Abstract: The morphogenesis of long bones is a multistep process that generates a variety of genetically defined forms. The tarsometatarsal (TMT) long bone morphology in birds develops through lateral fusion of three initially independent periosteal bone cylinders (BCs). Previous studies have clarified the histological details and chronology of the changes occurring during development. The present study investigated the temporospatial distribution of osteogenic and osteoclastic cells in the embryonic chicken using histochemistry for alkaline phosphatase and tartrate-resistant acid phosphatase, with particular reference to the radial growth of BCs and their subsequent fusion process. Osteogenic cells were localized preferentially in the periosteum of radially growing BCs, leaving open cancellous spaces in the BC wall. Osteoclasts observed later than embryonic day 10 were localized preferentially in the endosteal surface, and therefore the radial growth of BCs resulting from osteoblast activity was accompanied by endosteal resorption by osteoclasts, with progressive enlargement of the bone marrow spaces. During BC fusion, trabecular bridges were formed by periosteal osteogenic cells, with removal of the bone septum by endosteal osteoclasts. These findings suggest that fusion of BCs in the embryonic chicken is mediated by cellular events constituting ordinary long bone development, and not through a defined mechanism specific for fusion.

Keywords: bone evolution, development, fusion, morphogenesis, osteoblasts, osteoclasts

Introduction

The appendicular skeleton of tetrapod vertebrates comprises three distinct segments (stylopodium, zeugopodium and autopodium [1,2]), and the hindlimb autopodium basically consists of the tarsal, metatarsal and phalangeal bones. In humans, the five metatarsal bones in the middle of the foot are not long, and each of them articulates with a tarsal bone on the proximal side and with a phalanx on the distal side. In comparison, the avian tarsometatarsal (TMT) skeleton is quite long, especially in terrestrial birds such as raptors, fast runners, and even domestic fowl. The TMT skeleton in birds extends from the base of the toes to the intertarsal ankle joint, where the bone articulates with a tibiotarsal bone in the original lower leg [3]. This may represent recruitment of a length of the intertarsal ankle joint, where the bone articulates with a tibiotarsal bone.

A prime example of evolution is the development of the short multiple toes in early ancestors of the horse into a large and elongated foot with a single toe in the modern horse [5]. However, a feature peculiar to avian TMT in early ancestors of the horse into a large and elongated foot with a single toe in the modern horse [5]. However, a feature peculiar to avian TMT development is the lateral fusion of three initially independent bones [6]. The fossil records indicate that this lateral fusion during avian leg development appears to recapitulate the changes that occurred during the evolution of Theropoda to modern birds [7]. This involved the elongation of three independent TMT bones in theropod species, becoming tightly packed together, fusing laterally with each other, and eventually becoming a single compound TMT skeleton with a long bone morphology.

Our previous study using light and electron microscopy [6], which focused on embryonic development of the TMT skeleton in domestic fowl, revealed 1) the establishment of a columnar TMT segment through juxtaposition of three metatarsal cartilage rods in the autopodium, 2) the onset and propagation of bone collar formation from the mid-diaphyseal portion of the cartilage, 3) longitudinal and radial growth of the cylindrical periosteal bones, and 4) their lateral fusion to generate a single compound bone. Thus, the time course of these changes has been largely established. In addition, among the observations made by scanning electron microscopy, a large number of lacunae for osteoblastic cells were demonstrated in the outer surface of the TMT bone. Although this certainly implied active bone formation, osteoblastic and osteoclastic activities were not examined.

The present study was therefore undertaken to clarify changes in the temporospatial distribution of osteoblasts and osteoclasts in the developing TMT bone using histochemistry for alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), with particular reference to the radial growth of individual bone cylinders and their subsequent fusion in the domestic fowl at embryonic day 8-20.

Materials and Methods

Preparation of specimens

Fertilized eggs of White Leghorn (Gallus gallus) obtained from Oohata Hatchery (Shizuoka, Japan) were incubated at 39°C in a humidified incubator (MTI-201A; EYELA, Tokyo, Japan). Embryos without any gross developmental defects were staged according to the morphological criteria of Hamburger and Hamilton [8]. TMT specimens were excised from developing hindlimbs of 34, 36, 39, 43, and 46 embryos at embryonic days (EDs) 8, 10, 13, 17, and 20, respectively. The ED number plus 26 represents the stage-numeral at ED8 or later, and ED20 is equivalent to the time of hatching. Eight embryos for each ED were used for the study.

Specimens fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.35) for 2 h at 4°C were subjected routinely to hematoxylin-eosin (HE) staining, ovβ3 integrin immunostaining, and histochemistry for ALP or acid phosphatase (ACP). All procedures were carried out in accordance with the guidelines of the Animal Experimentation Committee of Nihon University School of Dentistry, compliant with the National Act on Welfare and Management of Animals.

Preparation of undecalcified bone sections

Fixed and rinsed specimens of TMT at ED10, 13, 17, and 20 were embedded in 5% carboxymethyl cellulose (CMC), according to the procedure of Kawamoto and Shimizu [9], and frozen in liquid nitrogen-cooled 2-methyl butane [10]. Frozen sections, 5 μm thick, were prepared without decalcification using adhesive Cryofilm type I (Sakura Finetek Co., Tokyo, Japan) according to the transfer film method [11]. Fixed and rinsed specimens at ED8, which showed minimal calcification, were frozen using the ordinary procedure for cryosections: briefly, specimens were cryoprotected in a graded series (10, 15, and 20%) of sucrose in PBS, embedded in OCT Compound (Tissue-Tek; Sakura Finetek Co.), and frozen in liquid nitrogen-cooled 2-methyl butane.

Transverse sections of the TMT skeleton were prepared at the mid-diaphysis (in most cases) and at the proximal metaphysis (in some cases).
Longitudinal sections of the TMT skeleton were also prepared, but the findings were generally comparable with those observed in transverse sections, and therefore only one case at ED8 is described in this report.

ALP and tartrate-resistant ACP (TRAP) staining
Undecalciﬁed cryosections were enzymatically stained for ALP or TRAP using reagents in the TRACP&ALP stain kit (MK300; Takara Bio Inc., Kusatsu, Japan). Briefly, the sections for examining ALP activity were equilibrated in 100 mM Tris-HCl buffer (pH 9.5) for 10 min, incubated in a substrate solution containing BCIP/NBT (for blue purple coloration), and the reaction (or color development) was terminated at an appropriate time point by adding 20 mM EDTA to the substrate solution. The sections used for examining ACP activity were incubated in a substrate solution (pH 5.0) containing NABP/FRVLB (for red purple coloration). For examining TRAP activity, 0.5 M sodium tartrate (pH 5.0) was added to the latter substrate solution immediately before use. Sections for observation at higher magniﬁcation were counterstained with hematoxylin. Color-developed sections with or without hematoxylin staining were mounted in 30% glycerol and photographed with a Nikon microscope (Eclipse E600; Nikon Inc., Tokyo, Japan) equipped with a CCD camera (Pro 600ES; Pixera, Co., Santa Clara, CA, USA).

Immunohistochemistry
Undecalciﬁed bone sections prestained for TRAP were equilibrated and blocked in 1% bovine serum albumin (BSA)-PBS for 1 h, reacted with 1:100-diluted mouse anti-αvβ3-integrin monoclonal antibody (MAB1976; Chemicon International, Inc., Temecula, CA, USA) [12] for 1 h and washed in PBS. Sections were subsequently incubated in ﬂuorescein-isothiocyanate-conjugated goat anti-mouse IgG for 1 h. After washing in PBS, sections were mounted with antifade (Prolong Gold; Thermo Fisher Scientiﬁc, Waltham, MA, USA) and examined. Bright-ﬁeld images for TRAP, ﬂuorescence images for αvβ3 and their superimposed images were recorded digitally with an epifluorescence microscope equipped with a CCD camera. Controls in which MAB1976 was omitted or replaced with non-immune mouse IgG yielded no speciﬁc immunofluorescence.

Results
Temporospatial distribution of cells positive for ALP
The changes in the distribution of ALP (+) cells are shown as an overview (Fig. 1) and in closer views (Fig. 2). Cells positive for ALP were observed initially on the surface of the mid-diaphyseal portion of rod-shaped cartilaginous TMT primordia earlier than ED8, but their distribution soon spread towards both proximal and distal metaphyses on the surface of the cartilage rods at ED8. Each of the cartilaginous primordia was therefore surrounded completely by ALP (+) cells in cross-sectional images at the mid-diaphyseal level (Fig. 1A). Closer cross-sectional views showed an ALP-positive blue-purple ring apparently containing a thin layer of bone with evident outward bony strut formation (Fig. 2A); each bony strut was a tiny pillar connecting two separate bone layers.

During ED10 to 20, growth of the three periosteal bone cylinders (BCs), referred to as a bone collar, proceeded rather quickly, and ALP (+) cells were localized mostly in the periphery of the radial growth. At ED10, BCs were separated from each other and their cross-sectional outlines were still roundish (Fig. 1B). Through further radial growth, the BCs came close to each other, and the two lateral cylinders became half-rounded in shape whereas the shape of the remaining central cylinder resembled a squared timber by ED13. Although at this stage of development the three BCs remained separated (Fig. 1C), at ED17 (Fig. 1D) they no longer appeared so. At ED20, the trabecular structure in the diaphyseal portion of the TMT was shared between neighboring cylinders to a considerable extent (Fig. 1E).

More detailed examination at ED10 (Fig. 2B) revealed that individual BCs had become double-layered, each consisting of an initial (inner) and a secondary (outer) layer, which were connected to each other by bony struts. ALP (+) cells were observed in the outer and inner surfaces of the secondary cylinder, but where the latter cylinder had not yet formed completely, cells in the outer surface of the initial cylinder were positive. Thus, the outermost part of BCs was always positive for ALP, whereas no ALP (+) cells were found in the tissue interposed between neighboring cylinders at ED10. Chondrocytes in the cartilage surrounded by BCs were also positive for ALP to various degrees.

At ED13 and later, double-layered cylinders that had been characteristic at ED10 became less discernible, and outward radial growth generated a highly trabeculated pattern in the thickened wall of the TMT bone (Fig. 2C-E). Cells positive for ALP persisted in the outer two to three layers of bone trabeculae but were absent in the remainder of the TMT wall. However, a subset of cells positive for ALP remained in the trabeculated TMT at ED17 and 20. These ALP (+) cells appeared to be those originally located in the outermost area of BCs (Fig. 2C) and then became entrapped in the fusion plane (Fig. 2D, E). Indeed, the blue-purple staining in these cells was substantially diminished by ED20, indicating a decrease of their ALP activity, i.e., their transition to bone lining cells, which are osteoblasts presumably in a resting or quiescent state.

Meanwhile, erosion of cartilage occurred in BCs at ED10 and later. The eroded portion was replaced by bone marrow which had been introduced

Fig. 1 Distribution of ALP-positive (blue-purple) cells in developing BCs of the TMT skeleton at ED8 (A), ED10 (B), ED13 (C), ED17 (D) and ED20 (E). Cross-sectional images of the undecalciﬁed TMT skeleton are shown at ED8-20, but a longitudinally sectioned image is included at ED8. Boxed areas are enlarged in Fig. 2. All images are at the same magniﬁcation, and the bar in E represents 500 μm.

Fig. 2 More detailed views of developing BCs stained for ALP at ED8 (A), ED10 (B), ED13 (C), ED17 (D) and ED20 (E). Boxed areas in Fig. 1 are enlarged here. Bony struts in A and B are indicated by arrowheads. Inner and outer layers of the double-layered BCs in B are indicated by single and double arrows, respectively. The interposing tissue between BCs in A-C is labeled with asterisks, and the plane of fusion in D and E with a row of double diamonds. Abbreviations: ca, cartilage; cs, cancellous space; bm, bone marrow. All images are at the same magniﬁcation and the bar in E represents 100 μm.
to the developing TMT via the blood supply. A few ALP (+) cells were observed in bone marrow as well as at the eroding surface of the cartilage inside the BCs (Fig. 2C-E).

Temporospatial distribution of TRAP-positive osteoclasts

Although positive staining for TRAP is known to indicate the presence of osteoclasts, two comparisons were made before applying TRAP staining to multiple sets of serial sections of TMT bone: 1) TRAP vs. ordinary ACP staining, and 2) TRAP staining vs. immunolocalization of transmembrane αvβ3 integrin, with which osteoclasts bind to bone surfaces [13]. The results of staining for TRAP and ACP on film-based undecalcified sections did not differ so much (Fig. 3A, B), but TRAP staining appeared more localized than that of ACP, whereas counterstaining with hematoxylin was as effective as that in HE-stained adjacent sections (Fig. 3C, D). The localization of the integrin receptor (αvβ3 directed to osteopontin in the bone matrix) corresponded well with TRAP staining (Fig. 3E-G). Some of the TRAP-positive cells lacked fluorescence for αvβ3 (arrowheads in Fig. 3G), suggesting that those were osteoclasts not yet anchored or in a migratory state.

Figure 4 shows an overview of osteoclast distribution in developing BCs of TMT stained for TRAP, and Fig. 5 shows a more detailed view with counterstaining at ED10-20. At ED8, no positive staining for TRAP was detected (not shown).

At ED10, positive staining for TRAP was mostly localized within the wall (or mural space) of BCs, each consisting of double layers connected to each other by bony struts; however, the outermost surface of each BC was negative for TRAP (Figs. 4A, 5A).

At ED13-20, TRAP (+) osteoclasts occurred exclusively as scattered cells along the bone marrow surface in BCs and were never found within highly trabeculated areas of the TMT wall or in its outermost surface (Figs. 4B-D, 5B-D). The latter negative staining was also evident at the sites of cylinder fusion; i.e., TRAP (+) osteoclasts were absent in the surfaces of bone facing the tissue interposed between neighboring cylinders (Fig. 5B, C). When the BCs fused laterally and the septal bone became marked, TRAP (+) osteoclasts were observed at the bone marrow surfaces on both sides of the septa but were absent within the mural spaces of the septa (Fig. 5D).

These features were evident at the mid-diaphyseal level of the TMT. Since BCs developed initially at the mid-diaphysis and extended towards the proximal and distal metaphyses, development at the metaphyseal level was slightly delayed in comparison. Figures 4E and 5E show osteoclast distribution in cross-sections of the proximal metaphysis at ED17 at low and higher magnification, respectively. In the mid-diaphysis, the cartilage was eroded and replaced by bone marrow tissue (Fig. 4C), but in the metaphyses, bone marrow had partially developed and cartilage was still present in areas where TRAP (+) osteoclasts were observed at the bone and cartilage surfaces exposed to bone marrow, but none were present at the edges of bone adhering to yet uneroded cartilage (Figs. 4E, 5E).

Discussion

Enzyme histochemistry performed on undecalcified sections has demonstrated the distribution of ALP-positive cells in the developing TMT skeleton. As reported previously in embryonic chick tibial bone [14], ALP-positive cells in the present study would have included mature osteoblasts and preosteoblasts in periosteal tissue, but neither osteocytes nor bone lining cells (quiescent osteoblasts) were present [15-17]. The periosteal osteogenic cells were always localized at the periphery of radially growing TMT bone. Our previous study using SEM [6] demonstrated a large
The changes in the distribution of osteogenic cells observed in the three developing BCs of the TMT were basically identical to those in stylopodial and zeugopodial bones such as the femur, tibia and fibula [14,23], and have been shown clearly in diagrammatic form by Caplan & Pechak (The cellular and molecular embryology of bone formation. In: Bone and Mineral Research/5, Peck WA ed, Elsevier Science Publishers B.V, Amsterdam, pp. 117-183). However, lateral fusion of three TMT BCs is a noteworthy event. In previous studies, we generated an antibody directed to the periosteal molecule, fibrillin [24], which was localized in the outer periosteal layer but scarce or absent in the inner osteogenic cell layer [3,6]. In the present study using ALP histochemistry, it was found that these cells independently covered the two opposite outer surfaces of neighboring cylinders. Fusion was initiated and mediated by a limited number of trabecular bridges, which resulted in containment of ALP-positive cells, followed by the disappearance of their positive staining. We assume that these changes did not represent retraction of the interposing periosteal tissue, but more likely indicated that osteoblasts located deep in the trabeculated TMT bone did enter a quiescent phase.

Contrary to osteogenic cells, osteoclasts visualized by TRAP staining were observed in the innermost surface of BCs (Figs. 4, 5). However, BCs at ED10 were an exception. At this stage, positive staining for TRAP was observed within the walls of the cylinders (Fig. 4A). This pattern of staining suggests that osteoclasts are deployed to the outer side of BCs exogenously via the blood supply, since osteoclastic perforations are initiated at ED9 and are barely underway at ED10 in the diaphyseal wall of TMT BCs (doctoral thesis of Namba Y; Article ID #S00000515766 in NII, Japan). Osteoclasts observed later than ED10 in the endosteal surface of BCs are recruited from bone marrow tissue introduced into the marrow cavity via intraosseous blood vessels. These osteoclasts actively absorb the trabeculae of endosteal margins and enlarge the bone marrow spaces progressively, since the luminal diameters of BCs did appear to increase as development proceeded (Fig. 4).

Although the distributions of osteogenic and osteoclastic cells are discussed separately above, in fact they appear to cooperate with each other at the macroscopic level; the radial appositional growth of TMT bone mediated by osteoblasts is accompanied by endosteal osteoclastic resorption in a temporospatially relevant manner. This principle might be also apply to the progression of fusion between neighboring BCs examined in this study. Formation of trabecular bridges between BCs resulted from osteogenic activity in separate areas of periosteum aligned back-to-back, and thus could be considered merely as a slight variant of radial growth. In addition, the removal of the bone septum at the plane of fusion could also be considered a variant of marrow cavity enlargement, since osteoclasts involved in this process remained at the endosteal surface and exerted resorptive activity on the septal wall.

In a previous report based on morphology and histology [6], it was assumed that lateral fusion of TMT bone occurred via a specific mechanism. However, the present study suggested that the process of BC fusion involved cellular events that ordinarily occurred during long bone development. In recent studies [25,26], a modular model of long bone development and patterning has been emphasized. This model appears well fitted to the reported evolutionary diversity of elongation, fusion and unification of the narrow cavities of BCs in the avian TMT skeleton [4,6,27]. The concept of “modularly regulated morphogenesis” might provide a clue for better understanding of the temporospatially specific lateral fusion of BCs investigated in this study.

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**Conflict of interest**

Nothing to declare.

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