distribution.

Discussion

Previously, in the journal "Bone and Joint Surgery", Yasui and co-workers reported a "third" ossification mechanism

producing"chondroid bone"[12]. In the paper, it was reported that in the third ossification mode, "chondroid bone", a tissue intermediate between bone and cartilage was formed directly by chondrocyte-like cells. This form of bone has not yet attracted much attention, although it was identified as an intermediate tissue between cartilage and bone some time ago. Previously it had been described that some hypertrophic chondrocytes undergo further differentiation into osteoblast-like cells and participate in the initial bone formation[13-15]. Furthermore, it was reported that chondrocyte-like cells and osteocyte-like cells coexisted in chondroid bone with no clearly distinguishable boundary. According to their investigations on BMP-induced heterotopic bone tissue, Nagai and coworkers also suggested that chondrocyte-like cells demonstrate the two-phase function of the chondrocyte and osteoblast and that the induced cartilage tissue should be classified as chondroid tissue rather than as normal cartilage[11]. In their paper, the intermediate tissue between cartilage and bone was called "chondroid bone" or "chondro-osseous tissue".

Although there is no direct evidence yet, I believe that the cells involved in "chondroid bone" temporally express cartilage phenotypes and then change directly into bone-forming cells which survive in the "condroid bone" untill the tissue resorped and remodelled by true bone tissue. Furthermore, TGF-β peptide and its mRNA were expressed in chondrocyte-like cells in the early phase of BMP-induced heterotopic bone formation model. Therefore, I believe thatTGF-β peptide might have some role in the chondrocyte-like cells undergoing further differentiation into cells showing bone forming phenotypes[17].

In conclusion, we view BMP-induced heterotopic osteogenesis to reflect Yasui's third mode of ossification, "transchondroid bone formation", based on our examination results and other published data.

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