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Age-related changes in bone morphology, function, and cell populations in inbred C57BL/6N mice

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Abstract

Bones play crucial roles in controlling motility, regulating electrolyte metabolism, and hematopoiesis. We examined age-related changes in bone morphofunction using 3-, 6-, and 13-month-old male C57BL/6N mice. Tibia weight and length generally increased with age. Phosphorus, hemoglobin, and hematocrit measurements in blood, indices of electrolyte metabolism and hematopoiesis, significantly decreased with age. Bone histology showed that osteocyte and osteoblast numbers in the tibia were significantly correlated with decreases in hemoglobin levels and hematocrit values. Furthermore, platelet levels in blood increased with age, negatively correlating with osteoblast and osteoclast number. Thus, we have demonstrated age-related changes in bone morphofunction in healthy mice, particularly quantitative alternations of bone-composing cells and hematopoietic activities.

Key Words: aging, bone, erythrocyte

The increasing age of the human population is a serious concern in developed countries such as Japan[1)]. Aging can increase the risk of developing chronic kidney disease, cardiovascular disease, and infectious diseases[3,8,19)]. Osteoporosis is an age-related disease in humans that can lead to accidental fractures and elderly populations becoming bedridden[10)]. The life span of companion animals is also increasing due to advances in veterinary medicine[13)], where osteoporosis is also seen in elderly dogs and cats[5,18)]. Aging and nutritive imbalance, rather than gonadectomy, is associated with pathogenesis in animals[12,15)].

Bones play an important role in assisting motor function and in maintaining electrolyte balance of circulating calcium (Ca), and phosphorus (P). Furthermore, bones play a crucial role in immunity as the bone marrow (BM) is located deep within them. The function of BM is strongly affected by cells that compose bones, such as osteoblasts, osteoclasts, and osteocytes, participating in the production of hematopoietic stem cells (HSCs) and/or progenitor cells[2,4,11)]. Therefore, any alterations in bone morphology

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Table 1. The altered parameters including bone, serum level and blood cells with aging.

Parameter		Unit	Age		
			3 months	6 months	13 months
Body weight		g	26.3	34.5*	34.8*
Tibia and fibula weight		mg	138.6	148.8	164.3*
Tibia and fibula weight/body weight		%	0.54	0.43*	0.46
Tibia length		mm	17.77	18.73*	19.39*
S/B		%	0.26	0.21	0.23
Serum analysis	Ca	mg/dl	8.63	8.66	8.25
	iP	mg/dl	11.29	12.58	9.5#
	PTH	pg/ml	374.2	284.8	458.4
Hematological analysis	WBCs	Number × 10/μl	514	574	332*
	RBCs	Number × 10^4/μl	978.8	976.8	922.8
	PLs	Number × 10^3/μl	1216	1670	1966
	HC	g/dl	15.1	14.0	13.0*
	HV	%	48.7	44.3	42.8*
	MCV	fL	49.7	45.3*	46.3
	MCH	pg	15.5	14.3*	14.1*
	MCHC	g/l	311.7	315.8	304.0
Bone histology	Bone area		0.88	0.71	0.71
	Osteocyte density	Number/mm^2	606.7	472.5*	505.6*
	Osteoclast	Number/mm	1.30	0.68	0.77
	Osteoblast	mm^2/mm	0.37	0.20*	0.11*

*: significant difference with 3 months, #: significant difference with 6 months (Kruskal-Wallis test followed by Scheffé's method, $P<0.05$). n = 4-5. S/B: Ratio of spleen weight to body weight, Ca: Calcium, iP: Inorganic phosphorus, PTH: Parathyroid hormone, WBCs: Number of white blood cells, RBCs: Number of red blood cells, PLs: Number of platelets, HC: Hemoglobin concentration, HV: Hematocrit values, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular HC, Bone area: Area ratio of bone to bone marrow, Osteoclast: Number of $TRAP^+$ osteoclasts, Osteoblast: Numerical value of $osteocalcin^+$ osteoblasts.

due to aging could affect bone function, suggested by anemia seen in the elderly[6]. Senescence-accelerated mouse (SAM) strains have been used as animal models to analyze these relationships. In particular, the SAMP6 substrain exhibits age-related osteoporosis, characterized by decreased bone weight, osteoblast hypoplasia, and osteoclast hyperactivity without any alterations in mineral components of the bones[16]. However, SAMP6 mice develop colitis, indicating a high susceptibility to immune-related diseases. Therefore, to evaluate the effects of aging on bone phenotypes and to exclude the effects of any disease-modifying factors, a healthy strain must be analyzed.

In this study, we analyzed bones of healthy 3-, 6-, and 13-month-old (mo) C57BL/6N mice, with a particular focus on morphology, osteocyte counts, osteoblast counts, osteoclast counts, hematopoiesis, and changes in blood mineral composition in order to evaluate functional activity of their bones. Animal use protocol was approved by the President of Hokkaido University after the review by the Institutional Animal Care and Use Committees, Hokkaido university and Faculty of Veterinary Medicine, Hokkaido University (Approval number: 16-0124). The number of white blood cells (WBCs), red blood cells (RBCs), and platelets (PLs) was measured

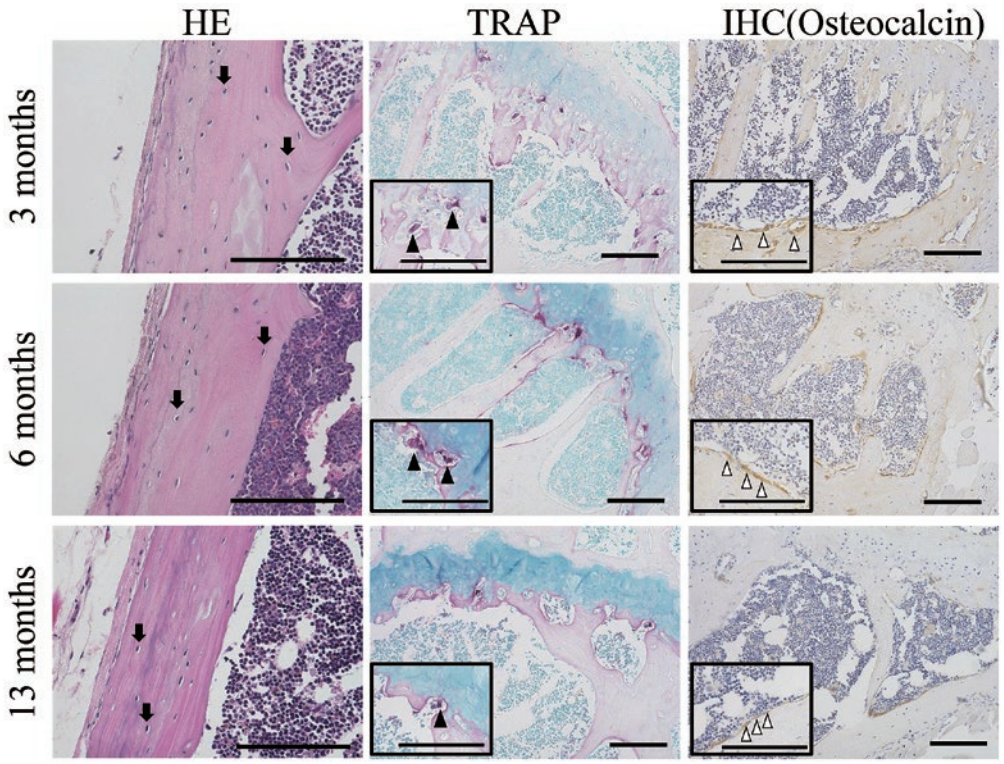

Fig. 1 Histological difference of bones. Tibia histology. In hematoxylin and eosin (HE) staining (left), the interval between osteocytes (black-arrow) increase. In tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts (middle, black-arrowhead) and immunohistochemistry (IHC) of osteocalcin for osteoblasts (right, white-arrowhead), the positive cells at 13 months tended to decrease compared with 3 and 6 months. Bars = 100 μm.

using an XT-1800i (Sysmex Corporation; Kobe, Japan). Hemoglobin concentration (HC), hematocrit values (HV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular HC (MCHC) were measured using an XT-1800i (Sysmex Corporation; Kobe, Japan). Serum Ca and inorganic P (iP) levels were measured using a Fuji Dri-Chem 7000v (FUJIFILM Medical Co., Ltd.; Tokyo, Japan). Serum parathyroid hormone (PTH) concentration was measured using a mouse PTH 1-84 ELISA kit (Quidel Corporation; San Diego, CA, USA). The spleen to body weight (BW) ratio was also calculated.

To assess bone morphology, the wet weights and lengths of the tibia and fibula were measured, and BW ratios were calculated. Bones were fixed with 4% paraformaldehyde, decalcified with the aqueous solution including 5% ethylenediaminetetraacetic acid, 7% sucrose and 0.5% sodium hydroxide, dehydrated with ethanol, and embedded in paraffin. To detect osteocytes or osteoclasts, paraffin sections were stained using hematoxylin and eosin (HE), or tartrate-resistant acid phosphatase (TRAP; FUJIFILM Wako Pure Chemical Corporation). Immunohistochemistry was performed with an osteocalcin antibody (1:3200, Cat No. M173, Takara Bio, Shiga, Japan) to detect the osteoblasts. The numbers of these cells were quantified by histoplanimetry.

The BW of C57BL/6N mice increased significantly with age (Table 1). The weights of tibia and fibula tended to increase with age, where 13 mo were significantly higher compared with 3 mo mice. Further, the bone to BW ratio was significantly decreased in 3 mo compared to 6 mo. The spleen to BW ratio did not differ among groups, indicating no significant age-

related changes in the systemic immune status. For the indices of blood mineral dynamics, the serum Ca and PTH levels did not differ with age. However, serum iP levels in 13 mo significantly decreased compared with 6 mo mice. Among hematopoietic parameters examined in this study, only PL levels tended to increase, whereas all the other parameters tended to decrease. In particular, HC, HV, and MCH were significantly lower in 13 mo than in 3 mo mice, while MCV and MCH were significantly lower in 6 mo than in 3 mo mice. These quantitative data indicated age-related alternation of bone morphology and hematopoiesis, particularly in erythrocytes.

Table 1 summarizes the quantitative histological analysis of the tibia. HE staining indicated that bone to BM area tended to decrease, and the number of osteocytes were significantly decreased in 3 mo mice compared to 6 mo (Fig. 1 and Table 1). $TRAP^+$ osteoclasts and $osteocalcin^+$ osteoblasts were observed mainly in the peripheral lumen of the BM; however, no morphological changes were observed with age (Fig. 1). Importantly, the number of osteoclasts and osteoblasts tended to decrease with age, where the latter was significantly decreased from 3 mo to 6 mo, indicating that bone remodeling might start to be low turnover from 6 mo.

We also evaluated correlations among bone morphology indices and hematopoiesis or blood mineral components by Spearman's correlation analysis ($P < 0.05$). No bone morphology indices correlated with changes in blood mineral components. However, significant positive correlations were observed in the number of osteocytes (compared to RBCs, HC, HV, MCV), osteoclasts (compared to HC, HV, MCV, MCH), and osteoblasts (compared to RBC, HC, HV, MCH). On the other hand, the numbers of osteoblasts and osteoclasts were significantly negatively correlated with PLs.

Thus, we found age-related reduction in bone-composing cells and altered hematopoietic properties in healthy mice. In this study, although bone morphology was evidently changed with aging, only serum iP, and not Ca, was significantly decreased in 13 mo mice. Serum Ca levels are restrictively controlled by absorption from the intestine and kidney, and is regulated by multiple hormones. This suggested that measuring serum iP was a more sensitive method and reflective of altered bone morphofunction. Importantly, age-related morphological changes of bone were closely related to hematopoiesis, characterized by microcytic and hypochromic features, decreased HC, and increased PL levels. Other reports have also indicated that both osteocyte counts and HC decrease with age in mice and humans[7,14,17]. One study revealed that osteocyte density was significantly lower in femurs at 22 months than at 5 months[17]. However, we observed significant differences in the tibias between 3 and 6 mo mice, indicating that osteocyte density started decreasing at an earlier age. Osteocytes have higher longevity than osteoblasts and osteoclasts, and are highly susceptible to accumulating cell injuries with age[9]. Furthermore, osteocytes can mobilize HSCs[2], and osteoblasts contribute towards HSC production[4]. Therefore, increasing age-related cell damage contributes to the decrease in the number of bone-composing cells, particularly those of osteocytes and osteoblasts. This may be associated with altered hematopoiesis in mice. Age-related increases in PLs (http://www.jslc.co.jp/pdf/mouse/004_C57BL6NCr2013.pdf), imbalances in PL production via humoral factors, and their destruction might relate to altered bone morphofunction.

In conclusion, we demonstrated that age-related changes in bone morphology and function in healthy mice were characterized by decreased numbers of osteocytes and osteoblasts, as well as, altered erythrocyte production. These findings are important when considering age-related changes in hematopoiesis and bone morphology in human and animals.

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