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Mechanical regulation of osteoclastic genes in human osteoblasts

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Abstract

Bone adaptation to mechanical load is accompanied by changes in gene expression of bone-forming cells. Less is known about mechanical effects on factors controlling bone resorption by osteoclasts. Therefore, we studied the influence of mechanical loading on several key genes modulating osteoclastogenesis. Human osteoblasts were subjected to various cell stretching protocols. Quantitative RT-PCR was used to evaluate gene expression. Cell stretching resulted in a significant up-regulation of receptor activator of nuclear factor-κB ligand (RANKL) immediate after intermittent loading (3 × 3 h, 3 × 6 h, magnitude 1%). Continuous loading, however, had no effect on RANKL expression. The expression of osteoprotegerin (OPG), macrophage-colony stimulating factor (M-CSF), and osteoclast inhibitory lectin (OCIL) was not significantly altered. The data suggested that mechanical loading could influence osteoclasts recruitment by modulating RANKL expression in human osteoblasts and that the effects might be strictly dependent on the quality of loading.

Keywords: Osteoblast; Osteoclast; Mechanotransduction; RANKL; OPG; OCIL; M-CSF

Skeletal mass homeostasis is maintained by the strictly coupled activities of bone-forming osteoblasts (OB) and bone-resorbing osteoclasts (OC) resulting in bone remodeling. Mechanical loading stimulates bone formation and remodeling, whereas unsuitable mechanical conditions may result in unbalanced bone resorption and reduced growth rate [1–3]. The functional responsiveness of bone derived cells to mechanical strain has been confirmed by many in vitro studies demonstrating that dynamic loading leads to anabolic effects by stimulating osteoblast proliferation and increasing extracellular matrix production [4–6].

Less is known about the influence of mechanical stimuli on bone resorption and the interaction of osteoblasts and osteoclasts in mechanically induced bone remodeling. It is generally accepted that osteoclast recruitment is mainly controlled by osteoblasts [7]. Osteoblasts express the key factors modulating osteoclastogenesis: receptor activator of nuclear factor-κB ligand (RANKL), a member of the tumor necrosis factor (TNF)-ligand family, and osteoprotegerin (OPG). RANKL binds to its receptor RANK on osteoclast progenitors and stimulates their differentiation and activity. OPG acts as a non-signaling decoy receptor, binds RANKL, and prevents activation of RANK resulting in decreased osteoclast recruitment. Osteoclastogenesis also requires the presence of macrophage-colony stimulating factor (M-CSF), which is released by osteoblasts as well. The RANK/RANKL/OPG and M-CSF/c-fms regulatory axes couple osteoblast and osteoclast activity controlling the balance between bone formation and resorption [7]. Osteotropic factors, e.g., parathyroid hormone, Vitamin D_3 ($1,25(OH)_2D_3$) or prostaglandin, increase the ratio between RANKL and OPG in favor of RANKL and can support osteoclastogenesis, whereas estrogens, for example, can inhibit osteoclast recruitment by changing the RANKL/OPG ratio in favor of OPG [7,8]. Another factor has recently been reported, osteoclast inhibitory lectin (OCIL), which is also expressed by osteoblasts and was shown to depress bone resorption [9,10] and osteoblast differentiation [11].

The strictly regulated interaction of osteoblasts and osteoclasts seems to be influenced by mechanical load. Several in vitro studies demonstrated that mechanical load could modulate the key factors controlling osteoclast

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recruitment. Rubin and co-workers reported a mechanically induced down-regulation of RANKL and an up-regulation of endothelial nitric-oxide synthase (eNOS), an enzyme producing the signaling molecule nitric-oxide, which is supposed to prevent bone resorption [12–14]. Saunders et al. found that mechanical stimulation significantly increased OPG levels and decreased M-CSF without affecting RANKL [15]. Both groups suggested that the observed effects could lead to decreased osteoclast activity and hypothesized that mechanical loading not only stimulates bone formation but also inhibits its resorption. In contrast to these studies, mechanical loading increased RANKL expression in vivo in the murine tibia [16], in rats during mandibular distraction [17], and also in vitro in human periodontal cells [18], and in primary murine osteoblasts [19] suggesting a stimulating effect on osteoclast recruitment.

The reason for these contradictory results might be varying experimental designs, e.g., different mechanical stimulation protocols and species. Most of the in vitro studies investigating mechanical effects on the RANK/RANKL/OPG and M-CSF/c-fms regulatory axes were performed with bone marrow stroma cells derived from mice [13,14,20] or with murine cell lines [21–23]. Beside the above-mentioned study of Saunders et al. using a human cell line [15] there is, to our knowledge, just one single study on primary human osteoblasts [24]. To better understand the effects of mechanical load on the interaction of osteoblasts and osteoclasts more studies on human derived cells are required.

The objective of the present study was to investigate the effects of mechanical strain on key factors regulating osteoclastogenesis. Primary osteoblasts derived from several human donors were subjected to various stretching protocols and mRNA expression of RANKL, OPG, M-CSF, and OCIL was evaluated. We hypothesized that cyclic strain would alter the expression profile of the investigated genes dependent on the quality of mechanical loading.

Materials and methods

OB were isolated from bone samples (femur or tibia) of 11 healthy patients undergoing surgery for fracture repair (1 female, 10 male, 15–87 years old) with informed consent (approved by the ethical committee at the University of Ulm). Cells were isolated by collagenase digestion as previously described [25]. Cell isolation was performed in Ca^{+2}-free Dulbecco's modified Eagle's medium (DMEM; Biochrom) to inhibit fibroblast growth. The cells were cultured and mechanically stimulated in 5% CO_2 at 37 °C and saturation humidity. Cells isolated by the described procedure express osteogenic markers and are regarded as osteoblasts [4]. Cells after passage 3 or 4 were seeded at a density of 20.000 cells/cm^2 on flexible silicone dishes (60 mm × 30 mm) and maintained in DMEM (F9050) containing 10% FCS and 10^{-8} M 1α,25$(OH)_2D_3$ (Sigma, D1530) for 3 days until subconfluency. The suitability of the dishes for cell cultures has been described before [6].

The cell stretching experiments were performed in a six-station stimulation apparatus driven by an eccentric motor [6]. The silicone dishes were stretched cyclically in the long axis at a frequency of 1 Hz and a magnitude of 1% or 8% (corresponding to 10,000 or 80,000 μstrain) for various time periods. Cells were harvested immediately or at different times after loading. The following mechanical parameters were tested:

(a) continuous stimulation (30 min, 6 h, 24 h, and 72 h) at a magnitude of 1%, cell harvesting immediately after stimulation.
(b) continuous stimulation (30 min) at a magnitude of 1%, cell harvesting at different time points (0 min, 30 min, 1 h, 3 h, and 5 h).
(c) continuous stimulation (6 h) at a magnitude of 1%, cell harvesting at 3 h and 18 h after mechanical stimulation.
(d) intermittent stimulation on 3 consecutive days (3 × 30 min, 3 × 3 h, and 3 × 6 h) at a magnitude of 1%, cell harvesting immediately after stimulation.
(e) continuous (30 min) or intermittent stimulation 3 × 3 h on 3 consecutive days at a magnitude of 8%, cell harvesting immediately after stimulation.

Mechanically unstimulated cultures were used as controls.

Analysis of gene expression. Effects on gene expression were examined as previously described [26]. Briefly, loaded and unloaded cultures were harvested. Cells were lysed and total RNA was isolated for cDNA synthesis. Specific primer pairs (Table 1) were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) and were synthesized by Thermo Electron (Ulm, Germany). Amplification products were cloned and used as standards for real-time RT-PCR on the iCycler system (Bio-Rad Laboratories, Munich). The amount of the respective amplification product was determined relative to the house-keeping gene GAPDH. Normalized values of mechanically stimulated cells were compared to unloaded controls.

Statistical analysis. Independent experiments with different donors were performed 5–8 times in triplicate cultures. A non-parametric Wilcoxon signed rank test was performed to evaluate differences between stretched cell cultures and unstimulated controls. Statistical significance was assumed if the p-value was 0.05 or lower. Results are shown as relation of gene expression of mechanically stimulated samples compared to mechanically unstimulated controls.

Results

To validate the successful transfer of the mechanical signals to the cells and their basic responsiveness, the expression of the immediate early gene c-FOS was investigated. It is well known that c-FOS expression is rapidly up-regulated as a response to mechanical stimulation and this can, therefore, be used as an internal control for the successful transfer of the mechanical signal [27]. c-FOS mRNA expression was measured immediately after continuous mechanical stimulation (frequency 1 Hz, magnitude 1%) for 30 min. c-FOS expression increased 5.0-fold (range 2.3- to 5.6-fold; $p = 0.05$) compared to mechanically unstimulated controls.

There were no significant effects on mRNA expression of RANKL, OPG, M-CSF, and OCIL after continuous application of cyclic strain of 1% for 30 min, 6 h, 24 h, and 72 h except a slight down-regulation of M-CSF expression after mechanical stimulation for 24 h (Table 2). To investigate the effect of a higher strain magnitude, osteoblasts were subjected to 8% cyclic strain for a period of 30 min. Again, no significant effects on the expression of the investigated genes were observed (Table 2).

To acquire effects, which did not occur immediately after mechanical stimulation due to slower gene regulation, we performed extensive time course experiments and