Bone 46 (2010) 167–175

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Nuclear factor of activated T cells mediates fluid shear stress- and tensile strain-induced Cox2 in human and murine bone cells

Ayse B. Celil Aydemir [a], Hiroshi Minematsu [a], Thomas R. Gardner [a], Kyung Ok Kim [a], Jae Mok Ahn [b], Francis Young-In Lee [a,*]

[a] Department of Orthopaedic Surgery, Columbia University Medical Center, 630 W 168th Street, Black Building 14-1412, New York, NY 10032, USA
[b] Hallym University, Chuncheon of Gangwon, Republic of Korea

ARTICLE INFO

Article history:
Received 25 November 2008
Revised 27 August 2009
Accepted 31 August 2009
Available online 11 September 2009

Edited by: D. Burr

Keywords:
NFAT
Fluid shear stress
Mechanical stimulation
Cox2
Osteoblast

ABSTRACT

Mechanical loading such as interstitial fluid shear stress and tensile strain stimulates bone cells, which respond by changing bone mass and structure to maintain optimal skeletal architecture. Bone cells also adapt to bone implants and altered mechanical loading. Osseous integration between host bone and implants is a prerequisite for the stability of implants. Fluctuating fluid pressure and interfacial strains occur between bone cells and implants due to mechanical loading during walking and other daily activities. In this study, we examined the signaling mechanism by which mechanical stimulation activates a novel transcription factor in human and mouse bone cells. Nuclear factor of activated T cells (NFAT) is one of the transcription factors that act downstream of the Ca^{++}/calcineurin (Ca^{++}/Cn) network: a well-known pathway of inflammation. In this study, we hypothesized that NFAT2 is activated in response to mechanical stimulation and mediates Cox2 expression. Fluid shear stress and tensile strain results in nuclear translocation of NFAT in cells of the osteoblastic lineage. A peptide inhibitor of the Cn/NFAT axis was found to block the mechanical stimulation-mediated Cox2 induction. Further, chromatin immunoprecipitation assay shows direct interaction between NFAT2 and the human Cox2 promoter region. Additionally, CnAβ knockout calvarial bone cells were found to be less sensitive than control bone cells to mechanical stimulation. Our study provides new evidence for a novel role for NFAT in bone mechanotransduction in the context of cytokine gene induction in bone cells.

Introduction

Bone is a complex and dynamic tissue that is able to regulate its own mass and architecture. Its metabolic demands are managed primarily through hormones, e.g., calcitonin, vitamin D, and PTH. To maintain its structural and biomechanical integrity, bone adapts to its environment [1]. The bone remodeling process is a result of the coordinated activity of osteoblasts, which make new bone, and osteoclasts, which resorb bone. When bone cells sense alterations in mechanical load, bone mass and structure are modified to respond to this change and maintain optimal skeletal architecture [2]. Mechanical loading at the physiologic level can stimulate a net increase in bone mass *in vivo* [3,4], whereas prolonged absence of loading as in the case of extended bed rest or weightlessness can result in bone loss [5].

Mechanical loading is known to elicit a response from bone cells, where deformation of skeletal tissue generates substrate strains that drive the oscillatory movement of interstitial fluid. Bone cells respond to interstitial fluid flow inside the canalicular–lacunar networks and trabecular spaces within bone tissue by increasing bone formation [6]. Several studies have been reported on the effects of oscillatory fluid flow on osteoblastic cells and osteocytic cells to include intracellular calcium transport, prostaglandin E2 release, increased osteopontin gene expression, increased MAPK activity, and inhibition of NFκB binding [7–9]. Studies have also shown that mechanical unloading leads to increased bone resorption, decreased bone mineral density, and decreased bone formation [8,10,11].

In addition to external loading, stimuli from the interfacial mechanical environment between host bone and implants also play a crucial role in the long-term clinical success of joint replacement arthroplasties. Altered mechanical stimulation exerts immediate effects on periprosthetic bone mass. Fluid flow in the effective joint space disseminates wear particle debris, and fluctuating mechanical stimulation caused by hip joint motion and ambulation contributes to implant failure [12–14]. Clinical studies suggest that mechanical stimulation may initiate periprosthetic bone loss prior to wear particle generation, and the instability associated with migration may cause locally high fluid pressure surrounding the prosthesis [15–17]. In addition, rat and rabbit experimental models support a role for oscillatory fluid pressure in the initiation of periprosthetic bone loss [15,18].

In progression to implant failure and periprosthetic bone loss, host-inflammatory reaction to interfacial motion may be regarded as a host-immune response. Factors that mediate the host-inflammatory

* Corresponding author. Fax: +1 212 305 2741.
E-mail address: fl127@columbia.edu (F.Y.-I. Lee).

8756-3282/$ – see front matter
doi:10.1016/j.bone.2009.08.061

reaction may contribute to periprosthetic bone loss. Although mechanical stimuli are known to activate calcium signaling and cytokine secretion in osteoblasts, the mechanisms by which transcriptional factors transactivate target cytokine gene promoter regions have not been well elucidated. One such transcription factor is the nuclear factor of activated T cells (NFAT) in immune cells [19,20]. Initially identified in T cells, the role of NFAT in other cell types is emerging [21,22]. This family of transcription factors includes five members. NFAT1, NFAT2, NFAT3, and NFAT4 are under the regulation of the calcium/calcineurin (Ca^{++}/Cn) pathway and are collectively called NFATc. NFAT5 responds to osmotic pressure changes [23]. NFAT proteins exist in a phosphorylated form in the cytoplasm but translocate to the nucleus upon dephosphorylation by Cn and bind to their consensus sequence on the promoter of several cytokines. Deletion of NFAT2 results in embryonic death, while NFAT1/NFAT2 deletion results in anergy [24].

The functional role of NFAT2 in osteoblasts and osteoclasts is recently being explored. NFAT2 is expressed in osteoclast precursors and osteoblasts. NFAT2 is also essential in RANKL supported osteoclastogenesis and its overexpression can result in osteoclast formation in the absence of RANKL [25,26]. NFAT2 may also regulate osterix transcriptional activity [27]. In addition to its functional role in bone cells, NFAT has an important role in mechano-sensing of cardiac myocytes [28,29] as a downstream signaling component of the Ca^{++}/Cn axis. Mechanical stimulation elevates intracellular calcium in osteoblasts and the Cn signaling axis acts downstream, thus making the Cn/NFAT2 network a compelling candidate for mechanotransduction in osteoblasts. The role of the Cn/NFAT axis in bone cells is also clinically relevant because cyclosporine A, an inhibitor which blocks the binding of Cn to NFAT, is commonly used for immunosuppression in patients with allogenic organ transplantation. Further, the Cox2 gene promoter is known to have the consensus sequence −CGAAA-, which is a binding site for NFAT. We hypothesize that the NFAT signaling mediates Cox2 in response to mechanical stimuli in osteoblasts.

The goal of this study was to show that NFAT2 is a mediator of mechanotransduction in human and mouse bone cells and that clinically relevant mechanical stimulation activates NFAT2 and induces Cox2 expression. Furthermore, we show that NFAT2 directly binds the Cox2 promoter and mechanically induced Cox2 is inhibited upon blocking NFAT signaling with a synthetic peptide (VIVIT). These results suggest a novel role for NFAT in mechanobiology of bone cells.

Materials and methods

Preparation of cells

Human mesenchymal stem cells (hMSC) (Cambrex Inc., Walkersville, MD) were maintained according to the manufacturer's recommendations. CnA$\beta^{-/-}$ mice [30] were kindly provided by Dr. Molkentin, University of Cincinnati (Cincinnati, OH). Primary osteoblast cells were isolated as previously described [31]. Briefly, parietal bones of calvaria were isolated from 4-day-old CnAβ +/+, +/−, or −/− mice and then the parietal bones were cut into small pieces and treated with 2 mg/ml of collagenase (Sigma-Aldrich, St. Louis, MO) and cultured in MEMα supplemented with 10% FBS and 1% antibiotic/antimycotic solution.

Mechanical loading

Two methods of clinically relevant mechanical loading were used in the studies: cyclical tensile strain and sinusoidal fluid shear stress (FSS). The cells were cultured on 6-well plates with a silicone elastomer membrane (Flexcell Inc., Hillsborough, NC) for tensile loading. Cells were subjected to physiologic and superphysiologic sinusoidal (0.5% and 5%, respectively) tensile stretching at 1 Hz for 15 min (FX4000T; Flexcell Inc.). For physiologic and superphysiologic FSS, the cells were cultured on type I collagen-coated glass slides (Flexcell Inc.) and loaded at 16 dyn/cm^2 (1.6 Pa) at 1 Hz [32] and/or 1.03×10^6 dyn/cm^2 (103 kPa) hydrostatic pressure for 15 min in parallel plate fluid flow chamber. The sinusoidal flow profile was generated by a Masterflex L/S 7550-30 computerized pump (Cole-Parmer, Vernon Hills, IL) controlled by a custom-written LabVIEW program (National Instruments Corporation, Austin, TX). The sinusoidal fluid flow waveform had an accuracy of $\pm$0.4 dyn/cm^2 as determined by a TS410 Flowmeter with an ME4PXN201 flowsensor (Transonic Systems Inc., Ithaca, NY). For superphysiologic stress, the hydrostatic pressure was applied via a pressurized reservoir connected in parallel with the tubing from the peristaltic pump and flow chamber. The reservoir was kept approximately half-full with 200 ml of sterile media. Laboratory-compressed air at approximately 50 psig was passed through a two-stage 50 SCFM extractor/dryer with a 5-μm filter to remove up to 99.9% of any in-line oil or water (Model 105; La-Man, Port Orange, FL) and then regulated by a precision pressure regulator (Type 700 Pressure Regulator; Control Air Inc., Amherst, NH, rated accuracy $\pm$0.03 psig). The hydrostatic pressure level was monitored with a 0- to 30-psig precision pressure gauge (WIKA Instrument Corporation, Lawrenceville, GA) with resolution of 0.5 psig and a manufacturer-rated accuracy of $\pm$0.3 psig. An in-line HepaVent # L0650 HEPA filter (Whatman; GE Healthcare, Piscataway, NJ) was placed downstream of the pressure regulator to ensure a sterile airstream. The loading regimens used in this study represent normal physiologic and superphysiologic mechanical loading, which are observed in the periprosthetic interface between host bone and the implant [33–35]. Control sham cells were cultured under the same conditions with the omission of mechanical stimulation. For NFAT2 inhibition, cells were treated overnight with 0.5 μM VIVIT peptide (Calbiochem, San Diego, CA) or control VEET peptide (Rockefeller Proteomics Center, New York). VIVIT is a cell-permeable peptide inhibitor of NFAT (RRRRRRRRRRR-GGG-MAGPVIVITGPHEE), which does not inhibit Cn activity, and VEET is the control peptide for VIVIT (RRRRRRRRRRR-GGG-MAGPPHIVEETGPHVI) [36,37].

Gene expression

RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and cDNA was synthesized with the Superscript III kit from Invitrogen (Carlsbad, CA). Real-time PCR (RT-PCR) reactions were conducted with gene-specific primers using the Roche Lightcycler Fast Start DNA Master^{Plus} SYBR Green kit (Roche Applied Sciences, Indianapolis, IN). An Eppendorf Realplex^4 system (Hamburg, Germany) was used for the RT-PCR reactions. The primers used were as follows: human Cox2 forward 5′-GAATGTTCCACCCGCAGTACA-3′; human Cox2 reverse 5′-GCATAAAGCGTTTGCGGTAC-'3; human GAPDH forward 5'- GAAGGTGAAGGTCGGAGTC-3′; human GAPDH reverse 5'-GAAGATGGTGATGGGATTTC-3′; human Alp forward 5'-CCGTGGCAACTCTATCTTTGG-3′; human Alp reverse 5'-GCCATACAGGATGGCAGTGA-3′; human Opn forward 5'-ACATGGAAAGCGAGGAGTTGA-3′; human Opn reverse 5'-CAATCAGAAGGCGCGTTCA-3′; mouse Cox2 forward 5'-ACATCGATGTCATGGAACTG-3′; mouse Cox2 reverse 5'-GGACACCCCTTCACATTATT-3′; mouse GAPDH forward 5'-AGAACATCATCCCTGCATCC-3′; and mouse GAPDH reverse 5'-AGTTGCTGTTGACGTCGC-3′.

NFAT2 Immunocytochemistry

Immunocytochemistry can visualize NFAT2 in the cytoplasm and in the nucleus. Slides were fixed with 4% paraformaldehyde and ethanol, rinsed with PBS, and air-dried overnight for immunocytochemistry. The slides were incubated in a blocking solution containing 5% mouse serum, washed and incubated with NFAT2 antibody (Abcam, Cambridge, MA) overnight at 4 °C. The slides were then washed and incubated with a secondary antibody Alexa 488 (Invitrogen) for 1 h