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Original

Vascular Invasion of Epiphyseal Growth Plate in Osteopetrotic (op/op) Mouse Tibiae

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Abstract: To clarify what type of cells lead vascular invasion of epiphyseal growth plate in developing long bones, we conducted immunohistochemical and electron microscopic studies on the op/op mouse tibia which has an inheriting deficiency of macrophages and osteoclasts.

Despite an absence of both TRAP-positive osteoclasts and F4/80-positive macrophages, resorption of epiphyseal cartilage followed by vascular invasion was evident in op/op mouse tibiae. Electron microscopic observation revealed that cells subjacent to the lowermost hypertrophic chondrocyte lacunae were almost exclusively vascular endothelial cells. Immunohistochemically, both cellular elements and extracellular matrix at the vascular invasion front of op/op mouse epiphysis were strongly positive for MMP-9. In situ hybridization revealed a distinct localization of mRNA for MMP-9 in cells located at the same region. From these findings, we hypothesize that vascular endothelial cells themselves are primarily responsible for resorbing the transverse septa of hypertrophic chondrocytes lacunae, and neither osteoclasts nor macrophages involve in this process.

Key words: op/op mouse, Epiphyseal growth plate, Endothelial cells, MMP

Introduction

Endochondral ossification in the epiphyseal plate is a highly orchestrated process that is temporally and spatially regulated. This process depends on the sequence of chondrocyte differentiation, matrix synthesis, and ultimate replacement of cartilage with bone. The initiation of bone formation is preceded by a breakdown of the transverse partitions of matrix between the lowermost lacunae of the zone of hypertrophic cartilage, after which capillary sprouts associated with osteogenic cells invade the space vacanted by the chondrocytes[1].

Classically, it has been described that the chondroclasts, as a counterpart of osteoclasts, are primarily responsible for degradation of the lowermost partition of the hypertrophic zone. However, there has been a controversy or confusion with regard to the entity of chondroclasts. Some reports have described that chondroclasts belong to the macrophage/osteoclast lineage[2,3], while others have postulated that resorption of cartilage matrix is solely dependent on perivascular cells and capillary penetration[4-6].

The osteopetrotic (op/op) mouse is one of the genetically distinct murine mutant strains showing a deficiency of the cells of macrophage/osteoclast lineage. Deckers et al. have reported that vascular invation can occur in the epiphyseal growth plate of op/op mouse caudal vertebrae in the absence of osteoclastic resorption[7], but they have not mentioned as to what type of cells involve in the resorption of cartilage matrix. Thus, we attempted to identify the cells primarily responsible for vascular invasion of epiphyseal growth plate in op/op mouse tibiae.

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Materials and methods

Animals and Tissue Preparation

Breeding pairs of B6C3Fe a/a-Csf1<op> mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and F2 mice were raised in our laboratory. Homozygous recessive mutants (op/op) could be distinguished from their phenotypically normal littermates by 10 days after birth by the failure of eruption of incisor teeth and a characteristic domed skull.

Under anesthesia with ether, the proximal tibiae, including their epiphyseal growth plate, were excised from op/op mice and their phenotypically normal littermates at 10 days of age and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4℃for 16 hours for histological and immunohistochemical examination. The fixed specimens were then decalcified in a 10% EDTA solution, dehydrated in a series of ascending concentration

of ethanol, and embedded in paraffin. For electron microscopy, the tibiae were fixed with 2% paraformaldehyde- 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at 4 ℃ for 3 hours. After decalcification in 10% EDTA solution and post fixation with 1% osmic acid, the specimens were embedded in epoxy resin.

Histology and histochemistry

For histological examination, paraffin sections were stained with hematoxylin eosin (H-E). For detection of tartrate-resistance acid phosphatase (TRACP) activity, a marker of the osteoclast lineage, paraffin sections were incubated with 50 mM of sodium acetate (pH 5.0) containing 0.016% naphthol AS-BI phosphate, 1.0% N-N-dimethylformamid, 0.14% Fast Red Violet LB salt, and 50 mM sodium tartrate (Sigma).

Immunohistochemistry

Paraffin sections were treated with 0.3% hydrogen peroxide in methanol to inactivate endogenous peroxidase for 30 min and normal goat or donkey serum to block the nonspecific sites with nonimmune serum. Then the sections were incubated with one of the following antibodies for one hour at room temperature; rat anti-mouse macrophage monoclonal antibody F4/80 (diluted 1: 100, Cedalane, Ontario, Canada), rabbit anti-mouse laminin polyclonal antibody (diluted 1:1,000, LSL, Tokyo), goat anti-human MMP-9 polyclonal antibody (diluted 1: 50, Santa Cruz Biotechnology, Santa Cruz, CA). The sections were next incubated with biotinylated goat anti rat IgG antibody (1: 100, CHEMICON, Temecula, CA), biotinylated goat anti rabbit IgG antibody (1: 100, CHEMICON, Temecula, CA), biotinylated donkey anti goat IgG antibody (1: 100, CHEMICON, Temecula, CA) for 30 minutes, respectively, and then with streptavidin-conjugated horseradish peroxidase complex (Histofine SAB-PO kit, Nichirei, Tokyo) for 30 minutes. After development of the peroxidase reaction with diaminobenzidine, the sections were counterstained with hematoxylin. Sections treated similarly, but incubated with normal mouse IgG instead of the primary antibody, served as a negative control.

In situ hybridization

Digoxigenin (DIG)-labeled anti-sense RNA probe for MMP-9 were synthesized from c-DNA (433bp) using T7 RNA polymerase. The primer sequence for cDNA amplification were 5'-CTTTGAGTCCGGCAGACAAT-3' (forward) and 5'-TCCTTATCCACGCGAATGAC-3' (reverse). In situ hybridization was performed according to the method described by Nomura et al.[8]. Briefly, hybridizations were performed under stringent conditions for 18h at 55℃. Following hybridization, sections underwent RNase A treatment to remove any nonhybridized probes. Subsequent washing steps included 2x SCC and 0.2x SCC twice for 20min at 50℃ each. The hybridized probes were detected by peroxidase-labeled rabbit anti-DIG antibody and GenPoint System (Dako Cytomation, Carpinteria, CA), following the manufactures protocol. After visualization of reacted sites, sections were counterstained with methyl green.

Electron microscopy

Prior to thin-sectioning, 1-2 mm thick sections were cut and stained with toluidine blue for light microscopic observation. Thin sections were cut on an Ultracut E ultra microtome (Reichert-Jung, Wine, Austria) with a diamond knife, stained with uranyl acetate and lead citrate, and observed with JEOL 1200 EX electron microscope at 80 kV.

Results

Histologic findings

There was no significant difference in the thickness and cellular arrangement of epiphyseal growth plates between op/op mice and phenotypically normal their littermates (Fig. 1a), although the longitudinal growth of tibiae of the former appeared to be retarded

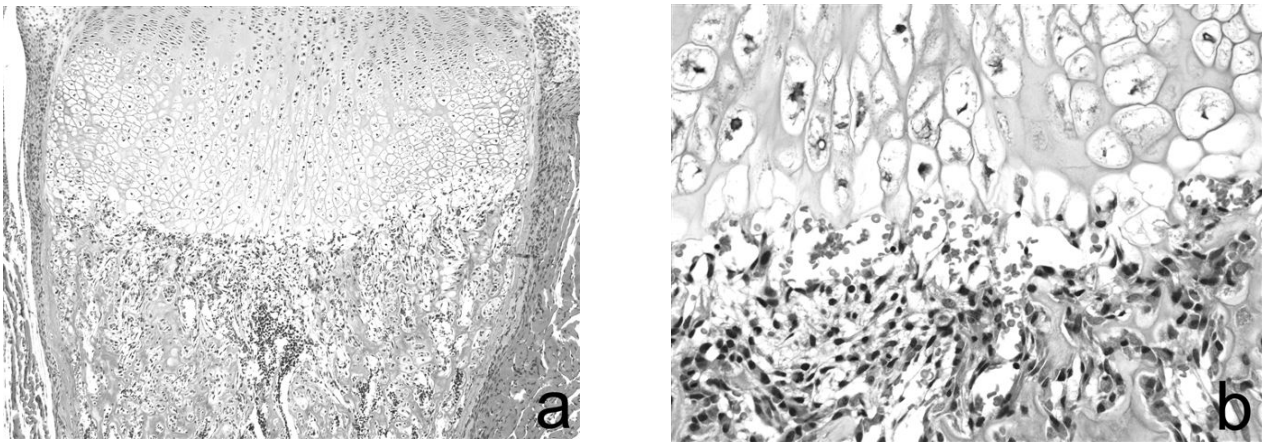

Fig. 1. H-E staining of 10-day-old op/op mouse proximal tibia. (a) Low power view of epiphyseal growth plate (x 100). (b) High power view of epiphyseal-metaphyseal junction (x 400).

to some extent. In op/op mouse tibiae, the lowermost transverse septae of hypertrophic zone were resorbed and capillary sprouts appeared to invade the space vacant by the chondrocytes (Fig. 1b). The metaphysis and the diaphysis of op/op mouse tibiae were filled by primary spongiosa, consisting of trabecular bone with persistent cartilage core.

TRACP histochemistry

In op/op mouse tibiae, no TRACP-positive cells were detected throughout the specimen (Fig. 2a). In their normal littermates, numerous TRACP-positive cells were present on the surface of bony trabeculae in the metaphysis (Fig. 2b), but no TRACP-positive cells were found at the vascular invasion front.

Immunohistochemistry

In tibiae from op/op mice, anti-mouse macrophage antibody F4/80-positive cells were not detected (Fig.3a). In their normal littermates, F4/80-positive cells were found scattered throughout the metaphysis (Fig.3b) but, as with TRACP staining, no F4/80-positive cells were detected at the vascular invasion front.

The endothelial identity of cells located at the vascular invasion front was confirmed by immunohistochemical staining using anti-laminin antibody. In both op/op mice and normal littermates, the positive reaction for laminin was observed along the surface of cartilage matrix at the vascular invasion front (Fig. 4).

The surface of cartilage matrix at the vascular invasion front revealed an intense immunoreaction for MMP-9 in a similar fashion to that of laminin (Fig. 5). Cells located in this area are positive for MMP-9 as well. In op/op mice, an expression of MMP-9 appeared to mostly localize at the vascular invasion front, whereas those in normal littermates were scattered throughout the metaphysis.

***In situ hybridizatio*n**

In op/op mouse tibiae, a strong expression of mRNA for MMP-9 was found in cells located at the vascular invasion front (Fig. 6). In their normal littermates, MMP-9 gene was expressed in cells scattered throughout the metaphysis including osteoclasts.

Electron microscopic findings

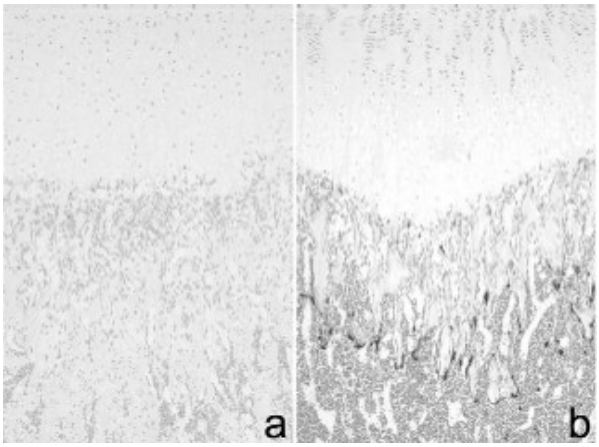

Fig.2. TRACP staining. (a) 10-day-old op/op mouse (x 100). (b) Normal littermate (x 100).

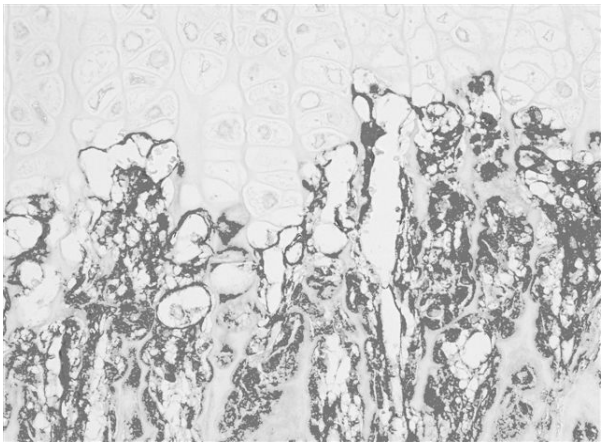

Fig.4. Immunostaining for laminin. 10-day-old op/op mouse (x 400).

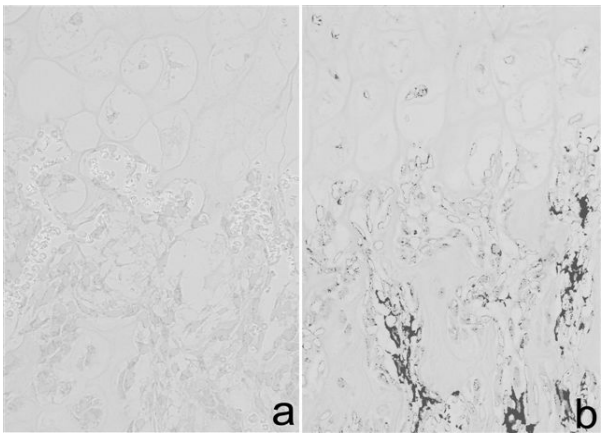

Fig.3. Immunostaining for F4/80. (a) 10-day-old op/op mouse (x 400). (b) Normal littermate (x 400).

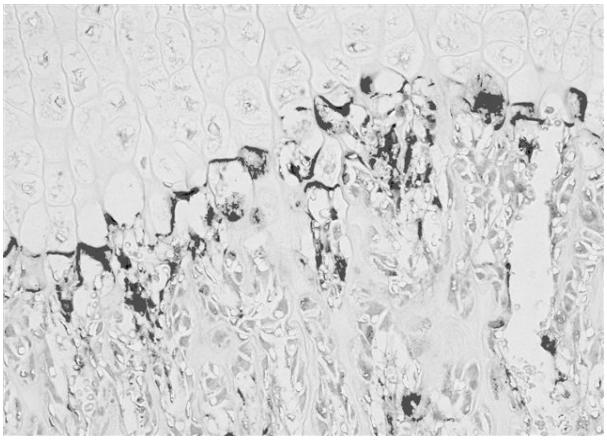

Fig.5. Immunostaining for MMP-9. 10-day-old op/op mouse (x 400).

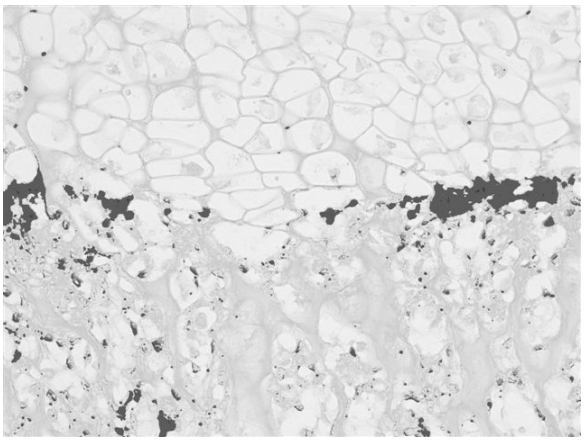

Fig. 6. In situ hybridization for MMP-9. 10-day-old op/op mouse (x 400).

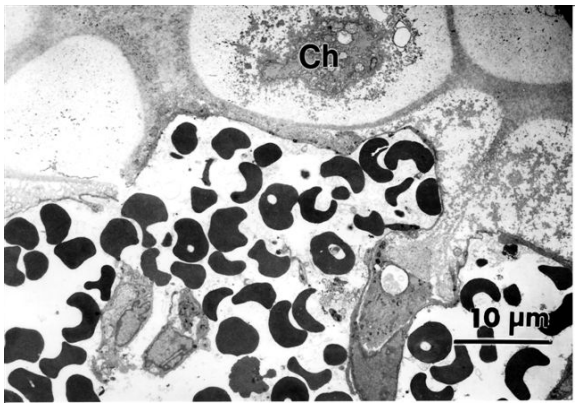

Fig. 7. Electron micrograph of epiphyseal-metaphyseal junction of op/op mouse tibia. Only capillary sprouts were noted next to chondrocyte lacunae. Ch: Degenerating hypertrophic chondrocyte. x 2,500.

In op/op mouse tibiae, cells subjacent to the lowermost intact chondrocyte lacunae were almost exclusively capillary endothelial cells (Fig. 7). Endothelial cells at the vascular invasion front were often highly attenuated and had no underlying basal lamina. Spindle cells and/or cytoplasmic processes were occasionally found intervening between endothelial cells and cartilage matrices, but none of them appeared to be located at the tip of capillary sprouts. The ultrastructural findings in normal littermates were essentially the same as those in mutants except an existence of macrophages and osteoclasts at a certain distance from the tip of capillary sprouts.

Discussion

In op/op mice examined in this study, despite a total lack of the macrophages/osteoclast lineage as shown by F4/80 and TRACP staining, resorption of the lowermost transverse partition of hypertrophic zone followed by capillary invasion was evident. No significant abnormality was found in the epiphyseal growth plate. This finding is consistent with that of Deckers et al.[7], who demonstrated vascular invasion of the epiphyseal cartilage in *c-fos* knock out mice which lack osteoclast differentiation. Electron microscopy revealed that neither macrophages nor osteoclasts existed in op/op mouse tibiae, confirming immunohistochemical features. In their normal littermates, macrophages and osteoclasts consistently appeared to lie at a certain distance from the capillary invasion front and were not found to precede the invading capillary sprouts. Thus, it is evident that neither macrophages nor osteoclasts are primarily responsible for cartilage resorption that leads to vascular invasion of epiphyseal growth plate.

An exposure of mineralized matrix is prerequisite for osteoclastic resorption. Studies on the mammalian epiphyseal growth plates have shown that the transverse septae of the cartilage in the lowermost hypertrophic zone remains unmineralized[4-6]. In this situation, osteoclast activity is not required for resorption of cartilage matrices. On the other hand, chondroclasts as a counterpart of osteoclasts play a primary role in breaking through the cartilage lacunae in the mandibular condylar cartilage because its hypertrophic chondrocytes are surrounded by mineralized matrix[9].

A question of what cell types are responsible for the resorption of epiphyseal growth plate, namely chondroclasts, has remained obscure. Several studies have described that the cells responsible for resorption of unmineralized cartilage matrix are perivascular cells[4, 5, 9, 10], a hypothesized cell type of obscure origin. Perivascular cells are located between invading capillary endothelial cells and cartilage matrix, and identified by histochemical staining for Dolichos Biflorus agglutinin (DBA) lectin. Their cell surface marker and ultrastructure are different from those of the macrophage lineage[10]. Electron microscopically, we found spindle cells or their cytoplasmic processes intervening between capillary endothelial cells and cartilage matrix, suggesting the existence of perivascular cells. However, it was not frequent but solely occasional and we failed to detect them at the vascular invasion front. Therefore, it is unlikely that these cells play a main role in resorbing cartilage matrix.

Angiogenesis requires localized proteolytic modification of the extracellular matrices and MMPs are implicated in these processes owing to their ability to cleave extracellular matrices. Specifically, MMP-9 is considered to be a key regulator of angiogenesis in epiphyseal growth plate and the lack of MMP-9 results in a delay in endochondral ossification[3]. MMP-9 has a major role in degradating cartilage matrix to allow accommodation of blood vessels [11]. Another function of MMP-9 is to generate angiogenic activators or to inactivate angiogenic inhibitors. A strong expression of both protein and mRNA for MMP-9 was seen at the epiphyseal-metaphyseal junction of op/op mice, as well as their normal littermates, particularly prominent at the vascular invasion front. Electron microscopic findings revealed that cells located in this zone were almost exclusively capillary endothelial cells. Thus, it is conceivable that cells expressing MMP-9 are vascular

endothelial cells. It has been shown that endothelial cells are capable of degradating nonmineralized matrix through the activity of proteolytic enzymes[12]. Studies on mammalian epiphyseal growth plate have shown that degradation of cartilage matrix is solely dependent on perivascular cells and capillary penetration[4, 6]. Thus, endothelial cells themselves possibly involve in cartilage resorption through the secretion of proteolytic enzyme MMP-9, *i.e.*,the true chondroclast. In this regard, it may be required to further clarify the existence and the role of perivascular cells in leading the vascular invasion front.

Acknowledgment

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