Histological evaluation of induced new bone formation by crude BMP

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Abstract: Crude bovine BMP was implanted into the thigh muscle pouch of mice. Tetracycline was injected 1 week and calcein was injected 2 weeks after implant. At 3 weeks after implant, the mice were sacrificed and reviewed histologically. Coexistence of calcified bone and osteoid was observed in Villanueva Goldner stained sections. Toluidine blue staining demonstrated cartilaginous matrix and adjacent locus, and spherical cells varying in shape and size were observed. Calcified lamellar bone was present in the border, and osteoid with osteoblast-like cells was found on the bone marrow side. Calcein labeling appeared as a strong line in the margin and was definitely observed as weak fluorescence in the center under fluorescence microscopy. These results suggest the presence of ossification mode different from the intramembranous and endochondral ossification modes.

Key words: crude BMP, ossification, histological evaluation

Introduction

Heterotopic bone formation induced by bone morphogenetic protein (BMP) is generally considered to follow an endochondral ossification process. However, some studies have demonstrated intramembranous ossification when certain carriers are used. Furthermore, recent reports advocate a third mode of calcification, termed “transchondroid ossification”. In the present study, we examined the modes of ossification in heterotopic bone formation induced by a crude BMP preparation.

Materials and Methods

Bone preparation

BMP was extracted by the following procedures. A block of bovine bone of approximately 1 mm^3 was pulverized. The powder was decalcified with 0.6 N hydrochloric acid, and treated with calcium chloride and EDTA. Protein from the decalcified bone was extracted using 6 M urea. The water insoluble fraction was collected and crude purification was conducted according to the method of Urist.

Animal experiment

Five week-old male Std. ddy mice were used. With the animal under Nembutal anesthesia, 5 mg of the BMP extract packed in a gelatin capsule was implanted between the fascias in the femoral region. Fluorescent-labeled tetracycline (25 mg/kg) was injected intraperitoneally (i.p.) after one week of implantation and calcein (15 g/kg) was injected i.p. after two weeks. The animals were sacrificed at three weeks after implantation.

Histologic Study

Non-calcified sections were prepared by the following procedures. The new bone formation site was removed and immediately fixed in 70% alcohol. After fixing and staining in Villanueva bone stain (VB) solution, the tissue block was embedded in methyl methacrylate. Ground sections less then 10 μm in thickness were prepared and stained with Villanueva Goldner (VG) stain and toluidine blue O (TB).

Laser scanning microscopy

Labeling was examined with a confocal laser scanning microscope LSM 410 (Carl Zeiss Inc.) at wavelengths 543 and 488 nm, using LP-515 and BP510-525 filters.

Results

In the labeling studies, no tetracycline labeling was observed, while calcein labeling was observed as a clearly defined line in the border, but as mottled labeling with variable intensity in the center (Figs. 1 and 2). In VG-stained section, immature matrix and undifferentiated mesenchymal stem cells were observed scattered in the central region. Early-stage lymphocytic bone marrow-like tissue was observed in the transitional region adjacent to the cartilage, calcified lamellar bone was evident in the border, and many osteoids with osteoblast-like cells are present on the bone marrow side. Most of the osteoblast-like cells could be classified as cuboidal or intermediate type according to Villanueva. Hematopoietic cells were observed in the bone marrow formed between the bone trabeculae, which partially became fatty marrow (Fig. 3). In the region adjacent to the cartilaginous matrix, coexistence of calcified bone and osteoid was observed, and cells morphologically different from the ovoid osteocyte-like cells were also found at this site (Fig. 4). At the border, the calcification front is evident at the boundary between the osteoid and calcified bone, but the calcification front is not clear in regions with a mixture of osteoid and calcified bone. In the TB-stained sections, a calcification front was observed between osteoid and calcified bone in the border, but the calcification front was not clear in areas with a mixture of osteoid and calcified bone (Figs. 5 and 6).

Discussion

Heterotopic bone formation induced by BMP is generally considered to proceed via an endochondral ossification process, in which cartilage is formed preceding bone formation and then replaced by bone tissue as a secondary step. Recently, however, other ossification modes have been reported. One of them is direct ossification, in which osteoblasts directly form bone matrix and become embedded in the matrix transforming into osteocytes. Another mode has been termed the third ossification mode, and includes “transchondroid ossification” in which “chondroid” tissue with intermediate characteristics between bone and cartilage is formed first and is subsequently replaced by bone tissue. Although

some studies have proposed that the different ossification modes are probably due to effects of the carrier, transchondroid ossification has been observed irrespective of the type of carrier. Sasano et al. used fibrous collagen membrane as carrier and observed cartilage with matrix expressing both type I and type II collagen. Furthermore, Kimura et al. used gelatin capsule as carrier and observed immunoreactivity for type I and type II collagen in the cytoplasm of chondrocyte-like cells and the surrounding cartilaginous matrix on the 10th postoperative day. They confirmed that these cells possess characteristics of both cartilage and bone, and named them chondroid bone-forming cells. The above data indicate that chondroid cells possessing characteristics of both chondrocyte and osteocyte participate in ossification different from the physiological endochondral ossification.

The heterotopic bone tissue induced by our crude BMP preparation showed only calcein labeling, indicating that calcification did not occur in the first week but started at the second week. The labeling pattern was a strongly stained line in the border and mottled staining with uneven intensity in the center, suggesting different modes of ossification. In the border, osteoblast-like cells with an active morphology were observed not adjacent to cartilaginous matrix, and TB staining demonstrated a clear calcification front, suggesting ongoing process of direct ossification. In the central region where osteoid co-existed with calcified bone, cells resembling chondroid bone-forming cells, which were morphologically different from the ovoid osteocyte-like cells, were found adjacent to cartilaginous matrix, suggesting ossifucatuib by the transchondroid mode advocated by Yasui et al5). These findings suggest that heterotopic bone formation induced by BMP proceeds not only by a single ossification process, but via at least three ossification modes. However, the BMP used in the present study was a crudely purified preparation. The results may not be the same when synthetic human BMP is used. Further studies are required to examine the relation of various ossification modes with cytokines such as LANKL2.

References

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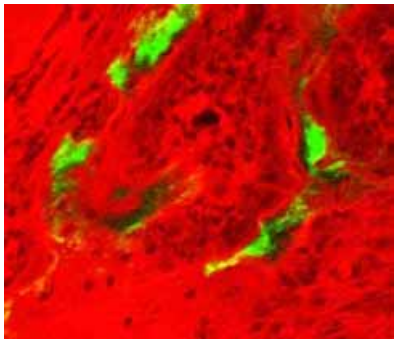

Fig. 1: Clearly demarcated line of calcein labeling can be observed at the superficial region of the heterotopic bone tissue

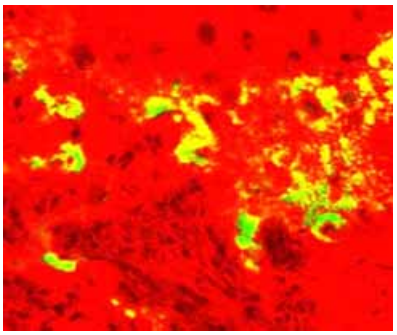

Fig. 2: Mottled calcein labeling with unclear demarcation can be observed in the central region of the heterotopic bone tissue.

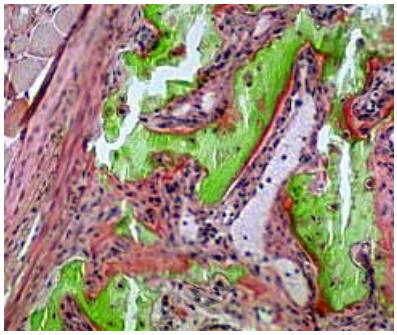

Fig. 3: At the border, bony matrix is observed, and osteoid and active osteoblast-like cells can be seen on the bone marrow side. O: osteoid, C: calcified bone

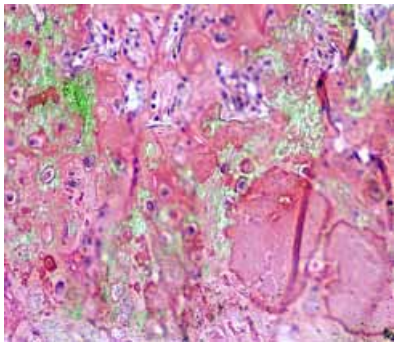

Fig. 4: In the central region, bony matrix and osteoid co-exist and cells morphologically different from the ovoid osteocyte-like cells are also present.
→: cells morphologically different from the ovoid osteocyte-like cells

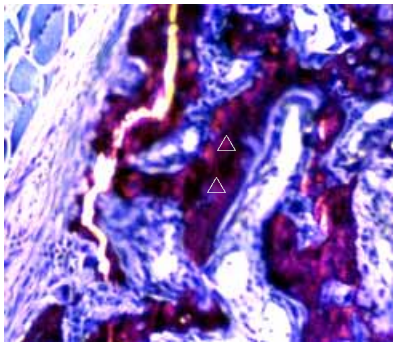

Fig. 5. At the border, the calcification front is seen at the boundary between osteoid and calcified bone. △: calcification front

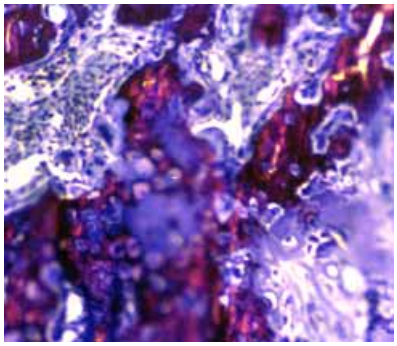

Fig. 6. In the central region where a mixture of bony matrix and osteoid co-exists, the calcification front is not sharp.