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<Short Communication>

Appearance of osteoporosis in rat experimental autoimmune encephalomyelitis

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Abstract : Experimental autoimmune encephalomyelitis (EAE) in Lewis rats is characterized by transient paralysis followed by recovery. To evaluate whether transient paralysis in EAE affects bone density, tibiae of EAE rats were morphologically investigated using micro-computed tomography and histology. The parameters of bone health were significantly reduced at the peak stage of EAE rats relative to those of controls ($p < 0.05$). The reduction of bone density was found to remain unchanged, even in the recovery stage. Collectively, the present data suggest that osteoporosis occurs in paralytic rats with monophasic EAE, possibly through the disuse of hindlimbs and/or autoimmune inflammation.

Keywords : autoimmunity, experimental autoimmune encephalomyelitis, micro-computed tomography, osteoporosis

Experimental autoimmune encephalomyelitis (EAE), an animal model of human autoimmune disease including multiple sclerosis (MS), is caused by autoimmune T cells, and consequently characterized by paralysis [1]. The neuropathological characteristics of rat EAE consisted of infiltration of T cells and macrophages into the subarachnoid space during the early stages of lesion development [13], with subsequent activation of microglia, immune cell apoptosis, and reactive astrogliosis during the peak stage of the disease [1]. Both pro-inflammatory and anti-inflammatory cytokines are important mediators during the induction of recovery from EAE [14]. Prior to neuroinflammation, proliferation of T cells and migration of T cells and macrophages have been occurred in the lymphatic tissues and circulating blood, respectively. Peripheral inflammation has been deeply associated with bone metabolism, increasing bone resorption through the induction of osteoclastogenesis or inhibition of osteoprogenitors [9].

MS is a chronic inflammatory-demyelinating disease of the nervous system [4]. There has been mounting evidence showing that MS is associated with increased risk of osteoporosis and fractures [4]. The development of osteoporosis in MS patients can be related to the cumulative effects of various factors including majority reduced mechanical load on the bones (offsetting gravity), low vitamin D levels, and use of medications such as glucocorticoids and anticonvulsants [4].

Osteoporosis, a condition characterized by low bone mass and deterioration of the skeletal microarchitecture, is a degenerative bone disease that can develop in accordance with reduced estrogen levels during and after menopause and following long periods of disuse [3]. The absence of mechanical loading in rats decreases bone formation and increases bone resorption [12]. Hindlimb unloading induces bone loss or reduces bone gain in cortical and cancellous bones, in addition to osteocyte apoptosis in cancellous bone [10].

Even though rat EAE is behaviorally characterized by the hindlimb paralysis, little is known on the bone structure. The aim of this study was to evaluate whether Lewis rats with acute monophasic EAE is associated with bone loss.

Lewis rats were obtained from Harlan (Indianapolis, IN, USA) and were bred in our animal facility. Rats of both sexes (7–8 weeks old; 160–200 g) were used. All animal experiments were conducted in accordance with the Jeju National University Guide for the Care and Use of Laboratory Animals (permit No. 2013- 0018).

Experimental procedures, including EAE induction, were conducted as described in our previous studies [1]. Following immunization, rats were observed daily for clinical signs

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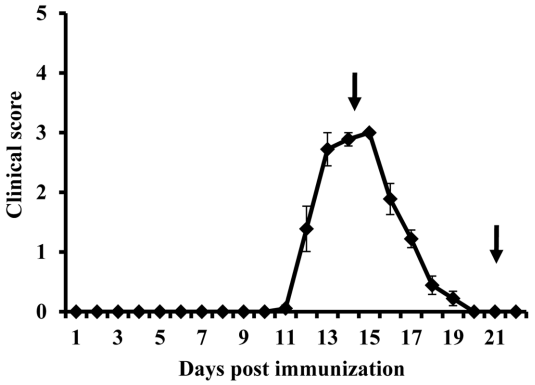

Fig. 1. Clinical scores in Lewis rats with experimental autoimmune encephalomyelitis (EAE). Behavioral changes of EAE rats were scored using a scale ranging from 0 to 5. Animals were sacrificed at each time point ($n = 5$ per group, PI days 14 and 21, indicated by arrows). Data are presented as mean paralysis scores ± SE.

of EAE, and body weight was recorded. EAE progression was subdivided into eight clinical stages: grade 0 (G.0), no sign; G.0.5, mild floppy tail; G.1, completely floppy tail; G.2, mild paraparesis; G.3, severe paraparesis; G.4, tetraparesis; G.5, moribund condition or death; and R.0, recovery.

After euthanasia under CO_2 inhalation, spinal cords and hindlimbs were sampled at the disease peak (G.3, day 14 post induction [PI]) and recovery stages (R.0, day 21 PI; $n = 5$ per time point). Normal and CFA-immunized rats were used as controls ($n = 5$ per group). The left tibiae were obtained and assessed for bone mineral density (BMD) and structural analysis using micro-computed tomography (CT). The serum levels of osteocalcin were measured with ELISA kit according to the manufacturer's instruction (Takara Bio, Japan). Paraffin wax-embedded tissues were cut into 6-μm-thick sections using a rotary microtome (Leica, Germany). Tissue sections were stained routinely with hematoxylin–eosin to evaluate inflammation and measure the height of the upper tibial epiphyseal plate.

The height of proximal tibial epiphyseal plates was determined in approximately five randomly chosen fields, taken from five representative sections per group, followed by 100× microscopic evaluation in conjunction with a digital camera (ProgRes CFscan; Jenoptik, Germany). Measurements were made using the Prog Res Capture Pro software package (ver. 2.5; Jenoptik).

Morphological measurements were calculated using the micro-CT scans from each mouse and the Skyscan 1172 apparatus (Skyscan, Belgium). Image slices were reconstructed and analyzed using CT and Skyscan software (Skyscan).

For all three independent experiments, data are presented as means ± SE. Results were analyzed using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls *post hoc* test for multiple comparisons. A p value < 0.05 was taken to indicate statistical significance.

MBP-immunized Lewis rats developed floppy tails (G.1) on PI days 9–11 and exhibited progressive hindlimb paralysis (G.2 or G.3) on PI days 12–14; they were recovered from paralysis after PI day 17 (R.0; Fig. 1).

The mean epiphyseal plate height decreased by 39.33% during peak stage EAE, which represented a significantly greater reduction compared with controls ($p < 0.001$) (Fig. 2A). During recovery stage EAE, epiphyseal plate height of tibia decreased by 16.6%, which did not represent a significantly greater decrease relative to the age-matched controls. Histological examination revealed that the mean epiphyseal plate height of the proximal tibiae differed between control and EAE rats (Fig. 2B–D). During peak-stage EAE (G.3, PI day 14), BMD was decreased significantly compared with controls ($p < 0.01$), which perseverated into the recovery stage (R.0, PI day 21) even though the rats had recovered from paralysis (Fig. 2E). In addition, osteocalcin, one of bone turnover markers, was significantly increased at the peak-stage EAE (G.3, PI day 14) compared with those of controls ($p < 0.05$), followed by decrease at the recovery stage (R.0, PI day 21) (Fig. 2I).

Table 1 summarizes the results of the micro-CT analysis in normal controls and EAE-affected rat tibia. Bone volume density, trabecular thickness and number were decreased significantly in EAE rats compared with controls ($p < 0.01$). Trabecular separation was increased significantly in EAE relative to control rats ($p < 0.01$). The cortical bone volume and cortical polar pattern factor of EAE rats were decreased significantly compared with controls ($p < 0.01$). Proximal tibial metaphysis images were obtained using micro-CT in control rats and during each disease stage in EAE rats (Fig. 2F–H). Compared with normal controls, EAE rats exhibited reduced cancellous bone mass and structural density.

This is a first study to demonstrate altered bone mass and structure in the tibiae of EAE affected rats during the course of paralysis and subsequent recovery of rat EAE. Our data indicate that BMD and epiphyseal plate height in the tibial metaphysis were significantly decreased at the peak stage of EAE compared with those of age-matched controls. The serum level of osteocalcin was significantly increased at the peak stage of EAE. The reduced thickness of each value remained unchanged even at the recovery stage of EAE.

The increased level of serum osteocalcin has been known to be associated with decreased BMD in postmenopausal women [8], and also confirmed in osteoporotic rat models [7]. Taken into considerations, we postulated that decreased level of BMD in tibia of EAE affected rats has an inverse relationship with increased level of osteocalcin as far as acute monophasic rat EAE is concerned.

Even though the mechanism underlying bone loss in EAE rats is not elucidated yet, we postulate that pro-inflammatory mediators including TNF-alpha would be involved in EAE-related bone loss. These findings are further supported by previous studies including rheumatoid arthritis [5] and ankylosing spondylitis [15] and periodontitis [6], in which periph-

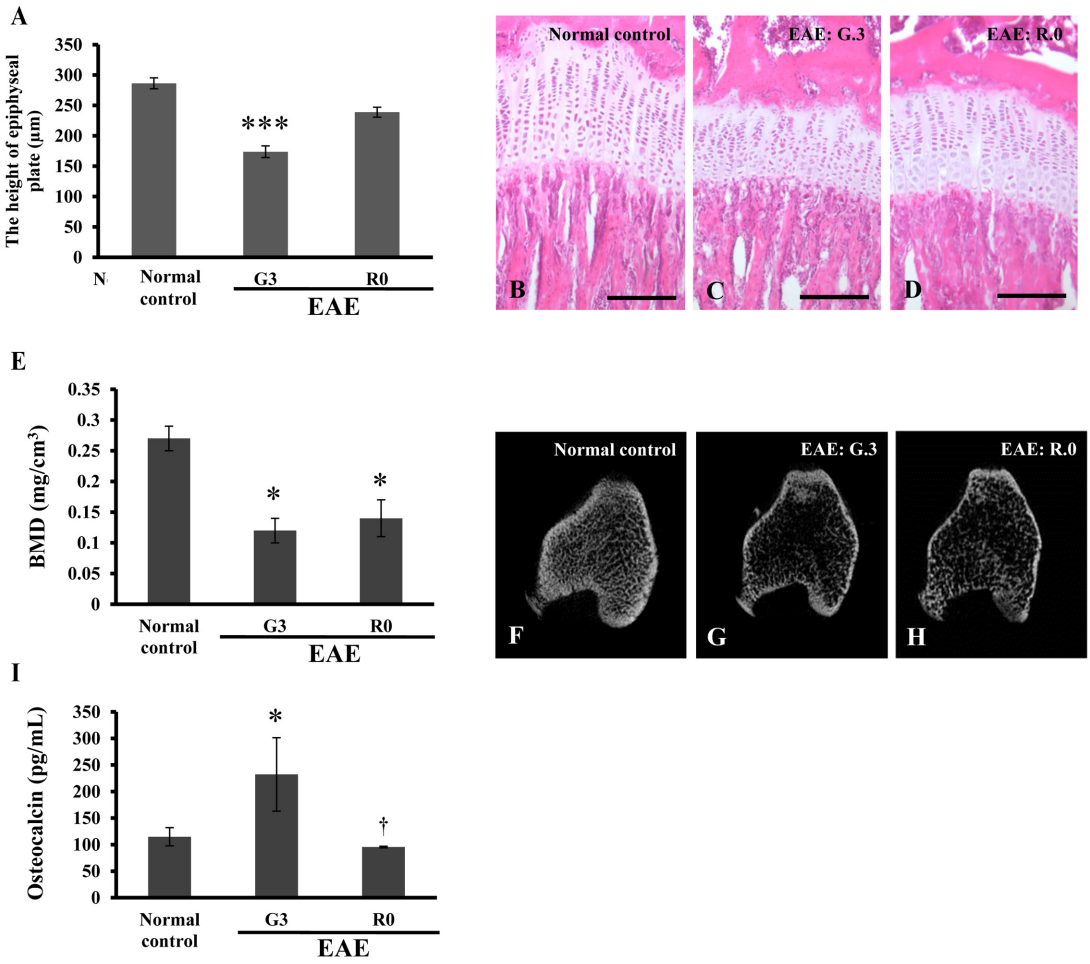

Fig. 2. (A) The height of proximal tibiae epiphyseal plate in rats with EAE was measured under a microscope. ***$p < 0.001$, compared with age-matched controls. Histological findings in (B) controls and (C) during peak- (G.3, PI day 14) and (D) recovery-stage EAE (R.0, PI day 21) Rat proximal tibiae. (E) Bone mineral density at the tibial metaphysis in control and EAE rats. *$p < 0.05$ *vs.* controls. *Ex vivo* 2-D micro-computed tomography (CT) images of a proximal tibial metaphysis demonstrating cancellous bone mass and structure of EAE rats during paralysis (G.3) and recovery (R.0). (F) Controls. (G) EAE G.3. (H) EAE R.0. (I) Serum level of osteocalcin in control and EAE rats. *$p < 0.05$ *vs.* controls; †$p < 0.05$ *vs.* peak stage of EAE. Scale bars = 100 μm (B–D and F–H).

Table 1. Evaluation of structural cortical geometric and trabecular microstructural properties of tibial metaphyses *ex vivo* using micro-CT

Parameter		Control	EAE : G3	EAE : R0
Trabecular	Bone volume density (%)	12.71 ± 2.57	4.44 ± 1.04**	5.83 ± 1.39**
	Thickness (μm)	63.69 ± 2.77	59.17 ± 4.87	57.04 ± 1.10**
	Number (1/mm)	1.99 ± 0.37	0.75 ± 0.17**	1.02 ± 0.24**
	Separation (μm)	213.71 ± 20.88	461.38 ± 51.01**	395.12 ± 79.15**
	Bone moment of inertia (1/mm)	17.01 ± 3.59	22.21 ± 2.61	20.64 ± 4.26
	Structure model index	1.87 ± 0.13	1.91 ± 0.08	1.81 ± 0.19
Cortical	Bone volume (mm^3)	7.84 ± 0.19	6.35 ± 0.23**	5.89 ± 0.12**
	Polar pattern factor (mm^4)	8.13 ± 0.59	5.71 ± 0.25**	5.29 ± 0.30**

*$p < 0.05$, **$p < 0.01$ *vs.* control group ($n = 4$ per group).

eral inflammation partly suppressed osteoblasts as well.

The absence of mechanical loading in rats decreases bone formation and increases bone resorption [12]. Hindlimb unloading induces bone loss or reduces bone gain in cortical

and cancellous bones, in addition to osteocyte apoptosis in cancellous bone [10].

In unloading models of osteoporosis [2, 10, 12], it has been known that the absence of mechanical load results in a reduction in bone formation, inhibition of longitudinal growth, and a decrease in the number of osteoblasts and osteoprogenitors in cancellous bone following increased osteocyte apoptosis. One possible factor in unloading associated osteoporosis has been supposed as the production of nitric oxide (NO) and increased expression of NO synthase (NOS) [2]. Even though NO involvement was not evaluated in the present study, we postulate that NO, via NOS, would also be associated with BMD in EAE rats, because NOS and NO are upregulated consistently in EAE-affected central nervous system tissues [11].

Taken together, the present evidence suggests that transient paralysis in rat acute monophasic EAE directly and/or indirectly influences bone metabolism leading to osteoporosis, possibly through the action of pro-inflammatory mediators and/or unloading.

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