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Osteoclast differentiation in ectopic bone formation induced by recombinant human bone morphogenetic protein 2 (rhBMP-2)

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Abstract Osteoclast differentiation in the process of ectopic bone formation induced by recombinant human bone morphogenetic protein 2 (rhBMP-2) was examined to clarify the relationship between osteoclast development and rhBMP-2-induced bone formation. A combination of rhBMP-2 with a porous microsphere (PMS) and blood clot was implanted subcutaneously on the bilateral chest muscles of rats. Tartrate-resistant acid phosphatase (TRAPase) activity, cathepsin K (cath K), and calcitonin receptor (CTR), as markers of osteoclasts and their precursors, were examined using enzyme and immunohistochemical analysis up to 7 days after implantation. Mononuclear cells positive for TRAPase, cath K, and CTR first appeared on day 3 in connective tissue surrounding the PMS after implantation of rhBMP-2. Simultaneously, alkaline phosphatase activity became detectable in mesenchymal cells in the connective tissue. Electron microscopy demonstrated some mononuclear cells with abundant mitochondria and poorly developed rough endoplasmic reticulum in the proximity of mesenchymal cells. However, there was no evidence of cartilage or bone matrix formation on day 3. Osteoclasts in various stages of development, classified by the pattern of immunoreactivity for cath K, were observed by day 7. The polarized intracellular distribution of cath K was found only in osteoclasts attached to bone matrix. In conclusion, we have demonstrated for the first time the appearance of osteoclast precursors before bone matrix formation induced by rhBMP-2, suggesting that bone matrix is not a prerequisite for osteoclast precursor recruitment. Furthermore, we suggest that differentiation into polarized functional osteoclasts is accomplished when the osteoclasts attach to the bone matrix.

Key words rhBMP-2 · osteoclast precursor · ectopic bone formation

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Introduction

Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-β superfamily, are known to be among a number of factors that influence osteoblast differentiation and induce rapid bone formation [1]. Recombinant techniques have made BMPs accessible for biological and preclinical evaluation, and it has been well documented that local administration of recombinant human BMP 2 (rhBMP-2) induces ectopic bone formation [2] and improves the healing of fractures and various bone defects in animals [3–6]. It is also demonstrated that rhBMP-2 stimulates bone formation by human bone marrow-derived stromal cells in vivo [7]. Accelerated osteoblast differentiation and an increased rate of bone formation at an early stage of osteogenesis and osteoregeneration have been demonstrated in these experiments. In ectopic bone formation induced by rhBMP-2, rapidly induced bone matrix was remodeled to become highly mineralized mature bone, a process resembling that seen in normal bone development [8]. Also, this process is inhibited by bisphosphonates [9].

Because osteoclastic bone resorption is indispensable for bone remodeling and it is well established that osteoclast differentiation and activation occur with the support of stromal/osteoblastic cells [10–12], osteoclast differentiation and bone resorption in ectopic bone formation must also be harmonized with osteoblast differentiation and bone formation. In this context, it is worthwhile to investigate the possible role of rhBMP-2 in osteoclastic differentiation and bone resorption [13–15]. Indeed, there have been several reports concerning the influence of BMPs on the differentiation of osteoclasts and their ability to resorb bone. Kaneko et al. [16]

have demonstrated type IA and II BMP receptors in highly purified mature rabbit osteoclasts. Itoh et al. [17] reported that BMP-2 enhances the survival of purified osteoclasts. However, most of these reports focusing on the effect of rhBMP-2 on osteoclasts were examined in vitro; there have been few reports focusing on in vivo osteoclast development during ectopic bone formation induced by rhBMP-2. In this study, therefore, histological, histochemical, and ultrastructural means were used to examine osteoclast differentiation in the process of ectopic bone formation induced by subcutaneous implantation of rhBMP-2 in rats.

Materials and methods

Forty male, 5-week-old Long Evans rats were used. In the experimental group, 20 μg rhBMP-2 (Yamanouchi Pharmaceutical, Tokyo, Japan) with 200 μl carrier, consisting of a blood clot and porous microsphere (PMS) made of polylactic acid-polyglycolic acid copolymer, was implanted subcutaneously on the bilateral chest muscles. A number of animals were killed every day for up to 7 days after implantation, and the specimens were prepared as described below. In the control group, only the carrier was implanted subcutaneously.

Anesthetized animals were perfused through the left ventricle first with Ringer's solution, and then with an aldehyde mixture. The implants were removed en bloc with the underlying muscles and ribs. The specimens were immersed in the same fixative for 2 h at 4°C, followed by decalcification with 10% ethylenediammetetraacetic acid (EDTA) for 1 week at 4°C.

For histological and histochemical observation, a 4% paraformaldehyde and 0.1% glutaraldehyde mixture in 0.1 M cacodylate buffer (pH 7.4) was used as the fixative. The specimens were trimmed and embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany), paraffin or Tissue-Tek (Sakura Finetechnical, Tokyo, Japan). Technovit 8100-embedded 2-μm sections were used for the detection of tartrate-resistant acid phosphatase (TRAPase) activity. Localizations of cathepsin K (cath K) and calcitonin receptor (CTR) were detected immunohistochemically in the paraffin sections. The localization of alkaline phosphatase (ALPase) activity was determined by enzyme histochemical analysis using the azo-dye method with cryostat sections embedded with Tissue-Tek.

For immunohistochemical detection of cath K, the paraffin sections were treated with 0.3% hydrogen peroxide in methanol for 30 min followed by incubation with PBS containing 1% bovine serum albumin for 1 h. Then, the specimens were incubated with primary antibody, mouse antihuman cath K monoclonal antibody (F-92; Fuji, Takaoka, Japan), at a dilution of 1:100 in PBS for 3 h. The sections were then incubated with biotin-conjugated antimouse IgG followed by horseradish peroxidase (HRP)-conjugated streptavidin from a Histofine SAB-PO(M) kit (Nichirei, Tokyo, Japan) for 30 min each. Immunoreactivity was detected by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3′-diaminobenzidine and 0.002% hydrogen peroxide. For CTR immunohistochemistry, rabbit antirat CTR antiserum [18] (the courtesy of ASAHI KASEI, Shizuoka, Japan) at a dilution of 1:50 in PBS was used as the primary antibody, and HRP-conjugated antirabbit IgG at a dilution of 1:100 in PBS was used as the secondary antibody. All incubations were conducted at room temperature. Use of PBS instead of primary antibodies demonstrated non specific reactions in tissues.

For fine structural observation, a 2% paraformaldehyde and 2.5% glutaraldehyde mixture in 0.06 M cacodylate buffer (pH 7.4) was used as the fixative. Decalcified specimens were dissected and fixed with osmium tetroxide followed by dehydration in an ethanol series and embedded in Epok 812. Then, ultrathin sections were prepared for observation under a transmission electron microscope.

Results

In the control group, in which only the carrier was implanted, bone formation and osteoclast differentiation were not observed during the entire experimental period. Therefore, we mainly describe the events observed in the BMP-PMS-implanted animals, especially at the interface between the implanted BMP-PMS and underlying muscles, because this was the area in which initial ectopic bone formation appeared in the experimental model.

The histological events that occurred at the interface between the implanted BMP-PMS and the underlying muscles were as follows. On day 1, a thin layer of connective tissue was observed bordering the implanted PMS and the chest muscle fibers (Fig. 1a). By day 3, the intervening connective tissue layer had been thickened with a number of mesenchymal cells and a fibrous matrix (Fig. 1b). The cartilage matrix was first obvious at day 4 in the connective tissue between the implanted PMS and muscle fibers (Fig. 1c). The bone matrix appeared, mostly around the cartilage, at day 5 (Fig. 1d). By day 7, the bone matrix grew larger and occupied the major area of intervening connective tissue between the implanted PMS and the chest muscle.

Histochemically, on day 1, migratory round cells were found in the thin connective tissue layer; however, they did not show TRAPase activity (Fig. 2a). Mononuclear cells positive for TRAPase activity first appeared at day

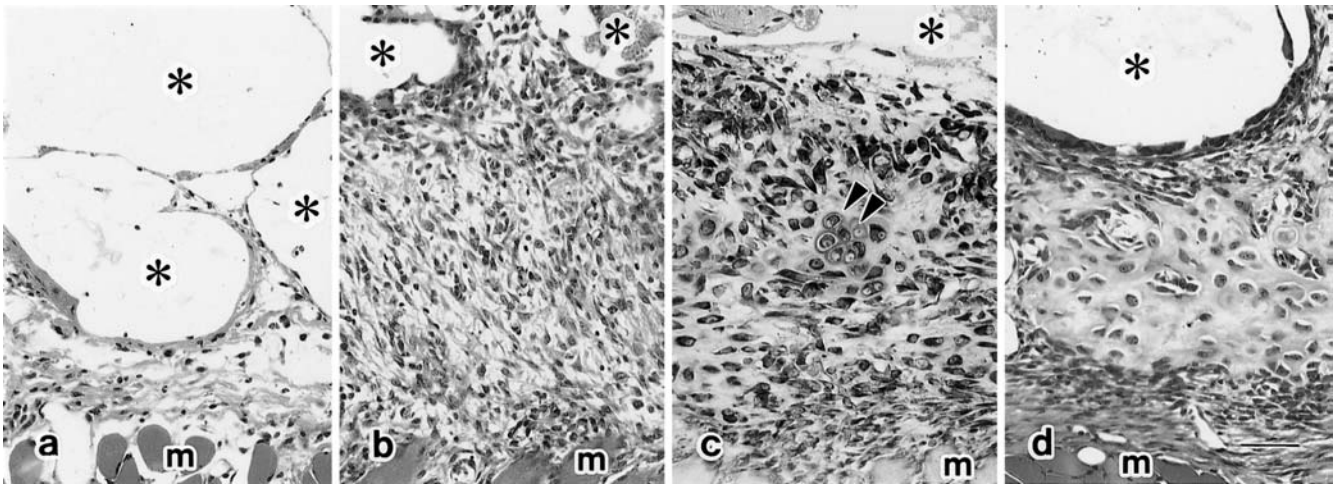

Fig. 1. Light micrographs illustrate changes in connective tissue intervening between implanted porous microsphere (PMS) (*asterisks*) and chest muscle fibers (*m*) in bone morphogenetic protein (BMP)-PMS-implanted animals. **a** Thin layer of connective tissue intervening between the implanted PMS and the chest muscle at day 1. **b** Connective tissue layer thickened by day 3 after implantation of BMP-PMS. **c** First appearance of cartilage (*arrowheads*) developed in the connective tissue layer at day 4. **d** Bone matrix is obvious at day 5. **a,b,d** Hematoxylin and eosin staining; **c** toluidine blue staining. *Bar* 50 μm

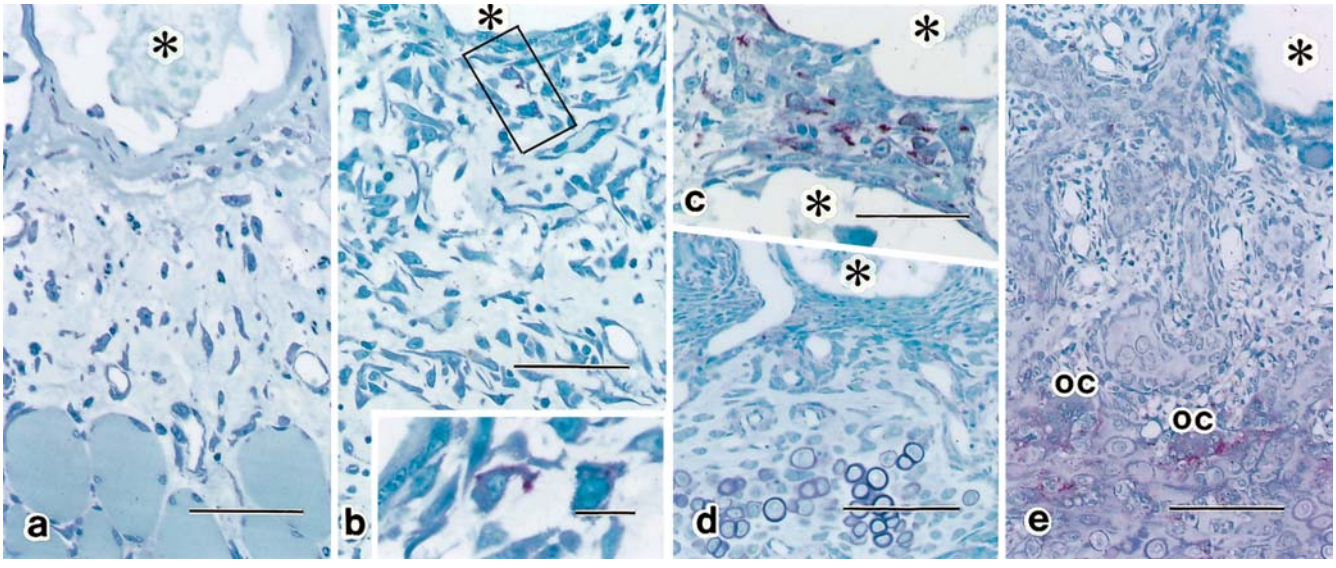

Fig. 2. Light micrographs show the development of cells positive for tartrate-resistant acid phosphatase (TRAPase) activity in the connective tissue layer. **a** At day 1, migratory round cells are found, but they fail to show TRAPase activity. **b** Mononuclear cells positive for TRAPase activity among mesenchymal cells near the PMS at day 3. *Insert*, higher magnification of a cell positive for TRAPase activity in the area indicated by *rectangle* in **b**. **c** At day 5, cells positive for TRAPase activity are seen in connective tissue surrounding the PMS. **d** Newly formed cartilage and bone at day 5. Note that no cells positive for TRAPase activity were seen on the newly formed bone surface. **e** Multinucleated osteoclasts (*oc*) on bone surface at day 7. TRAPase activity in osteoclasts is intense in the cytoplasm facing the bone, making a clear polarity of osteoclasts. *Asterisks*, PMS. *Bars* **a–c** 100 μm; **d,e** 50 μm; insert 10 μm

3, among the mesenchymal cells located close to the implanted PMS (Fig. 2b). By day 5, the number of cells positive for TRAPase activity in the connective tissue surrounding the PMS were increased (Fig. 2c). There were no cells positive for TRAPase activity in the bone matrix growing in the connective tissue layer (Fig. 2d). By day 7, multinucleated cells positive for TRAPase activity were found in the newly formed bone matrix. TRAPase activity was intense at the interface between the osteoclasts and the bone surface (Fig. 2e).

In the cryosections, ALPase activity was also detectable at day 3 in mesenchymal cells in the connective tissue (Fig. 3a). The activity became more intense at day 5 (data not shown) when bone formation was obvious. Ultrastructurally, mononuclear cells found close to mesenchymal cells at day 3 showed abundant large mitochondria and poorly developed rough endoplasmic reticulum and were indistinguishable from osteoclast precursor cells (Fig. 3b).

Immunohistochemically, mononuclear cells immunoreactive for CTR antibody were also first found at day 3 among the mesenchymal cells near the PMS (Fig. 3c). During the experimental period, various patterns of intracellular immunolabeling for cath K were detected. Immunoreactivity for cath K was also first detected at day 3. At that time, immunolabeling for cath K in some mononuclear cells were found regionally in the cytoplasm (Fig. 4a), whereas by day 7, much of the cytoplasm was immunolabeled in most of the mononuclear cells seen among the mesenchymal cells as well as in close proximity to osteoblasts (Fig. 4b). Binuclear cells found on the bone surface showed immunolabeling for cath K in much of their cytoplasm (Fig. 4c). In multinucleated osteoclasts, immunolabeling was more intense on the side facing the bone matrix than the basal side, resulting in a polarized image (Fig. 4d). As described previously, TRAPase or ALPase activity, as well as immunolocalization of cath K or CTR, was not detected in the implanted area of the control group (data not shown) during the whole experimental period.

Discussion

In this study, we morphologically examined the development of osteoclasts and their precursors in ectopic bone formation induced by subcutaneous implantation of rhBMP-2. The first appearance of osteoclast precursors, determined by TRAPase activity, expression of cath K, and CTR, was on day 3 after implantation. The intracellular localization of cath K in this study is consistent with a previous report by Yamaza et al. [19]. Ultrastructurally, the mononuclear cells showed abundant mitochondria and poorly developed rough endoplasmic reticulum, consistent with the characteristics of osteoclast precursor cells [20–22]. At the time of the study, there was no evidence of cartilage or bone matrix formation. This observation is consistent with the events occurring in the normally developing rat maxilla, where the appearance of osteoclast precursors is defined by TRAPase activity before obvious bone matrix deposition [23]. Moreover, in a previous study we observed that calcitonin-binding cells, demonstrated using autoradiography, appeared before cartilage and bone formation in the area of ectopic bone formation induced by crude BMP (unpublished data). These findings indicate that, even in ectopic bone formation induced by rhBMP-2, osteoclast differentiation is initiated before the deposition of cartilage or bone matrix, a process resembling that occurring in normal bone formation.

It is well established that osteoclast differentiation and activation are supported by stromal/osteoblastic cells. Two key factors of osteoclast differentiation, marcrophage-colony stimulating factor and receptor activator of nuclear factor (NF)-κB ligand (RANKL), are well known to be under the control of osteoblastic cells. In this study, ALPase activity in the mesenchymal cell layer was detected at day 3 and thereafter. ALPase activity is one characteristic of osteoblastic cells that supports osteoclast differentiation and activation [24,25]. Together with our previous findings that immunolocalization of RANKL is consistent with the distribution of ALPase activity in bone marrow [26], it is suggested that osteoblastic cells induced by rhBMP-2 support osteoclast differentiation in this ectopic bone formation.

According to Cunningham et al. [27], osteogenin and rhBMP-4 have chemotactic activity for monocytes. Kanatani et al. [28] suggested a direct effect of rhBMP-2 on osteoclastic cell formation from hemopoietic blast cells. Kaneko et al. [16] demonstrated BMP receptors in highly purified rabbit mature osteoclasts, suggesting direct effects of BMP on osteoclasts. Therefore, in addition to the effects of rhBMP-2 on osteoclast development via osteoblast activity described here, it is possible to assume that rhBMP-2 has a direct effect on osteoclast precursor cells. In any event, this study indicates that osteoclast development is initiated before bone matrix formation.

The number of mononuclear cells positive for TRAPase activity increased gradually by day 5. At this point, plump osteoblasts were prominent on newly formed bone, and no cells positive for TRAPase activity were found on the bone surface. Mature and functional osteoclasts, judged from multinucleation and intracellular localization of cath K antigenicity, appeared on newly formed bone at a later stage. Krukowski and Kahn [29] demonstrated the importance of mineralized bone matrix in the formation of a clear zone and ruffled border, two specialized plasma membranes of osteoclasts. Thus, it is suggested that, even though osteoclast dvelopment is initiated before deposition of the bone matrix, final differentiation into a functional osteoclast requires attachment to the bone surface to receive information about the mineralized bone matrix. Moreover, osteoblasts, which actively produce bone matrix, do not seem to attract osteoclast precursors. In other words, osteoblasts have the contradictory functions of producing the matrix and supporting osteoclasts; this facilitates the production by osteoblasts of some bone

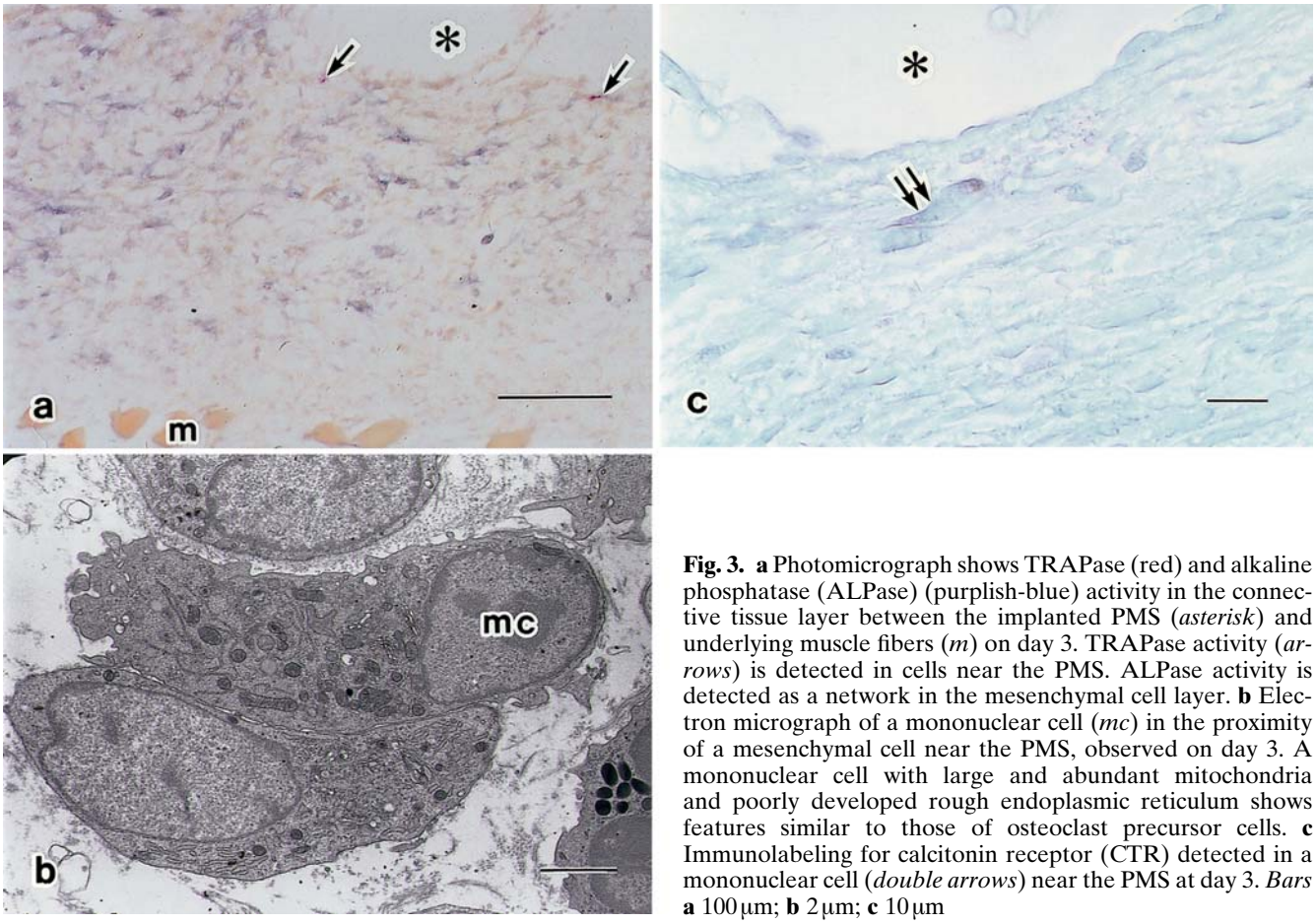

Fig. 3. **a** Photomicrograph shows TRAPase (red) and alkaline phosphatase (ALPase) (purplish-blue) activity in the connective tissue layer between the implanted PMS (*asterisk*) and underlying muscle fibers (*m*) on day 3. TRAPase activity (*arrows*) is detected in cells near the PMS. ALPase activity is detected as a network in the mesenchymal cell layer. **b** Electron micrograph of a mononuclear cell (*mc*) in the proximity of a mesenchymal cell near the PMS, observed on day 3. A mononuclear cell with large and abundant mitochondria and poorly developed rough endoplasmic reticulum shows features similar to those of osteoclast precursor cells. **c** Immunolabeling for calcitonin receptor (CTR) detected in a mononuclear cell (*double arrows*) near the PMS at day 3. *Bars* **a** 100 μm; **b** 2 μm; **c** 10 μm

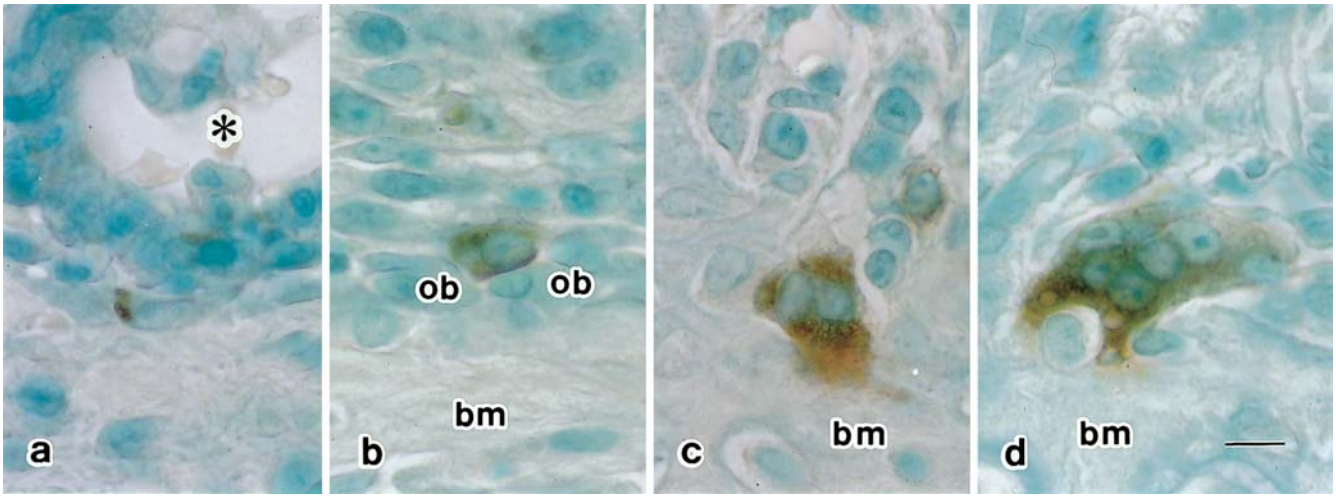

Fig. 4. Light micrographs show immunolabeling for cathepsin K (cath K) on day 3 (**a**) and day 7 (**b–d**). **a** Immunoreactivity for cath K is detected regionally in a mononuclear cell near PMS (*asterisk*). **b** The majority of the cytoplasm is immunolabeled in a mononuclear cell near the bone matrix (*bm*). *ob*, osteoblast. **c** A binuclear cell found on the bone surface with an even distribution of immunolabeling for cath K. **d** A multinucleated osteoclast showing polarity by immunolabeling of cath K, which is more intense on the side facing the bone matrix than on the basal side. *Bar* 10 μm

matrix without osteoclast interference at the initial stage of osteogenesis. Osteoclast precursor cells are preserved at a distance in the initial stages of osteogenesis, and will differentiate into osteoclasts when bone mass has been formed and bone needs to be remodeled.

Polylactic acid-polyglycolic acid copolymer with a blood clot was used as the carrier for rhBMP-2 in this study, and might have had some influence on the events that were observed. However, neither cells positive for TRAPase activity nor bone formation were detected in the control group. Therefore, we attribute the ability to induce cells positive for TRAPase activity in this study to the rhBMP-2 itself.

In conclusion, we have demonstrated the occurrence of osteoclast precursors before bone matrix formation and a delayed appearance of functional osteoclasts after bone formation in the process of ectopic bone formation induced by rhBMP-2. The appearance of osteoclast precursor cells before bone matrix formation suggests that bone matrix is not a prerequisite for osteoclast precursor recruitment. Furthermore, it is also suggested that differentiation into a polarized functional osteoclast is accomplished when the osteoclast precursor cell is attached to the bone matrix.

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