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Ibandronate concomitantly blocks immobilization-induced bone and muscle atrophy

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ABSTRACT

Both bone and muscle volume is concomitantly reduced under immobilization conditions; however, no single drug is currently available to block these outcomes simultaneously. Bisphosphonates are utilized clinically to inhibit osteoclast-dependent bone resorption, but their effects on muscle are largely unknown. Here we show that skeletal muscle is a direct target of the bisphosphonate ibandronate (IBN) and that reduced muscle volume and induction of *Atrogin-1* and *MuRF1*, both atrogenes, are significantly inhibited by IBN administration *in vivo* using a mouse model of muscle atrophy. IBN treatment also significantly blocked immobilization-induced bone loss *in vivo*. We also report that expression of *Atrogin-1* and *MuRF1* and accumulation of Smad2/3 proteins, which are upstream of atrogines, occurred following serum starvation of myogenic C2C12 cells *in vitro*, effects significantly inhibited by IBN treatment. Interestingly, IBN effects on C2C12 cells were abrogated by MG132, an ubiquitin/proteasome inhibitor, suggesting that IBN functions via the ubiquitin-proteasome system. Our findings lend new insight into the role of IBN in preventing muscle atrophy.

1. Introduction

Limb immobilization by casting following traumatic injury such as fractures and tendon or ligamentous rupture promotes bone and muscle atrophy [1]. Similarly reduced bone and muscle volume is seen in the elderly, suggesting that changes in the volume of both tissues are similarly controlled. Various mechanisms are proposed to underlie reduced bone mass due to either immobilization or unloading conditions, among them inhibited function of osteoblasts and osteocytes or accelerated osteoclast activity [2,3]. In elderly or postmenopausal women, bone mass is reduced by accelerated osteoclast-mediated bone resorption, leading to osteoporosis [4]. Muscle volume decreases following either immobilization or unloading due to proteasome-dependent degradation of muscle proteins by the E3 ligases Atrogin-1 and MuRF1 [5]. Accumulation of Smad2/3 protein was demonstrated induced even under Myostatin-depleted condition, and is required for immobilization-induced *Atrogin-1* and *MuRF1* expression, leading to muscle atrophy *in vivo* [6]. In the elderly, inactivity reportedly promotes muscle volume reduction [7]; however, drugs that prevent both bone and muscle atrophy are not available.

Bisphosphonates, which are worldwide the most frequently used drugs to treat osteoporosis, strongly inhibit osteoclastic bone resorption and elevate bone volume, thus preventing bone fragility fractures [8]. Ibandronate (IBN) is a nitrogen-containing

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bisphosphonate effective in preventing bone fragility fractures in osteoporosis patients [9]. Low bone mineral density (BMD) is a risk for hip fracture [10], and prevention of such fractures in osteoporosis patients by bisphosphonate treatment is attributable to elevation in bone mass following osteoclast inhibition.

Bisphosphonates have high affinity for bone tissue and are incorporated into osteoclasts during bone resorption [11]. These reagents then reportedly promote osteoclast apoptosis by inhibiting the geranylgeranyl pathway [12]. Thus, osteoclasts are considered the primary bisphosphonate targets. However, evidence suggests that bisphosphonates act on other cell types as well. For example, the bisphosphonates zoledronic acid and IBN reportedly induce apoptosis and inhibit cell cycling in several types of cancer cells by preventing prenylation of GTP-binding proteins such as Ras and Rho *in vitro* and *in vivo* [13,14], Furthermore, bisphosphonates reportedly act on immune cells or cartilage and chondrocytes by modulating production of pro- and anti-inflammatory cytokines in chronic inflammatory joint diseases [15]. Precise mechanisms underlying these activities remain to be elucidated.

Falls are the most frequent cause of hip fracture and cause more than 75% of those injuries. Reduced muscle volume and power is reportedly associated with falls [16–19]. Thus, interventions to prevent muscle weakness would be useful in preventing hip fracture. However, a potential effect of drugs known to be protective in the context of hip fracture on muscle tissue has not been demonstrated.

In this study, we demonstrate that IBN treatment significantly inhibits denervation-induced bone and muscle atrophy in mice model. Expression of *Atrogin-1* and *MuRF1*, both muscle atrophy-inducing factors upregulated by denervation, was significantly inhibited by IBN administration *in vivo*. Likewise, elevation of *Atrogin-1* and *MuRF1* by serum starvation of myogenic C2C12 cells was also significantly inhibited by IBN treatment *in vitro*. In C2C12 cells, Smad2/3 protein accumulation, which is known to be required for *Atrogin-1* and *MuRF1* upregulation and subsequent muscle atrophy [6], was abrogated by IBN treatment. Thus, our data provide new insight into the role of IBN in preventing atrophy of both bone and muscle.

2. Material and methods

2.1. Muscle atrophy animal model

Wild-type C57BL/6 mice were purchased from CLEA Japan (Meguro, Tokyo, Japan). An immobilized muscle atrophy model was generated in 8-week-old female C57BL/6 mice, as previously described [6]. Some mice were administered 0.01 or 0.1 mg/kg ibandronate sodium (IBN, Chugai Pharmaceutical Co., LTD, Tokyo, Japan) subcutaneously once at the time of denervation. Animals were maintained under specific pathogen-free conditions in animal facilities certified by the Keio University Animal Care Committee. Animal protocols were also approved by the committee.

2.2. Reagents

MG132 was purchased from Calbiochem (San Diego, USA).

2.3. Analysis of skeletal morphology

Bone mineral density (BMD) of whole tibiae was measured using Dual-energy X-ray absorptiometry (DEXA) using DCS-600R (Aloka Co. Ltd., Tokyo, Japan). Bone parameters were determined by bone morphometric analysis using micro-computed tomography (μCT40; Scanco Medical, Brüttisellen, Switzerland).

2.4. Real-time PCR analysis

Total RNA was collected from gastrocnemius tissue using Trizol reagent (Invitrogen Corporation, California, USA). Real-time PCR was performed using SYBR Premix ExTaq II (Takara Bio Inc., Otsu, Shiga, Japan) as described [6].

2.5. Western blotting

Whole cell lysates of C2C12 cells were prepared using RIPA buffer, and western blots were probed with the following antibodies: anti-phospho-Smad2 (3101, Cell Signaling), anti-phospho-Smad3 (9520, Cell Signaling), anti-Smad2/3 (3102, Cell Signaling), and anti-Actin (A2066, Sigma).

2.6. Immunohistochemistry

Tibial paraffin sections were stained with anti-Cathepsin K antibody (ab19027, 1:200, Abcam, Cambridge, UK) followed by Alexa-488-conjugated anti-rabbit Igs antibody (1:100 dilution; Invitrogen). Diamidino-2-phenylindole (DAPI) (Wako, Osaka, Japan; 1:2000) was used for nuclear staining. Sections were assessed by fluorescence microscopy (Biorevo; Keyence, Osaka, Japan).

2.7. Cell culture

C2C12 cells (ATCC, CRL-1772) were differentiated as myotubes by cultivation for 3 days in DMEM supplemented with 2% horse serum (HS) (PAA, B11-021). Myotubes were cultured in serum-free conditions for the muscle atrophy model [6] with or without IBN (0.2–5.0 μM) *in vitro*. Cells were then harvested after 6 or 12 h culture for protein or RNA preparation, respectively.

2.8. Statistical analysis

Data were analyzed using a two-tailed Student's *t*-test. For all graphs, data are represented as means ± standard deviation (SD) ($^{\#}p < 0.1$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3. Results

3.1. Denervation-induced immobilization reduces both bone and muscle volume in mice model

Immobilization is known to induce muscle and bone atrophy [20]. Here, to assess drug effects on both parameters we employed a denervation-induced immobilization model in mice [21]. To do so, we cut the sciatic nerve on one side, while the other, sham-operated side served as a non-immobilized control [22,23]. Seven days later, we observed significant reduction in muscle volume on the denervation-induced immobilization (DEN) compared with the sham-operated control (Ctrl) side (Fig. 1A). At that time point, bone mass in immobilization and control sides was comparable (Fig. 1B, top panel). However, over time relative loss of bone mass on the denervated side became apparent and was significant at 2 and 4 weeks after surgery (Fig. 1B, middle and lower panels).

3.2. Osteoclast activation is required for reduced bone mass seen following immobilization

To determine whether osteoclasts promote loss of bone mass after denervation, we stained proximal tibial bone sections from denervated and control sides with an antibody to the osteoclast marker Cathepsin K (Ctsk) (Fig. 2A). Formation of Ctsk-positive

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