

Original

Immunohistochemical Comparison of Ontogenic Development of Bone Marrow Hematopoiesis in Two Different Ossification Systems

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Abstract: It is well known that the body skeleton is formed by two different types of ossification systems, endochondral and intramembranous ossification. Bone marrow is the main site of active hematopoiesis after the formation of the bone marrow cavities. However, it is unclear whether the hematopoiesis in the bone marrow of two types of ossification is regulated by the same system or not. In this study, we focused on the ontogenic development of bone marrow hematopoiesis in two different ossification systems using mouse humeral bones and palatal process of maxillary bones. Immunohistochemical and RT-PCR analyses were performed to examine the development of hematopoiesis and the expression of cytokines related to hematopoiesis in the forming bone marrow (16-days gestation stage to 1-day postnatal stage). Immunohistochemical studies showed the sequential difference of hematopoiesis between two different ossification systems. In humeral bone marrow, granulopoiesis appeared first at E16, followed by erythropoiesis from E17. On the contrary, erythropoiesis preceded one day in the maxillary bone marrow at E18, one day before the detection of granulopoiesis. G-SCF and GM-CSF were expressed at every examined stage in both types of bones while M-CSF was not expressed in the humeral bone marrow at E16. Erythropoietin was detected in the endothelial cells and its expression was coincident with the onset of erythropoiesis. These results suggest the time kinetic and sequential differences of hematopoiesis in two different ossification systems, which might relate to the differences of hematopoietic microenvironment.

Key words: bone marrow, hematopoiesis, endochondral ossification, intramembranous ossification.

The skeletal system, characterized by a hard tissue component, is an extraordinarily dynamic system with disparate functions such as structural support, movement, soft-organ protection and the maintenance of calcium homeostasis. In addition, bone houses definitive hematopoiesis. During vertebrate ontogeny, hematopoiesis is established sequentially in different anatomical sites. Around birth time, hematopoietic site shifts from fetal liver to the forming bone marrow cavity which is the major site of hematopoiesis during adult life.^{1~3)}

Vertebrate bones are formed by two different types of ossification systems, endochondral and intramembranous ossification. Intramembranous bone formation is mediated by osteoblasts differentiated from the periosteal osteogenic cells, which form bone without the mediation of cartilage, while, in the case of endochondral bone formation, osteoblasts form bone on a calcified cartilage scaffold after epiphyseal and physeal cartilage have shaped and elongated.^{4~6)} In both of ossification systems, hematopoietic tissues are located inside the forming bones.

It has been indicated that the bone marrow micro-environment provides essential regulatory role in hematopoietic system. The hematopoietic micro-environment includes stromal cells and the cytokines and extracellular matrix which they secrete.⁷⁾ The bone marrow stromal cells include macrophages, endothelial cells, adipocytes, fibroblasts and osteoblasts.^{8,9)}

Erythroblastic islands consist of a central macrophage surrounded by a ring of erythroblasts that undergo terminal maturation leading to enucleation. It has been reported that erythroid cells mature to the late erythroblast stage but fail to enucleate in macrophage-depleted cultures.¹⁰⁾ Erythropoietin (EPO), a major regulator of erythropoiesis, has also been detected in the macrophages of fetal liver blood islands.^{11,12)} These results suggest that erythroblast-macrophage contact promotes proliferation and terminal maturation of erythroid cells.

Osteoblasts are differentiated stromal cell type unique to the bone marrow environment. It has been indicated that osteoblasts play the central and direct role in myelopoiesis by secreting numerous hematopoietic cytokines which support the terminal neutrophil maturation and the *in vitro* expansion of primitive long-term culture-initiating cells (LTCICs).^{13~15)}

Osteoblasts are differentiated from a multipotent mesenchymal stem cell (MMSCs), which have the ability to commit to osteogenesis, chondrogenesis, adipogenesis and myogenesis by the defined developmental programs.¹⁶⁾ It has been proposed that once MMSCs commit to osteogenesis the cells differentiate into a putative committed osteoprogenitor cells, then progress to preosteoblasts and osteoblasts.¹⁷⁾ Although the precise mechanisms for the regulation of this process are poorly understood, several studies indicate the inherent and/or functional heterogeneity of osteoblasts.^{18,19)} These results lead to suggest the heterogeneity of hematopoietic microenvironment in the bone marrow during the bone development and between two different ossification systems.

Therefore, in this study, we compared the ontogenic

development of hematopoiesis, erythropoiesis and granulopoiesis, in the bone marrow of two different ossification systems to know the heterogeneity of hematopoietic microenvironment in two different ossification systems.

Materials and Methods

Animals and antibodies

Pregnant BALB/c mice were purchased from Saitama Breeding Laboratory, Saitama, Japan. Mice from 15-day gestation (E15) to 1 day postnatal (1DPN) stage were used in this study.

Monoclonal antibodies, Gr-1 and TER-119, were purchased from BD Biosciences (San Jose, CA) and F4/80 was purchased from UK-serotec Ltd. (Oxfordshire, UK). A polyclonal antibody, goat anti-human Erythropoietin (EPO) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-labeled rat anti-mouse Gr-1 antibody was purchased from Biolegend (San Diego, CA), and Alexa Fluor 594-labeled goat anti-rat IgG antibody was purchased from Invitrogen (Carlsbad, CA). Biotinylated goat anti-rat IgG and rabbit anti-goat IgG antibodies were from VECTOR, Burlingame, CA.

Tissue preparation

The experimental protocol was approved by the Animal Care Committee of Showa University.

Humeral long bones and maxillary bones from E15 to 1DPN stage were dissected and fixed with 4% paraformaldehyde in PBS. After decalcification by 10% EDTA in PBS, the specimens were washed with 20% sucrose-PBS, embedded in CRYOform (International Equipment Co., Needham, MA), and quick-frozen in a mixture of acetone and dry ice. Some of the humeral and maxillary bones were processed for RT-PCR.

Immunohistochemical procedure

Frozen sections (8 μ m thick) were cut, placed on poly-L-lysine-coated glass slides and air dried. After incubation in 0.3% H₂O₂-methanol for 30 min, the sections were incubated with 5% normal goat serum in PBS containing 5% bovine serum albumin and

Table 1 Primer sequence used in this study.

M-CSF	Forward: 5'-CTAGGGGCCAGCATTAGACC-3'OH Reverse: 5'-GACACATACTACACCCAGAGG-3'OH
G-CSF	Forward: 5'-GGGACAAGACATCCCTGTTT-3'OH Reverse: 5'-CTGTGAGGACAGGAAACCCT-3'OH
GM-CSF	Forward: 5'-AAGCCCAGCAGATTCTCAAT-3'OH Reverse: 5'-TCTAGACGTTCCCTTGCACG-3'OH

0.025% Triton X-100, followed by incubation with each of the monoclonal antibodies. After several rinses, the sections were incubated with biotinylated goat anti-rat IgG antibody and then with avidin-biotin-horseradish peroxidase complex. After washing, the sections were transferred to and incubated with a mixture of 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml) (WAKO, Osaka, Japan) and H₂O₂ at a final concentration of 0.03% in 0.1 M Tris-HCl buffer at pH7.6. Counterstaining was produced by methylgreen. As control experiments, sections were incubated with either a normal rat serum or PBS instead of the primary antibody.

For the double labeling of granulopoiesis and erythropoiesis, sections were stained first with anti-TER-119 antibody coupled with Alexa Fluor 594-labeled goat anti-rat IgG antibody, and then with Alexa Fluor 488-labeled Gr-1. After several rinses with PBS, sections were mounted with AquaMount (Polysciences, Warrington, PA).

For the detection of erythropoietin, sections were stained with goat anti-erythropoietin antibody after the pretreatment described above. After thorough rinse with PBS, sections were incubated with biotinylated rabbit anti-goat IgG antibody and processed for the visualization of the immunoreaction products. Control sections were incubated with normal goat serum instead of the primary antibody.

RT-PCR analysis

Humeral and maxillary bones were isolated and homogenized. Total RNA was extracted from each sample using RNeasy Mini kit (QIAGEN, Tokyo, Japan). Four micrograms total RNA served as template for reverse transcriptase polymerase chain reaction (RT-PCR), using GeneAmp Gold RNA PCR (Applied

Biosystems, Tokyo, Japan) with mM-CSF, mG-CSF and mGM-CSF sequence aligned primers. Primers used in this study were listed in Table 1. Amplification conditions were as follows: RT reaction was performed at 25 for 10 min and at 42 for 12 min; PCR reaction start at 95 for 5 min, and then the 35 cycles were consisted of denaturing at 95 for 10 s, annealing at 60 for 30 s and extension at 72 for 2 min. The final extension was at 72 for 7 min. PCR products were mixed with 6×loading dye (TOYOBO Biologics, Osaka, Japan) at the ratio 6:1 (v/v) and separated by electrophoresis at 100 V for 20 min on a 1.0% agarose gel.

Results

Immunohistochemical detection of erythropoiesis and granulopoiesis during bone development

To examine when the primary bone marrow cavity of the humeral bones and the palatal process of maxillary bones was formed, specimens at E15 to 1dPN were sectioned and stained with hematoxylin-eosin. The bone marrow cavity was not formed in the E15 humeral bone; the bone rudiment was composed of cartilage tissue and no primary calcification center was detected. From E16, the bone marrow cavity inside the humeral bones was formed (Fig. 1a, d, g, j). The palatal process of maxillary bones are formed by intramembranous ossification. Bone formation was detected at the palatal region in the E16 mouse fetus (Fig. 3a, d, g, j). Therefore, we decided to start the immunohistochemical detection of hematopoiesis from E16 mouse fetus.

In the bone marrow of E16 humeral bone, Gr-1⁺ cells were detected at the extravascular space. These cells were solely distributed in the bone marrow cavity

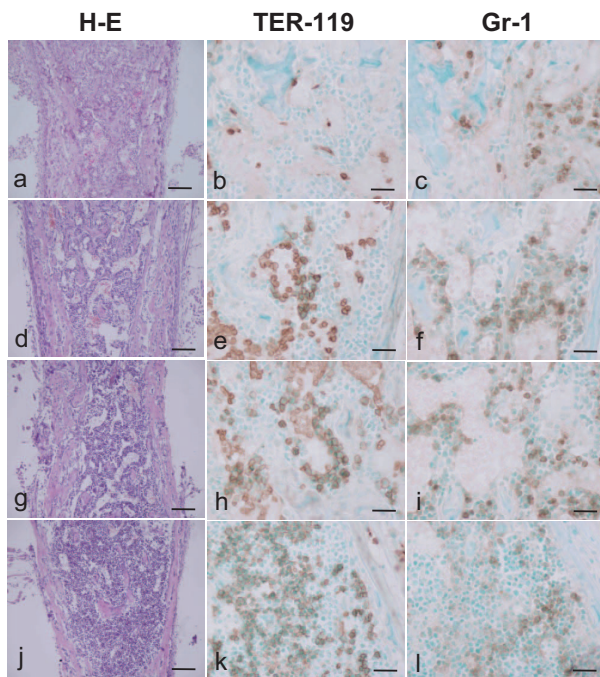


Fig. 1 Ontogenic development of endochondral ossification and hematopoiesis in humeral bones at E16 (a, b, c), E17 (d, e, f), E18 (g, h, i) and 0DPN (j, k, l). Histological features of bone development (a, d, g, i) and immunohistochemical localization of TER-119⁺ cells (b, e, h, k) and Gr-1⁺ cells (c, f, i, l) were represented. Bars=50 μm (a, d, g, h) and 25 μm (b, c, e, f, h, i, k, l).

(Fig. 1c). The number of Gr-1⁺ cells was increased with the development and formed the clusters near to the bone trabecular surface (Fig. 1f, i, l). In the case of erythropoiesis in the humeral bone marrow, TER-119⁺ nucleated erythrocyte-precursor cells were detected from E17 (Fig. 1e). With bone development, these cells formed clusters like Gr-1⁺ cells, which were mainly located adjacent to the vasculature (Fig. 1e, h, k).

These results indicated the difference of sequential development of erythropoiesis and granulopoiesis in two different ossification systems and the difference of the hematopoietic sites between erythropoiesis and granulopoiesis in the bone marrow. Double staining GR-1⁺ and TER-119⁺ cells confirmed clearly the different hematopoietic loci of erythropoiesis and granulopoiesis in the bone marrow. Gr-1⁺ cells were mainly located along the bone surface while TER-119 cells were detected in more central region of the bone

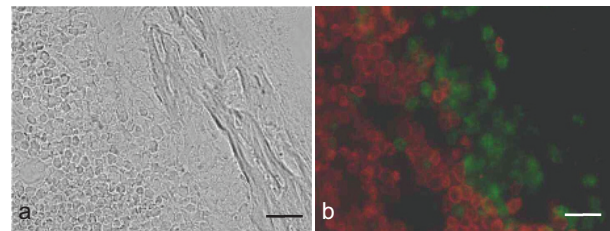


Fig. 2 Double staining of granulopoiesis and erythropoiesis in the humeral bone marrow at 0DPN. (a) A differential-interference image of the bone marrow. (b) Double staining of Gr-1⁺ cells (green) and TER-119⁺ cells (red). Bars=25 μm .

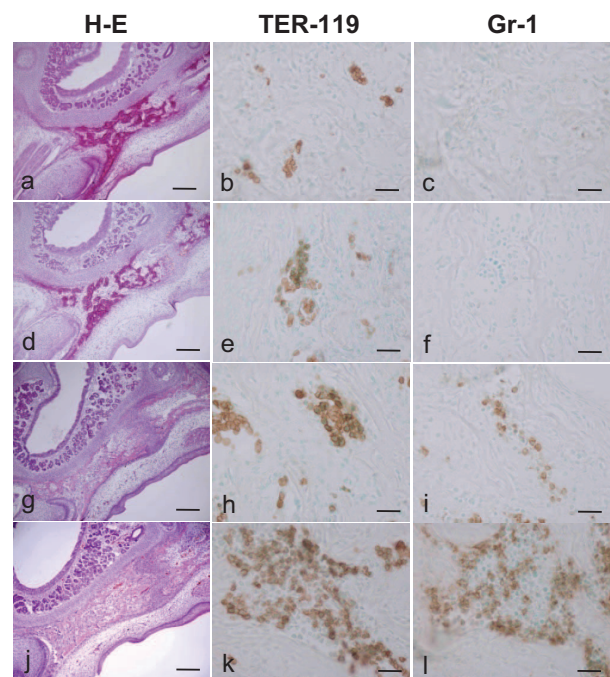


Fig. 3 Ontogenic development of intramembranous ossification and hematopoiesis in the palatal processes of maxillary bones at E16 (a, b, c), E17 (d, e, f), E18 (g, h, i) and 0DPN (j, k, l). Histological features of bone development (a, d, g, i) and immunohistochemical localization of TER-119⁺ (b, e, h, k) and Gr-1⁺ cells (c, f, i, l) were represented. Bars=100 μm (a, d, g, h) and 25 μm (b, c, e, f, h, i, k, l).

marrow (Fig. 2).

In the palatal process of the maxillary bone, TER-119⁺ nucleated cells were first detected at E17 when Gr-1⁺ cells were not yet detected (Fig. 3b, c). With bone development, the number of TER-119⁺ and Gr-1⁺ cells were increased and formed clusters, respectively (Fig. 3h, i, k, l). Hematopoietic loci of TER-119⁺ and Gr-1⁺

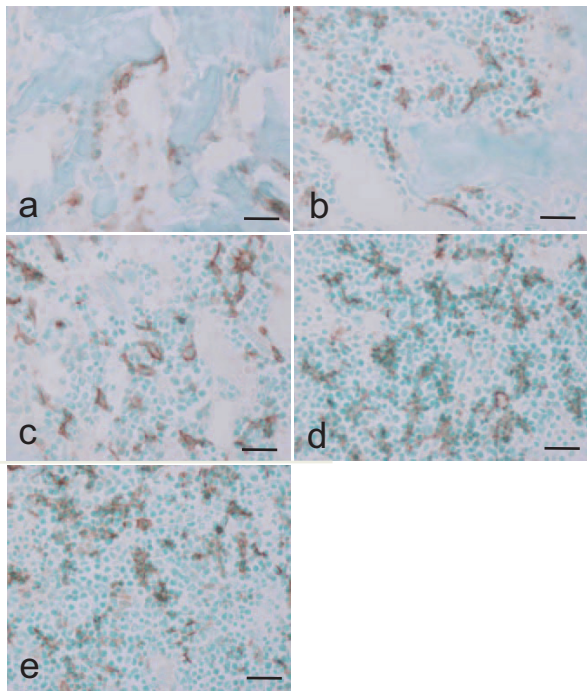


Fig. 4 Immunohistochemical localization of F4/80⁺ macrophages during the development of humeral bones. a: E16, b: E17, c: E18, d: 0DPN, e: 1DPN. Bars-25 μm

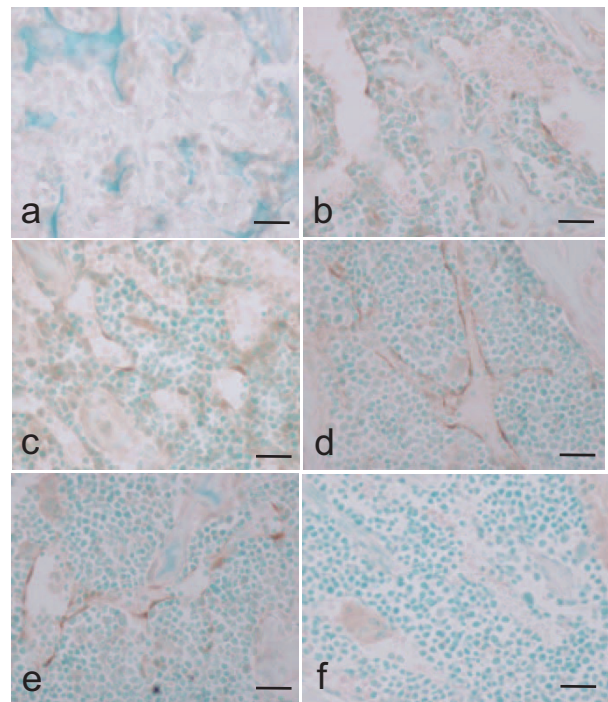


Fig. 6 Immunohistochemical localization of erythropoietin during the development of humeral bones. a: E16, b: E17, c: E18, d: 0DPN, e: 1DPN and f: negative control. Bars-25 μm

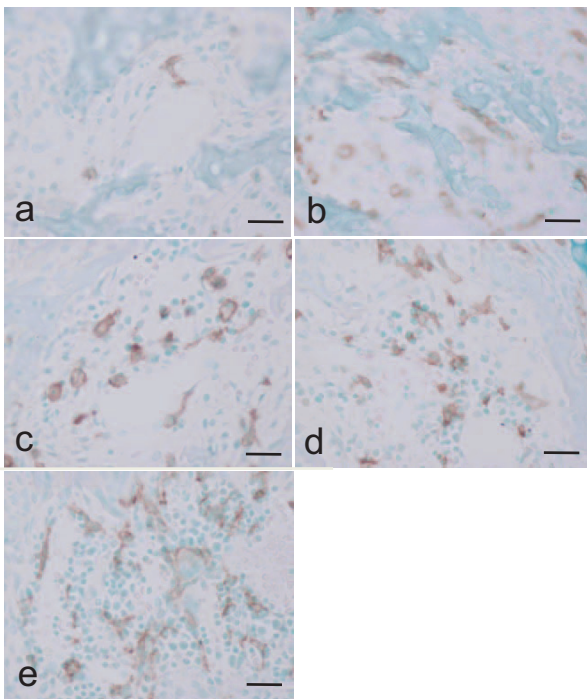


Fig. 5 Immunohistochemical localization of F4/80⁺ macrophages during the development of the palatal processes of maxillary bones. a: E16, b: E17, c: E18, d: 0DPN, e: 1DPN. Bars-25 μm

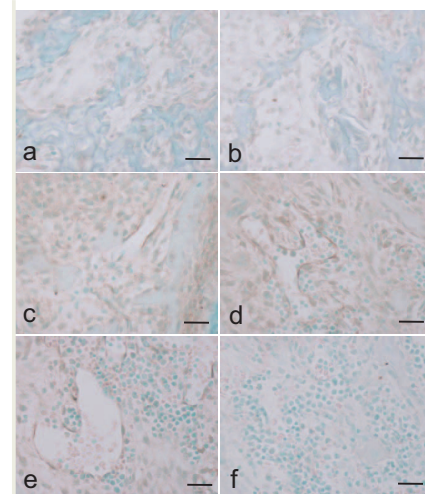


Fig. 7 Immunohistochemical localization of erythropoietin during the development of the palatal processes of maxillary bones. a: E16, b: E17, c: E18, d: 0DPN, e: 1DPN and f: negative control. Bars-25 μm

cells showed same distribution pattern with the blood formation in the humeral bone marrow.

Immunohistochemical detection of macrophages and erythropoietin in the bone marrow

Bone marrow resident macrophages and erythro-

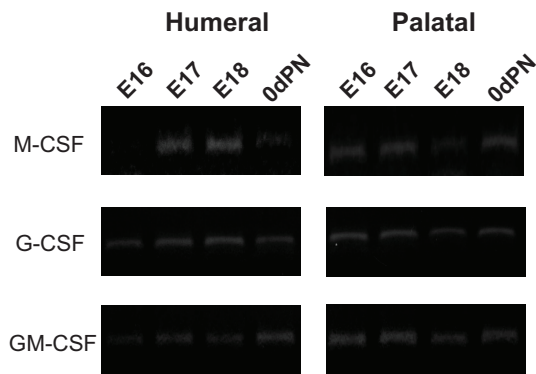


Fig. 8 RT-PCR analysis of the mRNA expression of M-, G- and GM-CSF during the development of two different types of bone ossification.

poietin are the main stroma cells and the key cytokine for erythropoiesis. Therefore, we next examined the immunolocalization of F4/80⁺ macrophages and erythropoietin.

In the humeral bone marrow, F4/80⁺ cells were detected from E16 (Fig. 4a–e). Macrophages at E16 and E17 showed the round or elongated shape and no cell processes (Fig. 4a, b) whereas macrophages from E18 developed the cell processes and were surrounded by hematopoietic cells (Fig. 4c, d, e). In the palatal processes of the maxillary bones, F4/80⁺ macrophages were also detected from at E16 (Fig. 5a). These cells had no cell processes until at E18 (Fig. 5b, c) and developed the processes from 0DPN (Fig. 5d, e).

The expression of erythropoietin was first detected in the vascular endothelial cells at E17 in the humeral bone marrow (Fig. 6b). The staining intensities increased with development (Fig. 6c, d, e). In the maxillary bone, the expression of erythropoietin was not detected at E16 and E17 (Fig. 7a, b). A weak immunoreaction of erythropoietin was detected in the endothelial cells at E18 (Fig. 7c) and then gradually stronger with age (Fig. 7d, e). No immunoreactions were detected in the control sections (Fig. 6f, Fig. 7f).

In both ossification systems, erythropoietin⁺ endothelial cells are mainly located apart from the bone trabecula and no immunoreactions were detected in the endothelial cells near to the bone trabecula (Fig. 6, 7).

RT-PCR analysis of cytokine expression in the bone marrow

Humeral bones and the palatal processes of maxillary bones from E16 to 0dPN were analysed for the expression of M-CSF, G-CSF, and GM-CSF (Fig. 8). From E17 to 0DPN, three cytokines were constitutively expressed in the humeral bones. At E16, G-CSF and GM-CSF were also expressed while M-CSF was not detected in the humeral bone. On the contrary, the expression of three cytokines was detected at all of the examined stages in the maxillary bones.

Discussion

In this study, we demonstrated the time kinetic difference and the sequential difference of erythropoiesis and granulopoiesis in the bone marrow during the ontogenic development of two different types of bone formation. In the humeral bones, myelopoiesis was first detected at E16 and then observed erythropoiesis one day after the initiation of myelopoiesis. However, in maxillary bones, erythropoiesis was first detected at E17 and erythropoiesis was detected from E18. Double staining with Gr-1 and TER-119 also indicated the difference of hematopoietic loci between two different ossification systems.

Humeral bone at E16 was the stage that the primary bone cavity was opened and osteoblasts initiated the bone formation on the calcified cartilage trabecula. Solely distributed Gr-1⁺ cells became to aggregate to form clusters with development. These clusters were localized near to the bone surface. As osteoblasts are one of the components of stromal cells in bone marrow by secreting cytokines such as G-CSF and GM-CSF.²⁰ The proliferation of myeloid-lineage cells might be regulated by the cytokines produced by osteoblasts.

In the case of intramembranous ossification, Gr-1⁺ cells could not be detected until E18 in spite of the active bone formation by osteoblasts and the expression of G-CSF and GM-CSF in the hematopoietic sites. Furthermore, erythropoiesis preceded myelopoiesis. These results indicated the

emigration of hematopoietic precursor cells to this hematopoietic site, while osteoblasts might have had no capability to support granulopoiesis even if cytokines related to granulopoiesis were already expressed at this stage. Although osteoblasts are the stromal cells for granulopoiesis, several studies indicated the phenotypic and functional heterogeneities of osteoblasts.^{21, 22)} It has been well understood the body skeleton is derived from neural crest and from mesoderm.²³⁾ Indicated the expression of priostin in the extracellular matrix during intramembranous but not endochondral ossification.²⁴⁾ Indicated that embryonic origin and Hox gene expression status distinguished neural crest-derived from mesoderm-derived osteogenic progenitor cells, and both characteristics influenced the process of adult bone regeneration. Runx1, a master regulator of gene expression in hematopoiesis, and Sox9 expressed in the region of craniofacial bone development but not detected in the area of endochondral bone formation.²⁵⁾ These results suggest the phenotypic and/or functional difference of osteoblasts between endochondral and intramembranous bones, and it might be, therefore, conceivable that osteoblasts in maxillary bone at E16 and E17 may function differently to hematopoiesis.

Macrophages and erythropoietin are the prerequisite cells and factor for erythropoiesis in the bone marrow. Resident macrophages in the bone marrow showed the well developed cell processes surrounding many erythrocyte-lineage cells.²⁶⁾ In general, macrophages are derived from monocytes that circulate in the blood and exude into the each specific region and function as resident macrophages. M-CSF and GM-CSF are critical for macrophage differentiation and function. GM-CSF-deficient mice develop alveolar proteinosis due to impaired surfactant catabolism by alveolar macrophages.²⁷⁾ In M-CSF-deficient osteopetrotic (*op/op*) mice, monocytes, resident macrophages and osteoclasts are deficient. In *op/op* mice the bone marrow cavities filled with spongy bone. Administration of M-CSF into *op/op* mice recovered the marked reconstruction of bone marrow cavities and

marrow hematopoiesis was expanded. The numbers of osteoclasts and bone marrow macrophages in *op/op* mice were also increased.^{28, 29)} During endochondral ossification, differentiated macrophages were already penetrated into the perichondrium before the formation of the primary bone cavity and migrated into the bone marrow cavity with the formation of the cavity.³⁰⁾ In this study, erythropoiesis could not be detected at E16 humeral bone marrow in spite of the presence of macrophages. M-CSF was not expressed at this stage. These results may suggest that macrophages at this stage have no capability to support erythropoiesis and that appearance of stromal macrophages to support erythropoiesis is initiated by the expression of M-CSF from E17.

In intramembranous ossification, erythropoiesis preceded granulopoiesis. Macrophages were localized in the hematopoietic regions between the bone trabecula from E16 when the expression of M-CSF was also detected while erythropoiesis was not initiated until E18. Therefore, these results lead to suggest the consideration of the other key factor(s) for the onset of erythropoiesis in addition to the homing of hematopoietic stem cells into this site.

An unexpected result in this study was the expression of erythropoietin in the bone marrow endothelial cells. In both types of forming bones, endothelial cells near to the bone and in the surrounding tissues did not stain with the antibody. It has been well documented that erythropoietin is produced mainly by interstitial fibroblasts in the kidney³¹⁾ and stimulates the formation and differentiation of erythroid precursor cells in the bone marrow.^{32, 33)} Recently, it has been also indicated that erythropoietin is produced by a variety of cells such as endothelial cells, myoblasts and central nervous system neurons and that erythropoietin has a broad spectrum of tissue protecting actions affecting other systems than hematopoiesis.^{34~38)}

Immunohistochemical study indicated that erythropoietic loci in both types of bone formation were localized close to the vasculature. These results may suggest that

the expression of erythropoietin by endothelial cells during the early development of hematopoiesis in the bone marrow may initiate the onset of erythropoiesis with the development of marrow resident macrophages by M-CSF.

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