データを集積してきた．今後もこの貴重なデータを用いて研究を進め，高齢者の健康増進へ大きく貢献する多くの成果を上げることができるものと期待している．

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Research Paper

Differential impact of the Bisphosphonate Alendronate on undifferentiated and terminally differentiated human myogenic cells

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Abstract

Objectives Alendronate, a nitrogen-containing bisphosphonate, is well established as a treatment for osteoporosis through regulation of osteoclast activity. Previously, the pharmacological effects of bisphosphonates on cells outside the bone environment have been considered irrelevant because of the bone-targeting property of bisphosphonates. However, the chronic effects of bisphosphonates on tissue-neighbouring bone, in particular skeletal muscles, should not be ignored because patients are treated with bisphosphonates for long periods.

Methods Here, we show that the impact of alendronate on immortalized human myogenic cells depends on growth and differentiation-inducing conditions.

Key findings Alendronate disrupted cytoskeletal structures and prevented migration, proliferation and differentiation of undifferentiated human myogenic cells that are involved in muscle regeneration. In contrast, alendronate did not affect the morphology, gene expression or survival of terminally differentiated human myotubes.

Conclusions The present results suggest that the muscle regeneration capacity of osteoporosis patients treated with bisphosphonates for long periods may be attenuated. The present research on the pharmacological effects of alendronate on cultured human myogenic cells will contribute to improvement of therapeutic strategies and optimization of rehabilitation programmes for locomotive activity in osteoporosis patients treated with bisphosphonates.

Introduction

Nitrogen-containing bisphosphonates (N-BPs) are well established as leading drugs for the treatment of osteoporosis through the regulation of osteoclast activity.[1,2] N-BPs have the ability to bind strongly to bone mineral. During bone resorption, the acidic pH increases the dissociation of N-BPs from bone surfaces. Therefore, osteoclasts are exposed to a locally high concentration of N-BPs and preferentially internalize them, allowing the selective uptake of N-BPs by their intended target cell osteoclasts, although cellular functions of osteocytes are also affected by N-BPs.[3,4]

N-BPs inhibit farnesyl pyrophosphate (FPP) synthetase, an enzyme of the mevalonate pathway.[1,2] Inhibition of FPP synthetase depletes isoprenoid lipids, FPP and geranylgeranyl diphosphate (GGPP), which are required for post-translational prenylation of small G proteins, such as those of the Ras, Rho and Rab families.[1] N-BPs disrupt localization of small G proteins because prenylation is required for their localization to plasma and subcellular membranes. N-BPs are assumed to have similar impacts on various types of cells other than osteoclasts, because FPP synthetase is required for commonly used signalling pathways. Actually, the N-BP alendronate (ALN) prevents prenylation of small G proteins, Rho, Rac and Cdc42 in macrophages, resulting in inappropriate control of downstream signalling pathways.[5] N-BPs have also been shown to act as antitumor reagents by inducing cell cycle arrest, inhibiting cell migration/invasion and causing apoptosis.[6,7] In addition, N-BPs induce apoptosis of untransformed cells,

including macrophages, keratinocytes and myoblasts.[8–13] These studies strongly suggest that N-BPs inhibit proliferation and have cellular toxicity in various cell types other than osteoclasts.

A previous study concluded that local concentration of ALN released from hydroxyapatite could achieve a concentration of 0.1–1.0 mM in the resorption space.[14] The bone-targeting property of N-BPs contributes to their selective uptake by osteoclasts and osteocytes and may minimize their effects on cells outside the bone environment. However, skeletal muscles are tightly connected to the skeleton and close to bone surfaces. Therefore, muscle tissues are likely to be chronically exposed to low concentrations of N-BPs that are released from bone matrix during bone resorption in osteoporosis patients treated with N-BPs for many years. Skeletal muscle function has a great impact on the quality of life of patients with osteoporosis. If N-BPs impair myogenic cell function, treatment with N-BPs will increase the risk of muscle dysfunction in patients, exacerbating their locomotive disability. However, previous studies on the effects of N-BPs on human myogenic cells are unknown. Even the results obtained by experiments with rats *in vitro* and *in vivo* are controversial. ALN-induced apoptosis of the rat myoblastic cell line L6 *in vitro*,[13] whereas another analysis *in vivo* showed that ALN does not affect skeletal muscle function of ovariectomized rats.[15]

In this study, to determine whether N-BPs impair human skeletal muscle cell function, we focused on the impact of ALN on growth and differentiation of immortalized human myogenic cells that retain a growth property similar to that of primary cultured human myogenic cells in the early passages, multipotentiality, and normal diploid chromosomes.[16,17] We found here that ALN impaired functions of undifferentiated human myogenic cells. The results suggest that the regeneration capacity of muscle is attenuated in osteoporosis patients treated with N-BPs for long periods. This study will contribute to improvement of therapeutic strategies and optimization of rehabilitation programmes for locomotive activity in osteoporosis patients treated with bisphosphonates.

Materials and Methods

Cell culture

Hu5/KD3 cells were maintained at 37°C under 10% CO_2 in dishes coated with type I collagen (Sumilon, Osaka, Japan) and containing primary cultured myocyte growth medium (pmGM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2% Ultroser G (Biosepra, Cedex-Saint-Christophe, France) and glucose (4.5 mg/ml).[17,18] For induction of myogenic differentiation, the medium was changed to primary cultured myocyte differentiation medium (pmDM) after 48 h of culture; pmDM consists of the chemically defined medium transferrin-insulin-selenite[19,20] supplemented with 2% FBS. The cells (5×10^4 cells) were plated evenly in 35-mm dishes for low density culture or in a micromass for high-density culture. To form a micromass of cells, 5×10^4 cells in 50 µl medium were plated and cultured for 4–8 h in a silicon ring (8 mm inner diameter, 1 mm thick); then the ring was removed, and 1.5 ml of medium was added. Alendronate (Sigma, St. Louis, MO) was dissolved in distilled water. Mevastatin (Sigma) was dissolved in dimethyl sulfoxide.

Time-lapse recording

Cells were cultured in a humid chamber (Tokai Hit, Fujinomiya, Japan) maintained at 37°C under 10% CO_2. Time-lapse images were taken using an inverted microscope (BZ9000; Keyence, Osaka, Japan) with a 20x Plan Apo Fluor objective lens (Nikon, Tokyo, Japan).

BrdU labelling and detection

Hu5/KD3 cells were cultured in pmGM with or without ALN (100 µM) for 2 days. The cells were incubated with 10 µM 5-bromo-2′-deoxyuridine (BrdU, Sigma) for the last 4 h of each culture, fixed in 4% paraformaldehyde for 10 min on ice, and then subjected to immunofluorescence analysis after denaturation of deoxyribonucleic acid (DNA) with 2 M HCl and neutralization with 0.1 M $Na_2B_4O_7$. The primary antibody was anti-BrdU monoclonal mouse antibody (Roche Diagnostics, Indianapolis, IN, 1 : 50). Secondary antibodies were AlexaFluor 488-labelled antibodies to mouse immunoglobulin G (Jackson ImmunoResearch Laboratory, Bar Harbor, ME, 1 : 1000). Cell nuclei and cytoplasm were stained with 2,4-diamidino-2-phenylindole dihydrochloride n-hydrate (DAPI) (Sigma) and Cell Mask Red (Life Technologies, Carlsbad, CA), respectively. Samples were visualized and numbers of both DAPI- and BrdU-positive nuclei were quantified using In Cell Analyzer 2000 (GE Healthcare, Piscataway, NJ). BrdU-positive nuclei were detected in three independent dishes. Data were statistically analysed using the Kruskal–Wallis test.

Immunoblotting

Sample preparation and immunoblot analysis were performed as previously described.[19,20] Immune complexes were detected by colorimetry with SIGMAFAST BCIP/NBT tablets (Sigma). Primary antibodies included mouse monoclonal antibodies to chicken sarcomeric myosin heavy chain (MyHC) (MF20, undiluted culture supernatant),[21] p53 (Merk, 1 : 500), Rb (BD Bioscience, San Jose, CA,1 : 1000), cyclin D1 (BD Bioscience, 1 : 1000), CDK4 (BD Bioscience, 1 : 250), RhoA (Cytoskeleton, Denver, CO, 1 : 100), Rac1

(Abcam, Cambridge, England, 1 : 100) and Cdc42 (ECM, 1 : 100), a rabbit polyclonal antibody to β-tubulin (Cell Signaling Technology, Beverly, MA, 1 : 1000). Secondary antibodies included alkaline phosphatase (DAKO, Carpinteria, CA)-labelled antibodies to mouse or rabbit immunoglobulin G. Immune complexes on the polyvinylidene difluoride membranes (Fluoro Trans W; Pall, Port Washington, NY) were scanned with a digital scanner (GT-9700F; Epson) and then post-processed using Adobe Photoshop.

Immunofluorescence and immunocytochemical analyses

Cultured cells were fixed with 4% paraformaldehyde at 4°C for 10 min and then incubated with primary antibodies. Primary antibodies included those to mouse monoclonal antibodies to mouse MyHC (undiluted supernatant), troponin T (1 : 1000 dilution, Sigma) and BrdU (1 : 50 dilution, Roche Diagnostics). Secondary antibodies were AlexaFluor 488-labelled antibodies to mouse IgG (Jackson ImmunoResearch Laboratory, Bar Harbor, ME) for immunofluorescence analysis. The peroxidase reaction was performed using an EXPOSE detection IHC Kit (Abcam). Cell nuclei were stained with DAPI (1.0 μg/ml) or haematoxylin (Wako). Samples were visualized using an inverted microscope (model IX71; Olympus) and a charge-coupled device camera (DP70; Olympus). Images were post-processed using Adobe Photoshop. Actin filaments were stained with AlexaFluor 546-labeled phalloidin (Life Technologies).

Results

Alendronate inhibits proliferation and migration of undifferentiated human myogenic cells

We have established the immortalized human myogenic cell line Hu5/KD3 derived from muscle of a healthy 42-year-old woman.[16,17] The culture of Hu5/KD3 cells provides an appropriate exo-vivo assay because they retain both proliferation capacity and differentiation potential. Hu5/KD3 cells divide at 24–28 h intervals and show lamellipodia with focal contacts in pmGM[18] (Figure 1a). In contrast, Hu5/KD3 cells became flattened and bipolar spindled in shape, and the lamellipodia disappeared when exposed to ALN (50 or 100 μM) for 2 days (Figure 1b). Mitotic figures were hardly found in ALN-treated cultures, suggesting that ALN inhibited cell proliferation. Low dosages (less than 25 μM) of ALN did not induce any morphological changes. To detect synthesizing DNA, cells were incubated with BrdU. The number of BrdU-positive nuclei significantly declined in ALN-treated cells (Figure 1c). In the next experiment, cell movement was sequentially observed by time-lapse recording. Untreated Hu5/KD3 cells migrated rapidly and divided for 18 h (Figure 1d and 1e, Movie S1). In contrast, ALN-treated Hu5/KD3 cells maintained the same position during 18 h of recording because ALN prevented proliferation and migration (Figure 1f and 1g, Movie S2). Finally, the cells detached from the culture dish (Figure 1g). We postulated that the effects of ALN were due to the inhibition of the enzymes of the mevalonate pathway (Figure 1h). Actually, mevastatin, a proven inhibitor of the mevalonate pathway, mimicked the effects of ALN (Figure 1i, Figure S1). The cellular damage caused by ALN was amplified when growth factors and FBS in the medium were reduced (Figure S2). The results suggest that ALN prevents proliferation, migration and adhesion to the substratum of undifferentiated human myogenic cells through inhibition of mevalonate pathway.

Alendronate does not inactivate retinoblastoma (Rb) gene product

To examine the cell cycle progression of human myogenic cells exposed to ALN, cell cycle-related proteins were analysed by immunoblotting (Figure 2a). Retinoblastoma (Rb) protein remained hyperphosphorylated in ALN-treated cultures as well as in controls. Activation of Rb protein plays a pivotal role in cell cycle arrest/exit under the control of cell cycle drivers. However, Rb protein was not activated in Hu5/KD3 cells treated with ALN even when the cells stopped proliferating. The amounts of p53, cyclin D1 and CDK4 were not affected by ALN. Therefore, the inhibition of cell proliferation caused by ALN was unlikely to be due to proximate suppression or activation of cell cycle regulator proteins. Disruption of cytoskeletal structures was considered as another possible cause of cell cycle inhibition by ALN because dynamic and ordered cytoskeletal rearrangement is essential to complete cell division.

Prenylation of small G proteins, Rho family members, was supposed to be inhibited by ALN. Thus, Rho family proteins, which play pivotal roles in cell morphology, migration, proliferation and survival, were the probable main target proteins affected by ALN. We found that the amounts of three Rho family proteins increased in ALN-treated cells (Figure 2b). In particular, RhoA protein levels increased markedly, whereas Rac1 and Cdc42 increased slightly. The increase of Rho family proteins might be involved in the cytoskeletal rearrangement and morphological changes of Hu5/KD3 cells. Actually, F-actin was accumulated at the leading edge of lamellipodia in human myogenic cells (Figure 2c). In contrast, ALN inhibited accumulation of F-actin beneath the plasma membrane (Figure 2d). The results imply that ALN inhibits proliferation of human myogenic cells independently of Rb

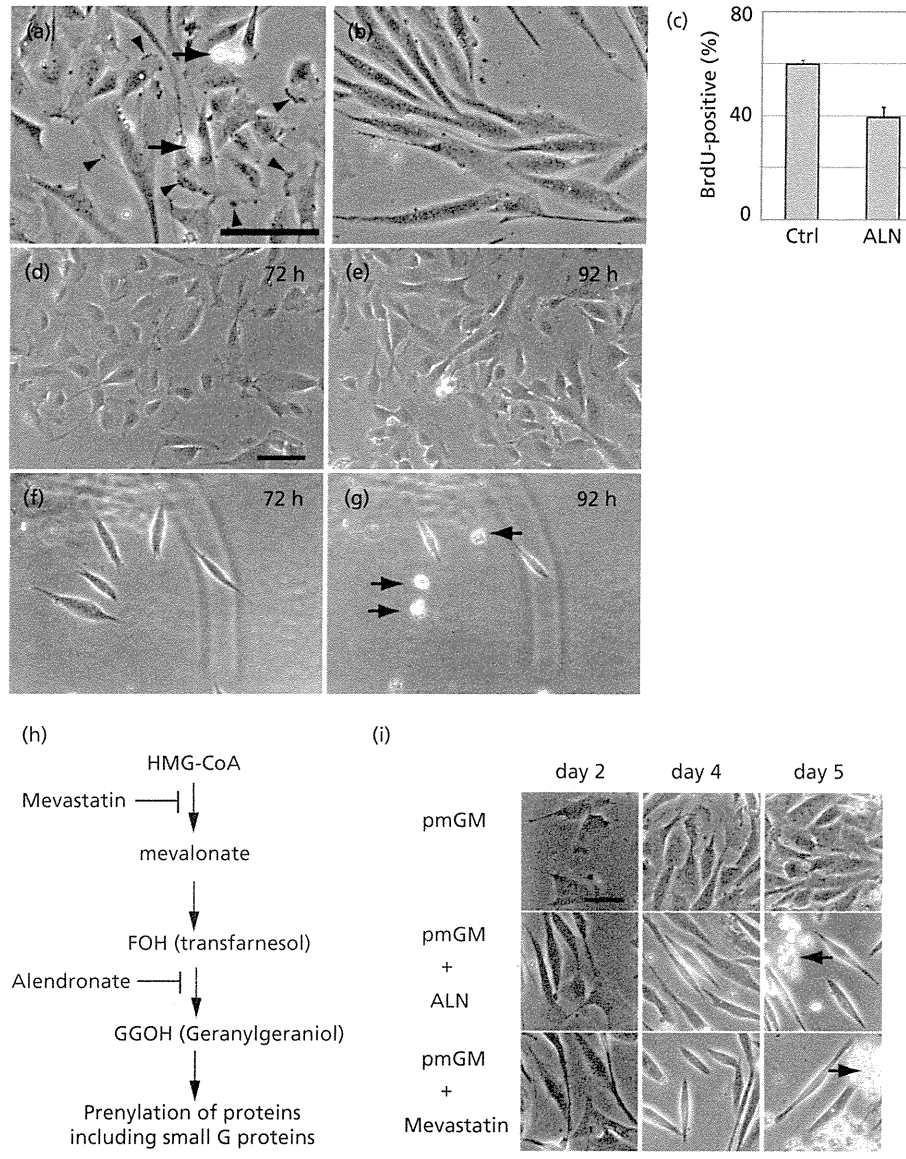

Figure 1 Alendronate inhibited proliferation of human myogenic cells. Hu5/KD3 cells were cultured in pmGM in the absence (a, Ctrl in c, d and e) or presence (b, ALN in c, f and g) of 100 μM ALN for 2 days (a–c) or 4 days (d–g). (a and b), phase contrast images were taken at the end of cultures. Arrows indicate mitotic cells, and arrowheads indicate focal contacts at lamellipodia. Scale bar, 100 μm. (c) Cells were incubated with BrdU for the last 4 h of culture. BrdU-positive nuclei were detected in three independent dishes. Average and standard deviation are shown and analysed using the Kruskal–Wallis test. *P*-value was less than 0.05. (d–g) Cell behaviour was serially recorded during 74–92 h of culture in pmGM with (f and g) or without ALN (d and e). Images of the same fields at the start and end of recordings are shown. Cells divided and migrated in pmGM (d and e). Cells kept the same positions during the recording (f and g). Arrows indicate the cells that shrivelled and became detached. (h) ALN and mevastatin block mevalonate pathway. (i) Hu5/KD3 cells (5×10^4 cells) were cultured in 20% FBS-hDMEM for 2 days, and then medium was switched to pmGM, pmGM with alendronate (ALN, 100 μM), pmGM with mevastatin (10 μM) for 5 days further. Arrows indicate clumps of detached cells. Scale bar, 10 μm.

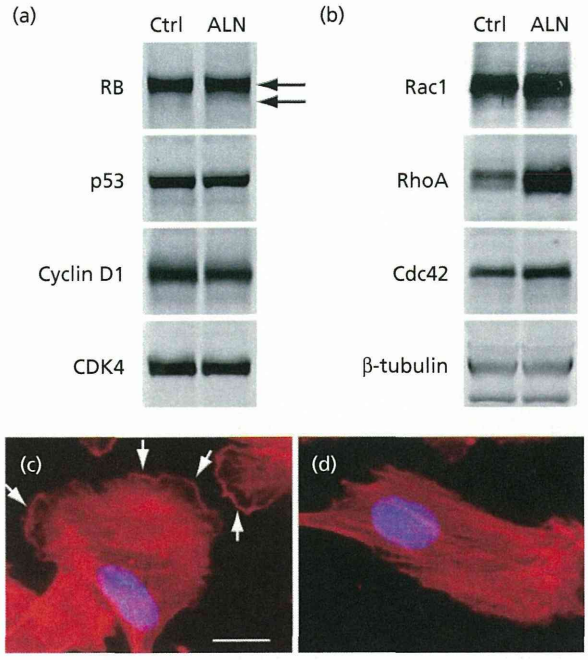

Figure 2 Alendronate impacted RB activation. Hu5/KD3 cells were cultured in pmGM in the absence (Ctrl in a and b, and c) or presence (ALN in a and b, and d) of 100 μM ALN for 2 days. (a and b) Total proteins (20 μg) were loaded in each lane. Arrows represent the positions of hyper- (upper) and hypophosphorylated (lower) Rb proteins. β-tubulin was used as a loading control. (c and d) Cells were fixed and then actin filaments were stained with AlexaFluor 546-labelled phalloidin (red). Lamellipodium formation was severely inhibited by ALN (d). Accumulation of F-actin on the leading edge of lamellipodia (arrows in c) was not observed in ALN-treated cells Nuclei were stained with DAPI (blue). Scale bar, 20 μm.

function, which is a main regulator of cell cycle arrest. The disruption of cytoskeletal structures, probably caused by dysfunction of RhoA, Rac1 and Cdc42 may impact cell cycle progression.

Alendronate inhibits terminal muscle differentiation

To determine whether ALN inhibits myogenic differentiation, Hu5/KD3 cells were cultured in differentiation medium pmDM with or without ALN. Hu5/KD3 cells began to fuse with each other and differentiate into myotubes on day 3 of differentiation culture (Figure 3a and 3c). When cultured at low cell density, the major fraction of cells were detached from the bottom of culture dishes and did not give rise to myotubes in the presence of ALN (Figure 3b). Both cell fusion and expression of differentiation markers myosin heavy chain and troponin T were prevented by ALN (Figure 3b and 3d). Terminal muscle differentiation depends on cell density.[22–24] To determine whether suppression of myogenesis is just a secondary effect due to the loss of cells caused by ALN, the cells were cultured under a high cell density condition. In untreated control cultures, Hu5/KD3 gave rise to myotubes that expressed differentiation markers (Figure 3e and 3g). Significant numbers of human myogenic cells survived during differentiation-inducing culture with ALN (Figure 3f and 3h), though a minor fraction of cells were detached (Figure 3f). Human myogenic cells did not differentiate into myotubes even though the cell detachment induced by ALN was attenuated under the high cell density culture condition. ALN also prevented the expression of the differentiation markers myosin heavy chain and troponin T in Hu5/KD3 cells (Figure 3h). ALN prevented myogenic differentiation even when many undifferentiated human myogenic cells survived. The results indicate that ALN prevents multiple steps of terminal muscle differentiation of human myogenic progenitor cells.

Alendronate damages only undifferentiated myogenic cells but not terminally differentiated myotubes

The previous results indicate that ALN severely impaired the function of undifferentiated human myogenic progenitor cells. Thus, we investigated the effects of ALN on terminally differentiated human myotubes. Hu5/KD3 cells were first cultured until they gave rise to prominent myotubes (Figure 4a). Then the medium was switched to fresh pmDM supplemented with or without ALN and cultured for 4 days further. In untreated controls, myotubes grew much larger and undifferentiated mononucleated progenitor cells remained between myotubes (Figure 4b). In contrast, undifferentiated cells were lost in culture when exposed to ALN (Figure 4c). To determine what happened to undifferentiated cells during exposure to ALN, the cells were serially observed by time-lapse recording. Undifferentiated human myogenic cells shriveled and then detached from culture dishes when exposed to ALN (Figure 4d). The majority of undifferentiated cells were detached during exposure to ALN for 70 h (Figure 4e and 4f). In contrast to undifferentiated cells, myotubes remained morphologically intact and expressed the muscle differentiation marker troponin T even during culture with ALN (Figure 4g and 4h). The results indicate that terminally differentiated human myogenic cells have acquired resistance to ALN during myogenesis.

Discussion

Bisphosphonates are well established as successful antiresorption agents for prevention and treatment of osteoporosis. Bisphosphonates target calcified tissues by virtue of their P-C-P backbone structure.[2] Then

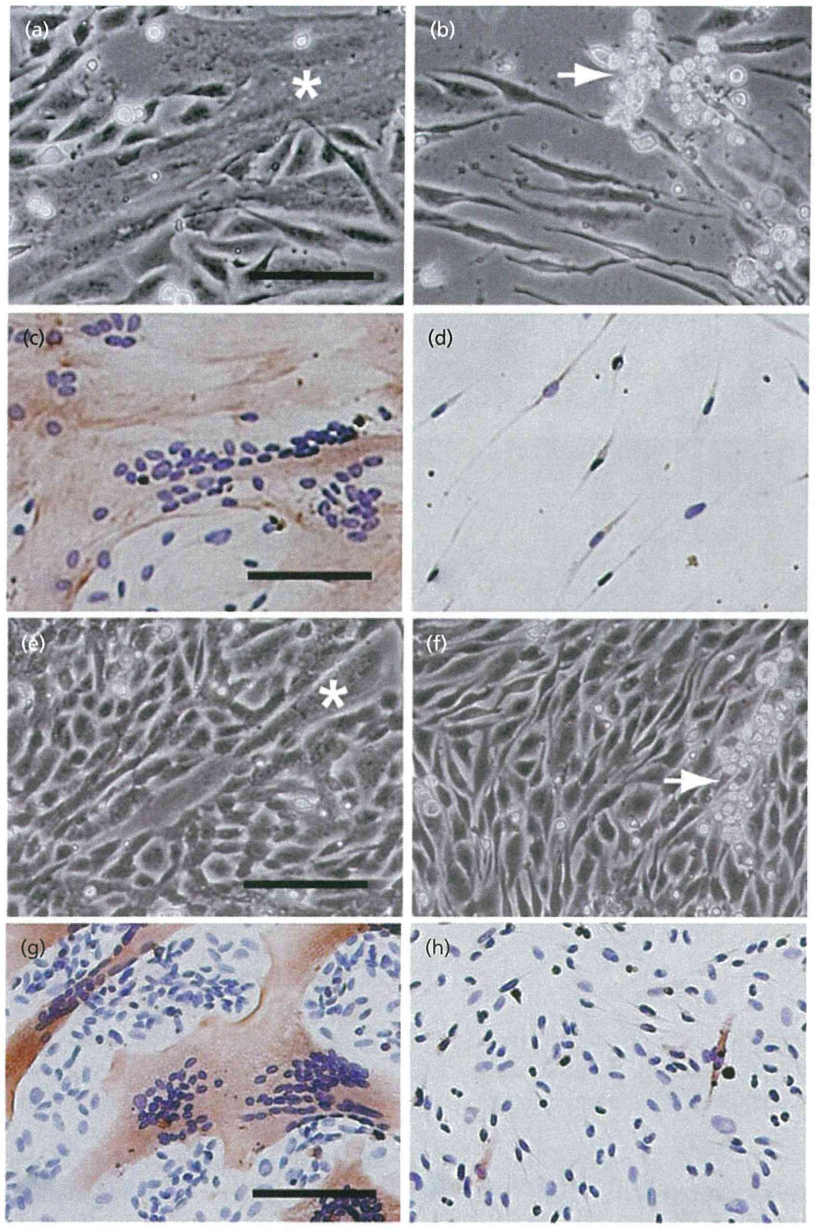

Figure 3 Alendronate inhibited myogenesis in a cell density-dependent manner. Hu5/KD3 cells (5 x 10^4 cells) were plated evenly (a, b, c, d) or per 50-µl spot (e, f, g, h) in a well of a 6-well plate, then cultured in pmDM in the absence (a, c, e, g) or presence (b, d, f, h) of 100 µM alendronate for up to 4 days. (a, b, e and f) Phase contrast images were taken on day 3. Asterisks indicate myotubes, and arrows indicate clumps of detached cells. (c, d, g and h) Cells were fixed on day 4 and probed with antimyosin heavy chain and troponin T (both signals were shown as brown) and haematoxylin (blue). Scale bars, 100 µm.

bisphosphonates are released and internalized by bone-resorbing osteoclasts. This feature enables highly specific interaction of bisphosphonates with the relevant cellular sites of action, in particular, osteoclasts, but also osteocytes and osteoblasts.[3,25] N-BPs impair osteoclast function by inhibiting the mevalonate pathway that prenylates the small G proteins required for cellular survival and then reducing bone resorption. However, it remains to be determined whether osteoclast apoptosis caused by N-BPs is required for inhibition of osteoclast resorption.[2] N-BPs also impair functions of cancer cells and untransformed cells.[6–13] Consequently, N-BPs are likely to be cytotoxic to various type of

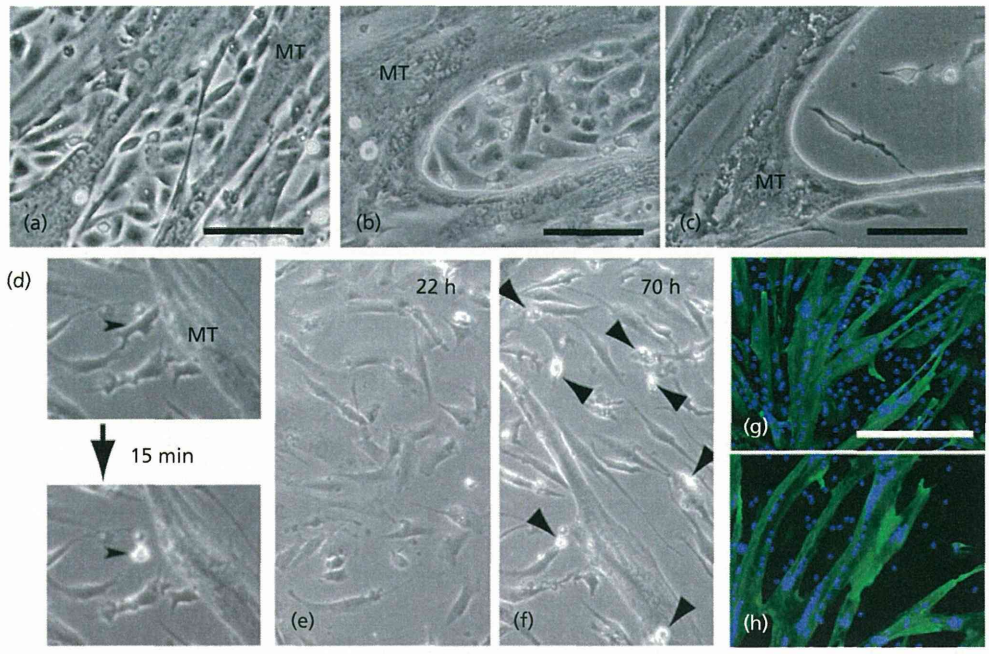

Figure 4 Terminally differentiated human myotubes acquire resistance to alendronate. Hu5/KD3 cells gave rise to myotubes during culture for 7 days in 10% FBS-hDMEM. Then the medium was switched to pmDM with or without ALN (100 μM) and cultured for 4 more days. (a) Both myotubes and undifferentiated mononucleated myoblasts were included in cultures before ALN exposure. (b and c) Myoblasts detached from the bottom of dishes and myotubes remained in culture exposed to ALN (c), whereas both myotubes and myoblasts remained in control cultures (b). (d–f) Time lapse recordings were done during 22–70 h after ALN administration. Only myoblasts shrivelled and detached, whereas myotubes survived in the presence of ALN (d). Many myoblasts (arrowheads) detached during 70 h of culture with ALN (e and f). (g and h) Cells were cultured in pmDM with (h) or without (g) ALN for 4 days. Differentiation marker troponin T (green) was detected by immunofluorescence. Nuclei were stained with DAPI (blue). MT indicates myotube; arrowheads in (d) indicate detaching myoblasts; arrowheads in (f) indicate detached myoblasts; scale bars, 100 μm (a–c) and 400 μm (g and h).

cells other than osteoclasts, although they inhibit osteocyte and osteoblast apoptosis in a connexin 43-dependent manner.[3,25] Previous clinical studies have not examined the pharmacological effects of N-BPs on cells outside the bone environment because of their bone-targeting property. However, the chronic effects of N-BPs on neighbouring tissues of bones cannot be ignored during the long-term N-BP treatment. Skeletal muscles are tightly and closely connected to bone. Hence, muscle cells may internalize N-BPs that are released from bone matrix during bone resorption in osteoporosis patients treated with N-BPs. For clinical use, it is necessary to determine the effects of N-BPs on human myogenic cells.

This study shows that ALN, one of most commonly used N-BPs, disrupted functions of undifferentiated human myogenic cells. ALN severely inhibited proliferation and migration of undifferentiated human myogenic cells. The time-lapse recording revealed that undifferentiated human myogenic cells developed a bipolar shape and then detached. We did not find any apoptotic features in the nucleus of human myogenic cells before cell detachment despite the fact that ALN induces apoptosis in the rat myoblastic cell line L6.[13] This study suggests that ALN does not induce apoptosis of human myogenic cells proximately. If apoptotic features were found in detached cells, it is supposed to be anoikis, apoptosis induced by disruption of cell-substrate adhesion.[26] The high cell density condition of micromass culture suppressed cell detachment, probably because the tight contact with neighbouring cells helps tether human myogenic cells to the substratum. Human myogenic cells in micromass culture were able to survive even in the presence of ALN but lost the ability to undergo myogenic differentiation independently of cell detachment.

We found here that terminal muscle differentiation can confer resistance to ALN on human myogenic cells. Terminally differentiated human myogenic cells, which are multinucleated myotubes, survived, avoiding detachment and retaining expression of myogenic differentiation markers. Myotubes lose an ability to migrate and proliferate after cell fusion following the loss of lamellipodia and membrane ruffles.[22,23,27] Therefore, small G proteins may play different roles in undifferentiated myogenic cells and

terminally differentiated myotubes. The resistance of differentiated myotubes/myofibers to ALN is consistent with previous results showing that ALN does not affect skeletal muscle function of ovariectomized rats.[15] The differential impacts of ALN on undifferentiated and differentiated myogenic cells account for the controversial effects of ALN on rat myogenic cells and muscles in the previous reports.[13,15]

This study provides us with two biological hypotheses on the possible effects of ALN on skeletal muscles. First, muscle regeneration capacity could be attenuated in osteoporosis patients treated with ALN for long periods because undifferentiated myogenic cells, which play essential roles in muscle regeneration are supposed to be susceptible to ALN. Thus, traumatic muscle injury should be avoided by patients being treated with N-BPs. Second, dysfunction of skeletal muscle need not be considered a limiting factor of ALN treatment because human myofibers may retain their function even when exposed to ALN.

Recently, a concomitant increase in muscle strength and bone mineral density was reported after alendronate–calcitriol therapy.[28] Vitamin D affects muscle differentiation and metabolism.[29] However, the contribution of ALN to muscle improvement still remains unknown. This study suggests that the improvement of muscle function would not be due to proximate effects of ALN on human skeletal muscle cells. Despite that, we cannot exclude the putative secondary effects of ALN that ameliorate muscle function. Ectopic accumulation of lipid in skeletal muscles is associated with metabolic disregulation, including type 2 diabetes[30] and attenuation of muscle function in the elderly.[31] Intramuscular lipid accumulation is a possible exacerbating factor in the muscle dysfunction caused by aging and muscular dystrophy. Osteoporosis patients include numerous elderly people that are likely to suffer from muscle wasting caused by decreased locomotive activity. Inhibition of adipogenic differentiation by ALN and a farnesylation inhibitor[32,33] leads us to hypothesize that N-BPs may ameliorate muscle exacerbation through inhibition of ectopic adipogenesis in skeletal muscle. From this point of view, it is worth-following changes in muscle mass, muscle strength and intramuscular lipid accumulation in osteoporosis patients treated with or without N-BPs for more than a couple of years. It should be determined whether there are relationships among muscle function, intramuscular lipid accumulation and bisphosphonate treatment. If that is indeed the case, prevention of ectopic lipid accumulation in skeletal muscle may be a novel therapeutic method to end the exacerbation of muscle dysfunction and ameliorate the locomotive ability of patients.

An unexpected increase in Rho family proteins, in particular RhoA, in ALN-stimulated human myogenic cells suggests that suppression of prenylation promotes the accumulation of RhoA protein. Whether degradation of RhoA protein is controlled in a prenylation-dependent fashion remains to be determined. The morphological changes in human myogenic cells induced by ALN look similar to those of myogenic cells when exposed to the Rac1 inhibitor NSC23766 or Src kinase inhibitor SU6656.[27] Both inhibitors impair the organization of microscale lipid rafts at the lamellipodia and ruffling membranes of myogenic cells, resulting in inhibition of cell migration, division and differentiation. Microraft organization may be involved in the ALN-induced dysfunction of human myogneic cells, although it remains unclear whether the marked accumulation of RhoA plays a role in ALN-induced inhibition of migration and proliferation.

Conclusion

Patients are treated long term with N-BPs because osteoporosis is a chronic disease. Thus, the effects of N-BPs on tissues-neighbouring bone, in particular skeletal muscles, should not be ignored. This study shows the differential impact of ALN on human myogenic cells: ALN disrupted cytoskeletal structures and prevented proliferation and differentiation of undifferentiated human myogenic cells, whereas it did not affect the morphology, gene expression or survival of terminally differentiated human myotubes. Therefore, skeletal muscle dysfunction would not be a limiting factor of ALN treatment. However, it is likely that the regeneration capacity of muscle is attenuated in osteoporosis patients treated with N-BPs for long periods. The use of immortalized human myogenic cells has opened new avenues for mechanistic research and will contribute to improvement of therapeutic strategies and optimization of rehabilitation programmes for locomotive activity in osteoporosis patients treated with bisphosphonates.

Declarations

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Mevastatin mimics the effects of alendronate on human myogenic cells. Hu5/KD3 cells (5 x 10^4 cells) were cultured in 20% FBS-hDMEM for 2 day and then medium was switched to pmGM, pmGM with alendronate (ALN, 100 μM), and pmGM with mevastatin (Mev, 10 μM) for 5 day further. Scale bar, 100 μm.