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Zebrafish scales respond differently to *in vitro* dynamic and static acceleration: Analysis of interaction between osteoblasts and osteoclasts

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ABSTRACT

Zebrafish scales consist of bone-forming osteoblasts, bone-resorbing osteoclasts, and calcified bone matrix. To elucidate the underlying molecular mechanism of the effects induced by dynamic and static acceleration, we investigated the scale osteoblast- and osteoclast-specific marker gene expression involving osteoblast–osteoclast communication molecules. Osteoblasts express RANKL, which binds to the osteoclast surface receptor, RANK, and stimulates bone resorption. OPG, on the other hand, is secreted by osteoblast as a decoy receptor for RANKL, prevents RANKL from binding to RANK and thus prevents bone resorption. Therefore, the RANK–RANKL–OPG pathway contributes to the regulation of osteoclastogenesis by osteoblasts. Semaphorin 4D, in contrast, is expressed on osteoclasts, and binding to its receptor Plexin-B1 on osteoblasts results in suppression of bone formation. In the present study, we found that both dynamic and static acceleration at 3.0 × *g* decreased RANKL/OPG ratio and increased osteoblast-specific functional mRNA such as alkaline phosphatase, while static acceleration increased and dynamic acceleration decreased osteoclast-specific mRNA such as cathepsin K. Static acceleration increased semaphorin 4D mRNA expression, while dynamic acceleration had no effect. The results of the present study indicated that osteoclasts have predominant control over bone metabolism via semaphorin 4D expression induced by static acceleration at 3.0 × *g*.

1. Introduction

Bone is not a stationary tissue even in the adult body, and continuous remodeling of bone occurs to fit the level of daily load (Frost, 1987). Previous studies have demonstrated the importance of acceleration loading on weight-bearing bone through immobilization, bed rest, and space flight (Klølner and Toft, 1983; Whedon, 1984; Vico et al., 1993). Mechanical stimulation in the form of vibration (dynamic acceleration) was shown to improve bone metabolism in both *in vivo* and *in vitro* studies (Rubin et al., 2001; Suzuki et al., 2007; Armbrecht et al., 2010). In studies of humans and some animals, hypergravity (static acceleration) has also been shown to positively affect bone metabolism in a site-specific manner (Naumann et al., 2001; Naumann et al., 2004). However, the differences in the effects and underlying mechanisms between dynamic and static acceleration on bone metabolism are not still fully understood.

Bone remodeling consists of a close coupling of bone resorption with osteoclasts and bone formation with osteoblasts (Harris and

Abbreviations: *g*, gravitational acceleration; RANK, receptor activator of nuclear factor kappa-B; RANKL, RANK ligand; OPG, osteoprotegerin; Dlx5, distal-less homeobox protein 5; ALP, alkaline phosphatase; NFATC1, nuclear factor of activated T cells, cytoplasmic 1; DCSTAMP, dendritic cell-specific transmembrane protein; Trap, tartrate-resistant acid phosphatase.

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Heaney, 1969; Parfitt, 1982; Martin and Rodan, 2001). Moreover, cell-to-cell communication molecules such as receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) produced by osteoblasts are positive and negative regulators of osteoclast development and function, respectively (for review, see (Silva and Branco, 2011)). On the other hand, the antidromic cell-to-cell communication molecule semaphorin 4D derived from osteoclasts inhibits osteoblastic bone formation through its receptor Plexin-B1, which is expressed by osteoblasts (Negishi-Koga et al., 2011). Therefore, the cell-to-cell communication that occurs between osteoclasts and osteoblasts is crucial for bone metabolism. Thus, an *in vitro* model involving osteoblasts and osteoclasts is essential for assessment of the effects of mechanical acceleration on bone metabolism.

Teleost fish, such as the zebrafish (*Danio rerio*), Japanese ricefish (medaka, *Oryzias latipes*), and goldfish (*Carassius auratus*), have cycloid scales containing calcified tissue consisting of osteoblasts, osteoclasts, and bone matrix proteins (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000, 2008; Yoshikubo et al., 2005; Pasqualetti et al., 2012; Yano et al., 2013). In addition, teleost scales have been shown to be a good model for studying acceleration responses (Suzuki et al., 2007, 2008; Kitamura, 2010; Kitamura et al., 2010; Yano et al., 2013). The availability of genome information for zebrafish allows us to investigate gene expression involved in bone metabolism in detail. In the present study, we elucidated the molecular mechanism underlying the effects on bone metabolism induced by dynamic and static acceleration using zebrafish scales as a bone model.

2. Materials and methods

2.1. Animals

Adult zebrafish (*Danio rerio*) ($n = 200$) were purchased from a commercial source (Yasuda Suien, Kanazawa, Japan) and used for the *in vitro* scale assay. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

2.2. Application of dynamic 3.0 × g loading

One hundred zebrafish were anesthetized in 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (14805–82; Nacalai Tesque, Kyoto, Japan) and scales were carefully collected under a dissecting stereomicroscope (SMZ645; Nikon, Tokyo, Japan) from either side of the body using stainless steel forceps (A-23; Natsume Seisakusho Co., Ltd., Tokyo, Japan). The scales of one side on each of 5 fish were collected and put into a 1.5-mL micro-tube into which 700 μL of Leibovitz's L-15 medium (11415-064l; GIBCO, Grand Island, NY, USA) supplemented with a 1% penicillin–streptomycin mixture (168–23191; Wako Ltd., Osaka, Japan) was added. The scales from the other side of the same 5 fish were also collected and put into another tube as controls. A cotton ball (diameter 1 cm) was placed into each micro-tube above the scales to prevent the induction of flow in the micro-tube during acceleration loading. The 20 micro-tubes containing scales were subjected to a dynamic 3.0 × *g* load (consisting of alternately vertical 2.0 *g* acceleration by vibration and 1.0 × *g* ground gravity) for 20 min at room temperature using the vibration loading system reported in our previous study (Suzuki et al., 2007). After acceleration loading, scales from the 20 tubes were split into 4 groups, consisting of 5 tubes each, and the 4 groups were incubated at 15 °C for 3, 6, 12, and 18 h, respectively. After incubation, scales were frozen at −80 °C until analysis. The data of dynamic 3.0 × *g*-loaded scales were compared with those of static 1.0 × *g* control scales. The loading magnitude of 3.0 × *g* in the present study was conducted according to the method of (Suzuki et al., 2007).

2.3. Application of static 3.0 × g loading

Scales were prepared as described for application of dynamic acceleration. Twenty micro-tubes containing scales collected from 100 zebrafish were subjected to 3.0 × *g* loading (consisting of 2.83 × *g* horizontal acceleration by centrifugation and vertical 1.0 × *g* ground gravity) in a centrifugal separator (LIX-130; Tomy Digital Biology Co., Ltd., Tokyo, Japan) for 20 min at room temperature. After acceleration loading, scales from the 20 tubes were split into 4 groups, consisting of 5 tubes each, and the 4 groups were incubated at 15 °C for 3, 6, 12, and 18 h, respectively. After incubation, scales were frozen at −80 °C until analysis. The data of static 3.0 × *g*-loaded scales were compared with those of static 1.0 × *g* control scales.

2.4. mRNA quantification

Total RNA was prepared from zebrafish scales using a total RNA isolation kit for fibrous tissue (74704; Qiagen Sciences, Germantown, MD, USA). Complementary DNA (cDNA) synthesis was performed with 1 μg of RNA using a reverse transcription kit (RR037A; Takara Bio Inc., Otsu, Japan). The synthesized cDNA samples were subjected to quantitative real-time PCR (qPCR) analysis of osteoblastic marker mRNA expression of distal-less homeobox protein 5 (Dlx5), type I collagen, alkaline phosphatase (ALP), osteocalcin, RANKL, and OPG, and of osteoclastic marker mRNA expression of nuclear factor of activated T cells, cytoplasmic 1 (NFATC1), tartrate resistant acid phosphatase (Trap), dendritic cell-specific transmembrane protein (DCSTAMP), cathepsin K, and semaphorin 4D using the SYBR® Premix Ex Taq™ II (Takara Bio Inc.) and Mx3005 P® qPCR System (Agilent Technologies, Santa Clara, CA, USA). The gene-specific primers used in qPCR analysis are shown in Table 1. The conditions for qPCR amplification consisted of 3 min of initial denaturation at 95 °C followed by 40 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 40 s, and a single cycle for dissociation curve analysis. Standards and samples were run in triplicate. To control for differences in the initial mRNA conditions, β-actin mRNA level was used as an internal standard. The relative values of target mRNA expression were calculated from the target threshold cycle values and β-actin threshold cycle values using standard curves.

2.5. Statistical analysis

The acceleration-induced effects on bone metabolism were compared by assessing control scales taken from one side of each fish and acceleration-loaded scales taken from the other side. Differences between the two sides were compared using paired t-tests, performed using the statistical software Excel Toukei 2010® (Social Survey Research Information Co. Ltd., Tokyo, Japan). All data are expressed as the means ± standard error of the mean (SEM).

3. Results

3.1. Effects of dynamic and static acceleration on osteoblast-specific marker mRNA expression

The results of dynamic and static acceleration are indicated in Figs. 1 and 2, respectively. After 20 min of dynamic 3.0 × *g* vibration, the expression of osteoblastic transcription factor Dlx5 mRNA was significantly increased at 3 h, 6 h, and 12 h after vibrational treatment. Moreover, the mRNA expression levels of osteoblastic functional genes, type I collagen, ALP, and osteocalcin, were also significantly elevated at 12 h after vibration. Twenty minutes of static 3.0 × *g* acceleration induced a significant increase in the level of ALP mRNA at 12 h compared to control. There were no significant increases in