学 位 論 文 の 要 旨

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学 位 論 文 名　TNX Deficiency Results in Bone Loss Due to an Increase in Multinucleated Osteoclasts

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論 文 内 容 の 要 旨

INTRODUCTION

Tenascin-X (TNX), a glycoprotein of extracellular matrix (ECM), is expressed in various tissues and plays an important role in ECM architecture. ECM is an integral component that not only provides physical support to the cells, but also a dynamic structure which controls tissue homeostasis. TNX encoded by the *TNXB* gene is the largest member in the tenascin family and its deficiency is known as the cause of classic-like Ehlers-Danlos syndrome (clEDS). EDS is a heterogeneous group of disorders, characterized by fragility of connective tissues and widespread manifestations in skin, ligaments, joints, blood vessels, and internal organs. Further, osteoporosis is also observed in a subset of patients with EDS. TNX has been found to play roles in dermal, muscular and obstetric disorders, but as yet its role in bone homeostasis has not been thoroughly investigated.

In this study, we aimed to clarify the contribution of TNX to bone metabolism, and investigated the bone phenotype and the osteoblastic and osteoclastic differentiation potentials of bone marrow cells in *Tnxb*-KO mice. Our data indicated that TNX deficiency enhances osteoclast maturation and activates bone resorption. This provides a new insight into understanding of bone metabolism and the pathology of clEDS in patients with TNX deficiency.

MATERIALS AND METHODS

Wild-type (WT) C57BL/6J mice and *Tnxb*-KO mice were used in the experiments. All

experiments with animals in this study were approved by the Ethics Committee for Animal Experimentation of Shimane University.

For histological analysis, the dissected femurs were fixed in 10% formalin and subjected for micro-computed tomography (micro-CT) and hematoxylin and eosin (HE) staining. Fixed femurs were immersed in 10–30% sucrose and embedded in 4% carboxymethyl cellulose (CMC), then sectioned (5 μm) and stained with HE. For micro-CT analysis, the femurs were wrapped in parafilm and scanned using a Skyscan 1174 micro-CT machine. Scanned three-dimensional images were reconstructed using the NRecon program. 3D analysis of trabecular and cortical bone was performed using the CTAn program and the images were generated in CTvol program.

For osteoblast and osteoclast differentiation, primary bone marrow cells isolated from femurs were seeded at $3.5x10^6$ cells for osteoblast and $1.5x10^6$ cells for osteoclast per well in 24-well plates, respectively. For osteoblast differentiation, the cells were cultured for 2 weeks in osteoblast differentiation medium, α-minimum essential medium (α-MEM) containing 10% foetal bovine serum (FBS) and 2 mM GlutaMAX supplemented with 0.1 μM dexamethasone, 10 mM β-glycerophosphate and 0.3 mM L-ascorbic acid. Afterwards, these cells were stained using a calcified nodule staining kit with alizarin red S. For osteoclast differentiation, the cells were stimulated with 50 ng/mL macrophage colony-stimulating factor (M-CSF) for 3 days and adherent cells were cultured in osteoclast differentiation medium containing M-CSF and receptor activator of nuclear factor kappa-B ligand (RANKL) for 4 days. Then these cells were stained using a tartrate-resistant acid phosphatase (TRAP) staining kit and subjected for bone resorption activity assay.

For gene expression analysis, total RNA from crushed bones and cultured cells were subjected for cDNA synthesis followed by RT-PCR and real-time PCR.

RESULTS AND DISCUSSION

To investigate the involvement of TNX deficiency in bone mass, we performed histological analysis of bone in *Tnxb*-KO mice. HE staining indicated that both trabecular bone mass and cortical bone thickness were reduced in *Tnxb*-KO mice compared with WT mice. Further, micro-CT analysis revealed that both bone mass and bone mineral density (BMD) of trabecular bone were significantly lower in *Tnxb*-KO mice than those of age-matched WT mice. Meanwhile, in cortical bone, BMD, cortical section and medullary area were mostly comparable, but bone volume, bone area and thickness were markedly lower than in WT mice at 8 months of age. These results indicate that TNX contributes particularly to the development of trabecular bone.

Next, we examined whether bone loss in TNX-deficient mice resulted from impairment in osteoblast or osteoclast differentiation. Although osteoblast differentiation was not affected by

TNX deficiency, the number of TRAP-positive osteoclasts were significantly increased in *Tnxb*-KO. Interestingly, the number of multinucleated osteoclasts was markedly increased compared with WT (2.9 ± 2.1 cells in WT vs 18.9 ± 7.4 cells in *Tnxb*-KO). These data were supported by increases in the expression of osteoclast markers in *Tnxb*-KO cells during osteoclast differentiation. In addition to osteoclast differentiation, the bone-resorbing ability of *Tnxb*-KO osteoclasts was also significantly higher than in WT cells. These suggest that TNX contributes to osteoclast maturation by suppressing the multinucleation of osteoclasts.

Finally, to examine whether TNX deficiency alters the expression levels of bone-associated genes *in vivo* as well as *in vitro*, we analysed the expression of osteoblast and osteoclast maker genes in bone. No significant differences in the expression of osteoblast or osteoclast markers were observed in bone from *Tnxb*-KO and WT mice, but those expressions did show a tendency to be higher in *Tnxb*-KO mice. Among them, DC-STAMP, which plays a critical role in cell-cell fusion of osteoclasts and macrophages, showed higher expression in *Tnxb*-KO mice compared with WT mice. This is in agreement with our findings in which TNX deficiency promoted the multinucleation of osteoclasts.

Taken together, these data suggest that TNX contributes to bone metabolism by suppressing the osteoclast maturation.

CONCLUSION

The present study showed that TNX-deficient mice exhibited significant bone loss. In addition, our data indicated that TNX deficiency promoted osteoclast multinucleation and increased bone resorption. These results indicate that increased multinucleated osteoclasts are the cause of bone loss in a TNX-deficient environment. Our findings provide a new insight into the mechanism of osteoclast differentiation mediated by TNX and the pathology of clEDS, but further investigation is needed for clarifying the molecular mechanism of bone metabolism in TNX deficiency.