Bone 50 (2012) 79–84

Contents lists available at SciVerse ScienceDirect

Bone

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

Original Full Length Article

Primary cilia modulate Ihh signal transduction in response to hydrostatic loading of growth plate chondrocytes

Yvonne Y. Shao [a,*], Lai Wang [a], Jean F. Welter [b], R. Tracy Ballock [a,c]

[a] Department of Biomedical Engineering, Cleveland Clinic, Cleveland OH, USA
[b] Department of Biology, Case Western Reserve University, Cleveland OH, USA
[c] Department of Orthopaedic Surgery, Cleveland Clinic, Cleveland OH, USA

ARTICLE INFO

Article history:
Received 21 June 2011
Revised 30 August 2011
Accepted 31 August 2011
Available online 10 September 2011

Edited by: Thomas Einhorn

Keywords:
Growth plate chondrocyte
Hydrostatic compression
Primary cilium
Indian hedgehog

ABSTRACT

Indian hedgehog (Ihh) is a key component of the regulatory apparatus governing chondrocyte proliferation and differentiation in the growth plate. Recent studies have demonstrated that the primary cilium is the site of Ihh signaling within the cell, and that primary cilia are essential for bone and cartilage formation. Primary cilia are also postulated to act as mechanosensory organelles that transduce mechanical forces acting on the cell into biological signals.

In this study, we used a hydrostatic compression system to examine Ihh signal transduction under the influence of mechanical load. Our results demonstrate that hydrostatic compression increased both Ihh gene expression and Ihh-responsive Gli-luciferase activity. These increases were aborted by disrupting the primary cilia structure with chloral hydrate.

These results suggest that growth plate chondrocytes respond to hydrostatic loading by increasing Ihh signaling, and that the primary cilium is required for this mechano-biological signal transduction to occur.

Introduction

Many studies of mechanical loading of cartilage and bone tissue have demonstrated clear effects at the level of gene expression, protein translation, extracellular matrix production, and in membrane transport processes [1–5]. These studies also suggest that there are multiple cellular pathways capable of responding to the physical stimulation resulting from mechanical forces.

It has been well-established that the hedgehog (Hh) family plays an important role not only during chondrogenesis and limb formation, but also during longitudinal growth at the growth plate [6–7]. Loss of Indian hedgehog in skeletal tissues results in severe dwarfism due to reduced proliferation and abnormal maturation of growth plate chondrocytes. When Ihh is over-expressed in mice, chondrocyte proliferation is enhanced [8–9]. Smoothened (Smo) is one of the two hedgehog receptor proteins involved in the Ihh signal transduction pathway. Binding of Ihh to its receptor Patched (Ptc) allows Smo to initiate the signaling cascade that leads to activation of the Cubitus interrupts (Ci) transcription factor family members Gli1, Gli2, and Gli3 [10–11].

Primary cilia have a unique hair-like structure, and act as a cellular sensory organelle. The intraflagellar transport (IFT) complex is required for ciliary function, which occurs through a complex of multi-subunit proteins resulting in transfer of precursors back and forth from the flagellar tip to the cell body. Recent studies have shown that a key step in Ihh activation occurs when Smo moves to the tip of the primary cilium [12–14], a translocation process that can be disrupted by the Smo antagonist cyclopamine [13–14]. Studies in mice have shown that mutations that cause IFT dysfunction result in the loss of the primary cilia, abnormal Ihh signaling, defects in limb growth and bone formation [15–19].

Primary cilia were originally linked to mechano-transduction in kidney epithelial cells, in which the cilia detect urine flow and transduce this fluid-flow signal into a transient intracellular calcium signal, resulting in increased cell proliferation. Other studies have shown that chloral hydrate treatment can abolish this cilium-dependent flow-induced Ca^{++} signaling event in different cell types [20–21]. Furthermore, studies of a core component of the IFT, the Tg737 protein, reveal that mice with a mutated Tg737 gene have shorter primary cilia, are unable to mount a fluid flow response, and develop unregulated cell proliferation and cyst formation (polycystic kidney disease) [22–23].

It has long been known that cartilage is sensitive to mechanical forces; however, no specific cellular mechano-transduction signaling pathway has been discovered in chondrocytes. In this study, we used our previously described [24] chondrocyte cell pellet model subjected to a hydrostatic compression loading system to determine if primary cilia transduce mechanical forces into biological signals in growth plate chondrocytes.

* Corresponding author at: Department of Biomedical Engineering, ND-20, Lerner Research Institute, The Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA. Fax: +1 216 444 9198.
E-mail address: shaoy@ccf.org (Y.Y. Shao).

8756-3282/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2011.08.033

Materials and methods

Epiphyseal chondrocyte culture

Primary epiphyseal chondrocytes were isolated from two day-old Sprague Dawley rats (Harlan, Indianapolis, IN) as previously described [24]. Animal use was according to a protocol approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic. The primary cells were cultured either as high-density monolayers (6.6×10^5 cells/cm^2) or as three-dimensional cell pellets (3×10^5 cells/pellet) in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 50 μg/ml L-ascorbic acid phosphate, 100 μg/ml sodium pyruvate, 1% penicillin–streptomycin, and ITS + premix (Sigma Chemical, St. Louis, MO) to a final concentration of 0.625 μg/ml bovine insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml BSA, and 5.35 μg/ml linoleic acid. Primary chondrocyte pellets were cultured for 5 days before being subjected to hydrostatic compression, with or without treatment with recombinant sonic hedgehog (Shh 5 nM, R & D System, Minneapolis, MN) or chloral hydrate (4 mM, Sigma) as described below. The control samples were cultured unloaded and without any pharmacological treatment.

Hydrostatic compression

A custom-designed mechanical loading system (Fig. 1) was used to apply an intermittent 1 MPa hydrostatic compression force (1 h on, 1 h off). The hydrostatic loading system consists of two separate compartments, a sterile bioreactor chamber which houses the chondrocyte pellets (up to 9 pellets at a time) and a non-sterile hydraulic section of the system. The two sections were separated by a gas-permeable flexible fluoro-ethylene-propylene membrane (McMaster-Carr, Cleveland, OH). Computer-controlled tandem high-speed micro-gear pumps (Micropump, IDEX Health & Science, Oak Harbor, WA) force water (maintained at 37 °C and equilibrated with 7.5% CO_2 in air) through the hydraulic section thereby generating hydrostatic pressures inside the bioreactor. Heat- and gas-exchange occurs through the membrane. The pressure in the chamber is monitored by a compensated transducer, and controlled in real time by modulating pump speed and a water flow outlet restrictor. Automatic control was achieved by using custom-written C software running on an IBM PC. Half of the culture medium was replaced every 12 h during the unloaded interval. At the end of the compression period, the pellets (loaded and unloaded control) were collected and terminal assays performed.

Cell proliferation

The cell proliferation assay was carried out immediately after the end of the compression period. Cell viability was evaluated using the CellTiter 96 Aqueous One assay kit following the manufacturer's protocol (Promega, Madison, WI). The reagent contains both a tetrazolium compound (MTS) and phenazine ethosulfate (PES), an electron coupling reagent. In metabolically active cells, reduced MTS is a soluble and colored formazan product, which is quantified by measuring the absorbance at 490 nm, and is directly proportional to the number of living cells.

Gene expression

To investigate the effect of hydrostatic loading on chondrocyte gene expression, total RNA was isolated from the cells at designed time points using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA was reverse transcribed using a SuperScript III Kit (Invitrogen, Carlsbad, CA), and quantitative real-time PCR was performed to measure Ihh and Smo gene expression. The expression of 18 s RNA was used for normalization. An ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) program was used for all measurements of minimum threshold cycle (Ct), and the differences in gene expression between control and treated sample were calculated using the manufacturer's protocol. The PCR primers were designed using the Primer Express Software (Applied Biosystems):

Gene	Primer pair
Indian hedgehog	5′-TGCCGACCGCCTCATG 5′-CATGACAGAGATGGCCAGTGA
Smoothened	5′-TTCTCTAAGCGGCGTGAACTG 5′-AAACCGGCAACAGGTCCAT

Gli-luciferase reporter assay

Ihh signaling was examined by measuring the activity of Gli, a downstream transcription factor in the Ihh pathway. Approximately 2×10^6 cells per 60 mm plate were transiently co-transfected overnight with an Ihh-responsive Gli-luciferase reporter plasmid and a Renilla luciferase control reporter plasmid (Promega) using Fugene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. The Gli-luciferase reporter was kindly provided by Dr. Jeremy Reiter, University of California [19] and contains an enhancer with 8 repeating sequences which include a Shh response element and a Gli binding site. The day after transfection, the cells were collected, counted, and then cultured as a three-dimensional cell pellet (3×10^5 cells/pellet) for 4 days. The chondrocyte cell pellets were then subjected to hydrostatic compression loading as described above for 2 days prior to measurement of luciferase activity (Dual

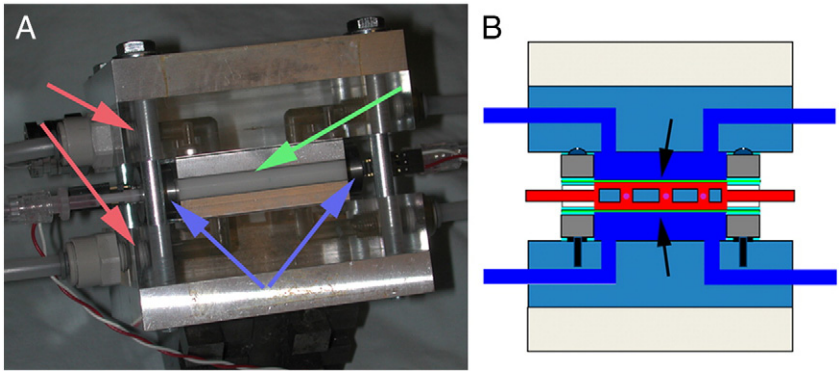

Fig. 1. Custom-designed hydrostatic loading system. The module consists of a PMMA/aluminum (A, red arrows) frame surrounding the bioreactor chamber (A, green arrow) with the intervening space filled with water (B, dark blue). When pressuring the bioreactor, the media ports are closed using valves (A, blue arrows), and pressure is applied to the sample across the flexible FEP membrane (B, arrows). A perforated insert holds up to nine aggregates. Automatic control was achieved by using custom-written C software running on an IBM-PC. This module allows the application of arbitrary hydrostatic pressure waveforms on the construct in the bioreactor.