Mechanical Signaling in NF1 Osteoblast Cell

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Abstract

Neurofibromatosis Type I (NF1) syndrome is characterized by neurofibromas and neural tumors but is also associated with skeletal abnormalities. The cellular pathophysiology of skeletal abnormalities in NF1 is not understood. These abnormalities result from constitutive active RAS and its downstream effectors, RASERK pathway, due to mutation of NF1 gene which converts active RAS-GTP into inactive RAS-GDP. In osteoblast cells, RAS-ERK pathway is involved in cell proliferation and differentiation and is also involved in mechanical signals transduction.

In this study, we propose that NF1 mutation in osteoblast cells will affect the response to mechanical stimulation through the RAS pathway. The Flexcell tension system was used to mechanically stimulate calvarial osteoblast precursor from conditional knockout mice, Nf1(ob-/-), and wild type calvarial osteoblast precursor cells, (WT). The protocol of cyclic mechanical strain was 2% to 4% elongation at 0.16 Hz (10 cycles per minute) for 24 h. Mechanically stimulated cells showed lower expression levels of the osteoblast marker gene, RUNX2, measured at 4 h and 8 h post-stretch. Mineralized matrix deposition, assessed by Alizarin red staining, was decreased in Nf1(ob-/-) compared to (WT) cells following mechanical stimulation. the Nf1(obl-/) and WT osteoblast precursor cells were then treated with RAS inhibitor (FTI-277), for 4 h and 8 h. RUNX2 expression level was increased in Nf1(obl-/) cells compared to non-treated cells. However, the opposite result was seen in (WT) cells. The FTI-277 treatment resulted in lower RUNX2 expression level and lower mineralized matrix deposition.

This response of (WT) cells was normal. However, the Nf1(obl-/) response showed that these cells although they have hyper-active RAS, but when it is exposed to stress, it loses its ability to express osteoblast markers or lay down mineralized matrix. Our results indicate that, the hyper-active RAS in NF1 mutant osteoblast will result in cells being stuck in proliferative state and unable to differentiate.

Keywords: Neurofibromatosis; Osteoblasts; Bone; Mechanical signaling; Craniofacial

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder caused by loss of function mutations in the NF1 gene with an incidence of approximately 1 in 3000, making it one of the most common genetic disorders [1,2]. NF1 syndrome is primarily characterized by subcutaneous neurofibromas and neural tumors. In addition, NF1 is associated with several skeletal abnormalities including, scoliosis, tibial bowing and sphenoid wing dysplasia [3,4]. Unfortunately, the cellular pathophysiology of the NF1 skeletal dysplasia is still not fully understood [5]. The main functional domain of NF1 gene is known to be located between exon 27 and 34, known as RAS-GAP domain which gives the NF1 gene it’s tumor suppressor property [6,7]. The NF1 gene encodes neurofibromin, a RAS-GTPase-activating protein (GAP) that promotes the conversion of an active RAS-GTP-bound form to an inactive RAS-GDP form and functions to negatively regulate the activity of RAS effectors, including the RAF–MEK–ERK signaling pathway [8,9]. Thus, NF1 mutations results in activation of canonical mitogen-activated protein kinase (MAPK) signaling [10,11]. Of relevance to skeletal development, NF1 expression has been reported in hypertrophic chondrocytes, which are an important intermediate step during endochondral ossification, and also in adult osteoblast and osteoclasts [12,13] either of which might explain the skeletal involvement in NF1. It is established that skeletal tissue can sense mechanical loading which induces bone remodeling activity, resulting in structural changes through different cellular pathways. Several studies have shown that, RAS-MAPK-ERK pathway is the main contributor in mechanical signaling response in osteoblast cells [14-17]. The role of MAPK signaling components have been shown to favor osteoblastic cell proliferation and differentiation. In particular, ERK1/2 is involved in cell proliferation, differentiation and the survival of several cell types, including osteoblasts [18,19]. ERK1/2 signals can promote the proliferation and anabolism of osteoblasts in order to facilitate bone turnover, thereby contributing to the homeostasis of bone tissue [20,21].
Knowing that neurofibromin is expressed in bone cells and acting on RAS signaling pathway and that bone cells can adapt to mechanical stimulation through activation of the RAS signaling pathway, we hypothesize that the response of NF1 mutant osteoblast to mechanical stress is defective which contributes to the skeletal tissue abnormalities in NF1 patients.

**Results and Discussion**

This result supports the previous finding of the lack of NF1 in osteoblast resulting in reduced bone mineral density (BMD) and reduced mechanical properties [40]. Blockade of RAS protein post-translation modification is shown to prevent its targeting to the cell membrane and abrogation of its downstream effects. FTI-277 is an anti-RAS treatment which prevents RAS isoprenylation [35,41]. We hypothesize that if we inhibit hyper-active RAS using FTI-277, then the NF1 mutant osteoblast cell should be able to differentiate normally. We find that, when treated with 5 µM of FTI-277 in OIM culture medium NF1(ob-/-) cells were able to produce more mineralized matrix. However, the NF1 (WT) osteoblast cells treated with 5 µM of FTI-277 produced less mineralized matrix when compared to untreated cells. This indicates that the level of RAS signaling is critical not only for osteoblast proliferation and differentiation but for normal function and matrix deposition. This is confirmed by the level of RUNX2 expression, which is known to be phosphorylated by ERK1/2 during matrix deposition [42,43].

Since we know that RAS-ERK pathway is involved in mechanical signal transduction [28,44]. We asked what is the role of NF1 in osteoblast during mechanical stress. Although different systems have been used to strain osteoblast cells, it is very difficult to compare in-vitro applied strains with those applied in-vivo because the characteristics of the strains are different, i.e. the 3D configuration and presence of interstitial fluid in-vivo. The Flexcell system has been a good platform to study the effect of mechano stimulation in many different contexts [45,46]. This system applies an equibiaxial strain to the cells using a flexible silicone bottom plate connected to a computer-controlled vacuum device. In the literature a wide variety of parameters have been used (i.e. the magnitude of stretch, the frequency of stretch and the duration of stretch, to mechanically stimulate different cell types). In terms of bone cells, there is a general concordance that the frequency is more important than the magnitude of stretch applied and 2% to 4% elongation is found to be enough to initiate osteoblast cellular response. At higher magnitudes (i.e. 10% to 12%) it is reported to be lethal to the cell [20,45]. In this study, we used the Flexcell system to apply a mechanical strain to NF1 (ob-/-) and NF1(WT) at 10 cycles per minute (0.16 Hz) for 24 h and 3% elongation. We found, in terms of protein expression that RUNX2 showed lower expression level in NF1(ob-/-) upon mechanical stretching compared to non-stretched control cells. However, the opposite was found in NF1(WT) cells with RUNX2 expression level increased with stretching in NF1(WT) cells. Also we tested the downstream effects of stretching on mineralized matrix formation. It has been shown that mechanical stimulation leads to increased matrix deposition [32,47].

**Conclusion**

Our results showed, NF1(ob-/-) cells upon mechanical stimulation were unable to responded normally and unable to form more matrix compared to NF1(WT). NF1(ob-/-) cells


32. Plotkin LI, Mathov I, Aguirre JI, Parfitt AM, Manolagas SC, et al. (2009) Mechanical strain may direct future studies to determine the response of osteoclast cells in NF1 to mechanical stimulation as this might bridge the gap and help to understand the cellular events leading to skeletal abnormalities in NF1 patients [48].