The Localization of Netrin 4 in Vivo

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Abstract

Background: Ntn4 derived from vascular endothelial cells inhibits differentiation of osteoclasts and Ntn4 administration prevents receptor activator of nuclear factor-κB ligand-induced osteoporosis in mice. In this study, the localization of Ntn4-expressing vascular endothelial cells located near osteoclasts and the level of Ntn4 in ovariectomized mice were determined.

Materials and methods: We performed drill-hole injury experiments and TRAP staining followed by immunostaining for PECAM-1 at day 14. We also investigated whether Ntn4 expression was changed in the serum of ovariectomized osteoporotic mice.

Results: TRAP-positive cells were also found in immediate vicinity of Ntn4-positive vascular endothelial cells during bone healing. We found that serum Ntn4 levels were significantly decreased in ovariectomized osteoporotic mice.

Conclusion: Taken together, Ntn4 may have a critical role for bone metabolism with characteristic localization.

Keywords: Netrin 4; Osteoclast; Vascular endothelial cells; Ovariectomy

Introduction

Bone regenerative treatment is one of the important methods in the region of dental medicine. Bone metabolism is orchestrated by various organs and systems, such as the nervous and vascular systems, via several hormones and cytokines [1]. Our previous studies and those reported by others demonstrated that netrins, which are involved in regulation of the nervous and vascular system, play a vital role in bone metabolism [2-5].

Mammalian netrin family, including netrin 1, netrin 3, netrin 4 (Ntn4), netrin 5, netrin G1 and netrin G2, regulates both axon guidance and angiogenesis. Among these netrins, Ntn4 is involved in lymphangiogenesis, negative regulation of corneal epithelial cell proliferation, retinal branching in ocular tissues and regulation of adhesion and differentiation of pancreatic epithelial cells. We have reported that Ntn4 derived from vascular endothelial cells inhibits differentiation of osteoclasts and Ntn4 administration prevents receptor activator of nuclear factor-kappa B ligand (RANKL)-induced osteoporosis in mice [2]. However, little is known about the localization of Ntn4 in vivo. In this study, the localization of Ntn4-expressing vascular endothelial cells located near osteoclasts and the level of Ntn4 in ovariectomized (OVX) mice were determined.

Materials and Methods

Mice

The C57BL/6 mice were obtained from Tokyo Laboratory Animals Science, housed in an environmentally controlled clean room and given a normal laboratory chow at the Department of Oral and Maxillofacial Surgery, Saitama Medical University. Ovariectomy

Seven-week-old female mice were OVX or sham-operated and subsequently, these mice were sacrificed at 9 weeks.

Measurement of Ntn4 level in the serum

Levels of Ntn4 in the serum were measured using ELISA kit according to the manufacturer’s instructions.

Drill-hole injury in the femur

Briefly, in the mid femur of mice, a straight longitudinal skin incision (5-mm long) was made on the front skin under ether anesthesia; after muscle splitting, periosteal membrane was stripped away to expose the bone surface. Then, a drill-hole injury was made by inserting a drill bit with a diameter of 1 mm fixed to a finger handle at the anterior portion of the diaphysis of bilateral femurs, 6 mm above the knee joint. The hole was drilled through the anterior cortical bone and bone marrow and thus a round defect measuring approximately 1.0 mm in diameter was made.

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Immunohistochemical staining

Mice (8-week-old, male) were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg/kg). Then the drill-hole was made in their right femur as described above. Six and 14 days later, they were sacrificed for immunohistochemical staining. Samples were perfused with 4% paraformaldehyde (pH 7.4). For immunohistochemical staining, the femurs were frozen in hexane using a cooling apparatus, embedded in a 5% carboxymethyl cellulose gel and subsequently cut using a Leica CM1900 cryostat (Leica Microsystems). The 5-µm-thick sections of non-decalcified femurs were prepared using Kawamoto's film method and fixed in ice-cold 5% acetic acid in ethanol. The sections were subjected to staining for platelet endothelial cell adhesion molecule-1 (PECAM-1) (R&D, MN, USA) or Ntn4 (R&D, MN, USA) using specific antibodies and subsequently, were subjected to staining for tartrate-resistant acid phosphatase (TRAP) and counterstaining with hematoxylin. Immunostaining of the femur was also conducted in unchallenged mice.

Histological and histomorphometric analyses

Undecalcified sections of the lumbar vertebrae were stained by von Kossa staining as previously described [2]. Static histomorphometric analyses were performed using OsteoMeasure Analysis System (OsteoMetrics, GA, USA) following nomenclature defined by the American Society for Bone and Mineral Research. Bone volume/tissue volume (BV/TV; %) was analyzed. Six mice were examined from each group.

Statistical analyses

Comparisons between two groups were analyzed using Student’s t-tests (#p < 0.05; ##p < 0.01). All values are represented as the mean ± S.E.M. Results are representative examples of more than three independent set of experiments.

Results

To determine the relation between vascular endothelial cells and osteoclasts in vivo, we performed drill-hole injury experiments, TRAP staining and subsequently immunostaining for PECAM-1 at day 14. As shown in Figure 1A, Ntn4 was found to be localized primarily in the blood vessels, which were stained with PECAM-1 in the unchallenged femur. TRAP-positive cells, which were present along the bone tissue, were found in immediate vicinity of vascular endothelial cells during bone healing (Figure 1B). TRAP-positive cells were also found in immediate vicinity of Ntn4-positive cells during bone healing (Figure 1B). These results indicate that TRAP-
positive cells are localized in immediate vicinity of vascular endothelial cells which express Ntn4.

Next, we investigated whether Ntn4 expression in OVX osteoporotic mice was changed in the serum. We confirmed that ovariectomy was successful by checking bone volume in the vertebrae (Figure 2A). By using ELISA kit for Ntn4, we found that serum Ntn4 levels were significantly decreased in OVX osteoporotic mice (Figure 2B). These results suggest that Ntn4 plays a vital role in bone metabolism physiologically.

**Discussion**

In this study, we showed that vascular endothelial cells are in proximity to osteoclasts during bone healing. This result indicates that regulatory mechanisms might be present between vascular endothelial cells and osteoclasts. Indeed, vascularization is essential for osteoclastogenesis [6].

Han et al. reported that osteoclasts express CX3C chemokine receptor 1 (CX3CR1) which is the only receptor for the unique CX3C membrane-anchored chemokine, fractalkine (CX3CL1) and CX3CL1 was dramatically upregulated in the vascular endothelium after ionizing radiation [7]. Their study also demonstrated that CX3CL1-CX3CR1 axis plays a pivotal role in bone resorption and Cackowski et al. showed that osteoclasts stimulate angiogenesis *in vivo* through matrix metalloproteinase-9 [8], suggesting that vascular endothelial cells positively regulate osteoclastogenesis. On the contrary, we have previously demonstrated that Ntn4, which is derived from vascular endothelial cells, inhibits osteoclast differentiation, indicating that vascular endothelial cells negatively regulate osteoclastogenesis. Moreover, it is possible that the inhibition of vascularization by Ntn4 results in the inhibition of osteoclastogenesis.

The balance between positive and negative regulators of differentiation derived from the vessels may be essential for osteoclastogenesis.

The pathological roles of Ntn4 in cancer have been reported. The decrease in Ntn4 expression is associated with breast cancer cell migration and invasion via regulation of epithelial mesenchymal transition-related biomarkers [9]. Ntn4 overexpression decreases tumor growth and carcinomatosis via an antiangiogenic effect [10]. Interestingly, our study demonstrated that Ntn4 expression is decreased after ovariectomy, suggesting that the downregulation of Ntn4 after menopause leads to break the inhibitory effect of osteoclastogenesis physiologically.

A limitation of this study is that as this was an animal study, no data on the serum levels of Ntn4 in postmenopausal women is available.

Our study also proposes the possibility that the Ntn4 administration is effective for osteoporosis of postmenopausal women because Ntn4 levels are decreased after ovariectomy in mice and that Ntn4 administration prevents bone loss in an osteoporosis mouse model by decreasing the osteoclast number [2]. Further investigation will be needed to determine whether Ntn4 administration is useful as a therapeutic approach for postmenopausal osteoporosis and periodontal disease.

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**Reference**


