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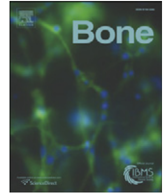

Original Full Length Article

Osteoclasts derived from patients with neurofibromatosis 1 (NF1) display insensitivity to bisphosphonates in vitro

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ABSTRACT

A total of 20 patients with neurofibromatosis 1 (NF1) were screened for NF1-related osteoporosis, and blood samples were collected for isolation of peripheral blood osteoclast progenitors. Patients with NF1 had higher levels of serum bone turnover markers (CTX and PINP) compared to controls. In addition, persons with high bone resorption in vitro on average had high levels of serum CTX. Of the 20 patients with NF1, 15 had low bone mineral density (osteopenia/osteoporosis), but these 15 patients did not have marked risk factors for low bone mineral density. Thus, we recommend screening for osteoporosis to all adult patients with NF1. Our aim was also to characterize the effects of bisphosphonates on NF1 osteoclasts in vitro. NF1 osteoclasts and osteoclasts from healthy controls in vitro were treated with zoledronic acid, alendronate and clodronate. These bisphosphonates caused a marked reduction in the number of normal control osteoclasts in vitro, while only a slight change was observed in the number of NF1 osteoclasts. Ras-inhibitor FTS counteracted this NF1-related insensitivity to zoledronic acid, suggesting that Ras may play a role in this phenomenon.
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Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant neuro-cutaneous-skeletal syndrome with an incidence of ~1/3000 [1–3]. The protein product of NF1 gene, neurofibromin, functions as a Ras-GTPase activating protein (Ras-GAP) negatively regulating Ras signaling pathway [4]. In bone, NF1 mRNA and protein have been detected in human chondrocytes, osteoblasts and osteoclasts [5,6]. Low bone mineral density (BMD) is a common feature in NF1, and is found in both sexes and also in children and adolescents with NF1 (Table 1) [6–12].

Low levels of serum vitamin D, high levels of serum parathyroid hormone, and increased collagen degradation products in urine have been reported in patients with NF1 [10–13]. In the subpopulation of patients with NF1 who have low BMD, high levels of serum parathyroid hormone, calcium, and tartrate resistant acid phosphatase 5b have been reported [12]. Analysis of whole-body subtotal age-matched BMD (Z-score), urine collagen degradation products and in vitro osteoclast formation capacity in 75 patients with NF1 aged 1–25 years showed no correlation between these parameters [13]. Bone biopsy histomorphometry of the patients with NF1 has revealed increased volume of osteoid, and increased number of both osteoclasts and osteoblasts compared to controls [10,11]. This suggests increased bone turnover in NF1, which can be assessed by bone turnover markers (BTMs), such as serum C-terminal cross-linking beta-telopeptide of type I collagen (CTX), tartrate resistant acid phosphatase 5b (TRACP5b), and the N-terminal propeptide of procollagen type I (PINP). CTX is representative of the catabolic rate of bone, TRACP5b has been used as an indicator of osteoclast activity, and PINP is secreted by osteoblasts during collagen synthesis, representing bone formation rate [14,15].

Amino and non-amino-bisphosphonates rapidly localize to bone and are used to treat osteoporosis. Non-amino-bisphosphonates, such as clodronate, generate toxic ATP analogues which disrupt the function of mitochondria, leading to apoptosis of osteoclasts [16–18]. Amino-bisphosphonates, such as alendronate and zoledronic acid, inhibit farnesyldiphosphate syntase, interfering with farnesylation of small GTPases including Ras, Rac and Rho. This leads to reduced ERK pathway signaling (downstream mediator of Ras), which in turn leads to mitochondrial membrane depolarization, activation of caspase cascade, and ultimately to cell death [18–21].

Osteoclasts are derived from haematopoietic progenitor cells, which can be isolated from peripheral blood samples [22,23]. In

Abbreviations: NF1, neurofibromatosis type 1; BMD, bone mineral density; CTX, collagen type I C-terminal telopeptide; PINP, procollagen type I N-terminal propeptide; TRACP5b, tartrate resistant acid phosphatase 5b; MCSF, macrophage colony stimulating factor; RANKL, receptor activator of nuclear factor kappa-beta ligand; FTS, farnesyl thiosalicylic acid.

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Table 1
Osteoporosis and osteopenia in neurofibromatosis 1 defined using dual energy X-ray absorptiometry.

Study	Patients with NF1	Osteoporosis	Osteopenia
Kuorilehto (2005)	12 females, 14 males, aged 21–73	7 (27%)	13 (50%)
Stevenson (2007)	39 females, 45 males, aged 5–18	BMD reported lower compared to controls	
Duman (2008)	16 females, 16 males, aged 3–17	BMD reported lower compared to controls	
Seitz (2008)	9 females, 5 males, aged 19–66	8 (57%)	Not reported
Brunetti-Pierri (2008)	47 females, 26 males, aged 3–59	24 (32%)*	41 (57%)
Tucker (2009)	43 females, 29 males, aged 18–72	14 (19%)	36 (50%)
Current study	10 females, 10 males, aged 23–76	4 (20%)	11 (55%)

* Osteoporosis was defined as Z-score −2.5 SD or less.

osteoclasts, Ras pathway functions as an anti-apoptotic and pro-osteoclastogenic pathway [24]. Osteoclasts derived from peripheral blood of patients with NF1 have been shown to be more numerous, resorb larger amounts of bone, display increased ERK and AKT activity, and tolerate serum-free conditions better compared to control osteoclasts [13,25–27].

Zoledronic acid has been shown to improve fracture healing in $Nf1^{+/-}$ mice [28]. Since NF1 protein functions as a Ras-GAP and since the functions of amino-bisphosphonates are mediated via Ras pathway, the aim of this study was to characterize the effects of zoledronic acid, alendronate and clodronate in osteoclasts derived from peripheral blood of 20 NF1 patients and their controls. An additional aim was to characterize BMD and BTMs in these patients with NF1.

Subjects and methods

NF1 patients

A group of 21 Finnish Caucasian patients with NF1 from 19 families was recruited from the NF clinic of the Department of Dermatology, Turku University Hospital, Turku, Finland. In the statistical analyses we included 10 males aged 23–76 years (average 45) and 10 females aged 23–62 years (average 38). One 56-year-old female patient was excluded because she was medicated with alendronate. All patients fulfilled the NIH diagnostic criteria for NF1 [29]. This study was approved by Ethics Committee of Southwest Finland Hospital District, and the participants gave their written consents to BMD measurement, laboratory tests and osteoclast cultures. The study was carried out in Turku University Hospital and University of Turku during the years 2010–2011.

Medical records of the patients were screened for the past 10 years. The body mass index of the patients was within normal range, three patients were smokers, and of 10 the female patients two were postmenopausal. Four patients were having vitamin D supplementation. Physical activity was evaluated by using a questionnaire [7]. No malignancies or other conditions known to affect bone were found, apart for alendronate in the one excluded patient, and lactose-intolerance in one patient. One patient with mild scoliosis and two patients with lytic bone lesions were found. Four patients had fractures during the past 10 years, which were located in ankle, distal radius, rib and patella. These fractures were noted in two patients with NF1-related osteoporosis and in two patients with osteopenia. In addition, the 56-year old female patient who was excluded from statistical analyses (see above) due to alendronate medication had a stress fracture of metatarsal bone during alendronate treatment.

Control persons

A group of 20 healthy volunteers was recruited from Turku University personnel. Controls were matched for sex, age and menopausal status. Osteoclast progenitors were isolated from peripheral blood samples for cell culture experiments. CTX and PINP serum measurements were available from 18 controls.

Bone densitometry

A dual energy X-ray absorptiometry scan of lumbar spine (L1–L4) and left proximal femur was obtained, using a Hologic QDR 4500 densitometer (Hologic Inc., Waltham, MA, USA) for 11 patients, or an Osteocore 3 densitometer (Medilink Inc., Mauguio, France) for 8 patients, or a Lunar Prodigy densitometer (GE Healthcare, Madison, WI) for one patient, with adult standard measurement software. Corresponding T-scores and Z-scores were calculated using the equipment's standard software and Finnish Caucasian demographic databases provided by the manufacturers. The densitometers were calibrated each morning with a calibration phantom. The image quality and measurements were analyzed by an experienced radiologist (ES). The diagnosis of osteoporosis was made according to the NIH criteria with T-score −2.5 SD or below either in lumbar or femoral region, and the diagnosis of osteopenia with T-score between −1.0 SD and −2.5 SD [30,31].

Serum samples

Laboratory analyses included total calcium, ionized calcium, inorganic phosphate, parathyroid hormone, 25-D3-vitamin, and alkaline phosphatase including its isoenzymes, and CTX. These measurements were purchased from Turku University Hospital, Turku, Finland, and carried out using Roche automatic analyzer (Roche diagnostics, Mannheim, Germany). TRACP5b was measured using BONETRAP immunofixation method, and analyses were purchased from Medix Laboratories, Helsinki, Finland. PINP measurements were purchased from Oulu University Hospital, Oulu, Finland, and were carried out using UniQ RIA analyzer (Orion, Espoo, Finland). All samples were collected in a similar way, and analyzed using a standardized protocol.

In vitro analysis of osteoclast cultures

In each case, osteoclast progenitors from 20 patients and 20 controls were isolated immediately after the patient's visit. Osteoclast progenitors were cultured as described initially by Yang et al. [25], with slight modifications [26]. Briefly, peripheral blood mononuclear cells were isolated using Ficoll-centrifugation (GE Healthcare, Uppsala, Sweden), and 0.5 million cells were seeded on bovine bone slices. Cells were cultured for 7 days in alpha-MEM (Gibco, NY) supplemented with 10% heat-inactivated fetal calf serum (Gibco, NY), receptor activator of nuclear factor kappa-beta ligand (RANKL, 20 ng/ml, Peprotech, Rocky Hill, NJ), macrophage colony stimulating factor (MCSF, 10 ng/ml, R&D systems, Minneapolis, MN) and antibiotics. This was followed by a 2-day culture with the same medium described above with RANKL and MCSF, and different bisphosphonates. We used amino-bisphosphonates zoledronic acid (Zometa ®, Novartis Finland, Finland) and alendronate (Sigma-Aldrich, Steinheim, Germany), and non-amino-bisphosphonate clodronate (Leiras, Finland). A Ras inhibitor Farnesyl Thiosalicylic Acid (FTS) 10E-6 M was purchased from Cayman Chemicals (Ann Arbor, MI) and dissolved in DMSO. For each drug 4 separate bone slices were prepared. Cells were fixed in formaldehyde and stained with tartrate resistant acid phosphatase staining kit (Sigma-Aldrich leukocyte acid phosphatase-kit, Steinheim, Germany), which is a direct osteoclast-specific staining method. Nuclei were visualized with Hoechst

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