Chapter 9

OSTEOCLASTS OF PATIENTS WITH NEUROFIBROMATOSIS 1 (NF1)

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ABSTRACT

Neurofibromatosis 1 (NF1) is an autosomal dominant neuro-skeletal cutaneous syndrome with an incidence of around 1/3000. Low bone mineral density (BMD) and osteoporosis/osteopenia are often associated with NF1. Bone dynamics include continuous bone formation and bone resorption, and imbalance in bone turnover may lead to low BMD. Bone is resorbed by osteoclasts which have been characterized in NF1 using peripheral blood-derived osteoclast differentiation assays. Peripheral blood mononuclear cells were isolated from patients with NF1, age, and gender-matched controls, and these cells were cultured into mature osteoclasts. The results showed that NF1 osteoclasts are more numerous, resorb larger amounts of bone, and display aberrant morphology compared to controls. NF1 osteoclasts also tolerate apoptotic signals, caused by serum deprivation or bisphosphonates, drugs used to treat osteoporosis. Taken together with the fact that osteoblast-mediated bone formation is impaired in NF1, this chapter provides insight on how mutation in the NF1 gene affects bone health, and these results may partially explain the low BMD in NF1.

SHORT COMMUNICATION

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen’s disease, is an autosomal dominant neuro-cutaneous-skeletal syndrome. NF1 is caused by mutations in the NF1 gene, NF1-protein, neurofibromin, and functions such as Ras-GTPase activating proteins or Ras-GAP, thus deactivating Ras [1-3]. The diagnosis of NF1 is based on clinical NIH criteria, and/or on mutation analysis of the NF1 gene [4,5]. The hallmarks of NF1 are benign neurofibroma tumors, café-au-lait macules, axillary freckling, optic gliomas, and typical
skeletal abnormalities, such as congenital pseudarthrosis of the tibia [6]. Skeletal abnormalities in NF1 are either focal or systemic. Focal bone abnormalities include congenital bowing and pseudarthrosis of long bones, fibrocystic lesions, scoliosis, and sphenoid wing dysplasia. The most common systemic bone abnormalities are short stature and reduced bone mineral density (BMD). Osteoporosis is found in 20-50% of patients with NF1 [7].

Bone turnover is a process of old bone being resorbed by osteoclasts and new bones being formed by osteoblasts. During the bone turnover, both bone formation and resorption products are released into the blood and subsequently into urine. These products are called bone turnover markers, including, for example, serum CTX, a collagen I degradation product [8]. Patients with NF1 have increased levels of serum CTX compared to controls, reflecting increased bone turnover [9].

Osteoclast progenitors can be isolated from peripheral blood samples, and cultured into mature multinuclear osteoclasts in vitro. Receptor activators of nuclear factor kappa-beta ligand (RANKL) and macrophage colony stimulating factors (MCSF) are required for the differentiation of mature osteoclasts, and the cell culture methods have been described in detail [10-12]. Briefly, peripheral blood mononuclear cells are isolated using Ficoll gradient centrifugation, and cultured on bone slices for 10-14 days. Osteoclasts are identified using tartrate resistant acid phosphatase (TRACP) staining, and TRACP positive cells with three or more nuclei are considered as osteoclasts.

NF1 osteoclasts have been demonstrated to be hyperactive by us [9,12] and others [11,13]. Specifically, culturing of osteoclast progenitors derived from peripheral blood of NF1 patients resulted in higher amounts of mature osteoclasts compared to controls. Thus, NF1 osteoclasts have increased formation capacity. In addition, the mature NF1 osteoclasts are larger in size and have more nuclei compared to controls, as shown in Figure 1 [11-13]. Moreover, the NF1 osteoclasts have enhanced resorption capacity, since NF1 osteoclasts resorbed higher amounts of bone compared to controls in vitro [11-13]. Bone resorption in vitro can be quantified by counting the resorption pits on the bone slice, or measuring the levels of collagen degradation product CTX in the osteoclast culture medium, which both give comparable results [12]. The increase in the number of NF1 osteoclasts is, however, not sufficient enough to explain the approximately two-fold increase in bone resorption. This leads us to speculate that other factors in addition to osteoclast numbers regulate the total bone resorption by NF1 osteoclasts [12].

![Figure 1](image-url)

Figure 1. A representative comparison of osteoclasts (arrow heads) derived from a NF1 patient and a healthy control person. The black areas represent tartrate resistant acid phosphatase (TRACP) staining. The nuclei appear bright, Hoechst staining. Scale bars 50 micrometers.
Actin is required for formation of actin rings in osteoclasts, which are essential for normal bone resorption and osteoclast migration [14]. NF1 osteoclasts display an enhanced migration capacity compared to control osteoclasts, and have increased content of actin [11,13]. Actin rings are more frequently found in NF1 osteoclasts, suggesting that a greater proportion of osteoclasts are resorbing in NF1 samples compared to controls [11,13]. Actin abnormalities also suggest that the cytoskeleton in NF1 osteoclasts may be different compared to controls. Since the osteoclast cytoskeleton changes during the different phases of the resorption cycle [14], it may be difficult to assess the cytoskeleton of NF1 osteoclasts.

Osteoclast death can be caused by a serum deprivation experiment. NF1 osteoclasts tolerate serum deprivation for up to 24 hours, while the number of control osteoclasts is markedly reduced in the same time [12]. This finding prompted us to evaluate the effects of bisphosphonates in NF1 osteoclasts in vitro. Bisphosphonates are pyrophosphate analogues that bind to bone induce osteoclast apoptosis, and are clinically used to treat osteoporosis [15,16]. Our recent data suggests that NF1 osteoclasts are insensitive to bisphosphonates alendronate, clodronate, and zoledronic acid [9]. Higher numbers and proportions of NF1 osteoclasts survived in vitro treatment with these drugs compared to controls. In addition, in NF1 samples, there was only a slight increase in levels of caspase-3, a marker of apoptotic activity, compared to a marked increase in control samples [9]. However, these in vitro results cannot be extrapolated to the NF1 patients taking these drugs.

It has been shown that NF1 osteoclasts display hyperactive Ras signalling pathways, which is in agreement with the fact that neurofibromin functions as a Ras-GAP [13]. In osteoclasts, Ras pathways have been shown to represent anti-apoptotic and pro-osteoclastogenic pathways [17]. The results on NF1 osteoclasts described above could thus be explained through hyperactive Ras. Also, the downstream signalling pathways ERK and AKT have been shown to be hyperactive in NF1 osteoclasts, supporting the role of Ras hyperactivation [11,13]. Inhibition of Ras in mature NF1 osteoclasts with farnesyl thiosalisylic acid (FTS) does not affect the number of osteoclasts in vitro. However, the addition of FTS counteracted the NF1-related insensitivity to zoledronic acid-induced apoptosis. Thus, the combination of FTS and zoledronic acid had an equal effect on both NF1 and control osteoclasts. This may suggest that the NF1-related insensitivity to apoptotic signals is mediated through Ras signalling [9].

The studies described in this chapter do not evaluate the osteoblast – osteoclast interaction in NF1, such as the role of RANKL/osteoprotegrin signalling. However, it appears that osteoclasts are affected by mutations in the NF1 gene, instead of being normal osteoclasts in abnormal conditions. Human NF1 osteoblasts derived from NF1 patients display impaired differentiation and osteogenic capacity in vitro compared to osteoblasts derived from control persons [18]. Taken together with findings of NF1 osteoclasts, it is not surprising that patients with NF1 often develop and/or progress to low bone mineral density, regardless of gender and/or age [7].

No correlation between osteoclast formation capacity in vitro, urine bone turnover markers, and bone mineral density was found in young (age 1-25 years) patients with NF1 [13]. Thus, some patients with NF1 have normal BMD and high osteoclast activity in vitro, which is in analogy with our data [9]. However, it may be possible that selected bone turnover markers, such as serum CTX, may correlate with BMD or osteoclast activity in vitro. A positive relationship between levels of serum CTX and the bone resorption activity in vitro
has been shown in both NF1 patients and healthy controls [9,12]. Our results on serum CTX are promising, but are limited to a small number of patients without prospective data.

Increased numbers of both osteoclasts and osteoblasts have been shown in bone biopsies from patients with NF1, suggesting that the findings on NF1 osteoclasts in vitro may also be operative in vivo. These biopsies also show larger amounts of osteoids in NF1 samples compared to controls, suggesting a mineralization disorder in NF1 [19]. Also, in other Rasopathies, rare syndromes of the Ras pathway, increased levels of urine bone turnover markers have been documented, suggesting increased bone turnover. This is in analogy with hyperactive Ras and low BMD in patients with different Rasopathies [20].

One may also speculate about the role of osteoclasts in NF1-related focal skeletal abnormalities. Tissue sections from NF1-related congenital pseudarthrosis sites show multiple multinuclear osteoclasts. These osteoclasts may also reside without direct contact to bone, within the fibrous pseudarthrosis tissue [12,18], and may be able to escape the apoptotic signals of the adjacent microenvironment.

In conclusion, NF1 and hyperactive Ras regulate human osteoclastogenesis in vitro. This is in analogy to low bone mineral densities often noted in the NF1 patients. In addition, our preliminary results on bisphosphonate insensitivity in NF1 osteoclasts in vitro suggest that patients with NF1 may clinically respond differently to treatments of osteoporosis. Thus, a study on the effects of bisphosphonates on NF1 patients with osteoporosis is called for. Also, the use of biomarkers to predict low BMD or bone loss in NF1 could provide valuable clinical benefit, if a suitable biomarker could be identified. Therefore, continuing research in both clinical and basic science is fundamental for acquiring better understanding of bone pathology in NF1 and other Rasopathies.

REFERENCES

Osteoclasts of Patients with Neurofibromatosis 1 (NF1)


