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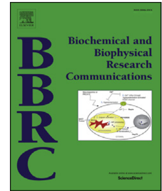

Mechanically stimulated bone cells secrete paracrine factors that regulate osteoprogenitor recruitment, proliferation, and differentiation

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ABSTRACT

Bone formation requires the recruitment, proliferation and osteogenic differentiation of mesenchymal progenitors. A potent stimulus driving this process is mechanical loading, yet the signalling mechanisms underpinning this are incompletely understood. The objective of this study was to investigate the role of the mechanically-stimulated osteocyte and osteoblast secretome in coordinating progenitor contributions to bone formation. Initially osteocytes (MLO-Y4) and osteoblasts (MC3T3) were mechanically stimulated for 24hrs and secreted factors within the conditioned media were collected and used to evaluate mesenchymal stem cell (MSC) and osteoblast recruitment, proliferation and osteogenesis. Paracrine factors secreted by mechanically stimulated osteocytes significantly enhanced MSC migration, proliferation and osteogenesis and furthermore significantly increased osteoblast migration and proliferation when compared to factors secreted by statically cultured osteocytes. Secondly, paracrine factors secreted by mechanically stimulated osteoblasts significantly enhanced MSC migration but surprisingly, in contrast to the osteocyte secretome, inhibited MSC proliferation when compared to factors secreted by statically cultured osteoblasts. A similar trend was observed in osteoblasts. This study provides new information on mechanically driven signalling mechanisms in bone and highlights a contrasting secretome between cells at different stages in the bone lineage, furthering our understanding of loading-induced bone formation and indirect biophysical regulation of osteoprogenitors.

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1. Introduction

Osteoporosis is a silent bone disease that can result in low-energy impact fractures with significant morbidity and associated mortality rates [1]. Treatment of osteoporosis has traditionally aimed to offset bone loss through medications that inhibit catabolism [2]. However, bone anabolic therapeutics may act as an important adjunct for advanced osteoporosis to regenerate lost tissue [3,4]. Given the limited lifespan of the osteoblast [5], bone anabolism requires the continued replenishment of the exhausted osteoblast from an osteoprogenitor population [6–8]. However, little is known regarding the biochemical cues that mediate replenishment from the stem cell niche. Therefore, an increased understanding of bone anabolistic regulatory mechanisms that enhance osteoprogenitor recruitment, proliferation and osteogenesis is required [9].

A potent regulator of bone anabolism is mechanical loading [10]. This involves a complex interplay between multiple cell types, with osteogenic cues believed to be propagated to osteoprogenitors via upstream mechanosensor cells. Recent evidence suggests that this upstream role is predominantly fulfilled by osteocytes [11,12], which are ideally numbered and positioned within their lacuno-canalicular network to coordinate mechanically-mediated secretion of biological signalling factors [12–14]. The mechanism by which osteocytes communicate with neighbouring precursor and/

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or effector cells is a topic of significant ongoing research [15]. It has been demonstrated that osteocytes communicate with osteoblasts via gap junctions [16], and osteoclasts via a paracrine mechanism [17,18], in both static and loaded conditions to regulate the bone remodelling cycle. Furthermore, it has also been shown that osteocytes can induce stem cell osteogenesis in static conditions [19], and recently we have demonstrated that mechanically stimulated osteocytes can induce osteogenic gene expression in MSCs via a paracrine mechanism [20]. These data taken together form a strong argument for the role of the osteocyte as a co-ordinator of bone formation via control of precursor cells, particularly in response to mechanical stimulation. In spite of the mounting evidence of the osteocyte as the key regulator of this process, there is little knowledge of its interplay with the stem cell niche, particularly under physically loaded conditions [15].

Interestingly, bone anabolism in response to loading is maintained in mouse models with depleted osteocytes suggesting a potential coordination by another mechanosensitive cell [21]. Such anabolism may be regulated by osteoblasts which are known to be mechanically sensitive [22] and interact with other cell types [23]. Osteoblasts at the site of loading would be expected to interact with neighbouring and recruited bone forming cells resulting in a co-ordinated anabolic response to loading [24]. Given the lineage progression of osteoblasts towards osteocytes one could infer a comparable secretome between osteoblasts and osteocytes although to date, there is an absence of data indicating the cellular effects of the osteoblast secretome upon bone forming cells.

The objective of this study was therefore to investigate the role of mechanically stimulated osteocytes and osteoblasts in directing the behaviour of bone forming cells. The hypothesis of this study is that soluble factors secreted by mechanically stimulated osteocytes will regulate MSC recruitment, proliferation and osteogenesis. We postulate that these same secreted factors will, in addition, regulate the recruitment and proliferation of osteoblasts. We also hypothesise that osteoblasts, in turn, will also regulate the recruitment and proliferation of bone forming cells in response to loading. By obtaining a greater understanding of the mechanisms underpinning bone formation through mechanical loading, we will further our understanding of bone physiology and which may ultimately direct us to novel anabolic bone therapeutics.

2. Materials and methods

2.1. Cell culture

Three cell lines were used in this study. Firstly, the MLO-Y4 cell line which is a murine derived model of an osteocyte (gift from Dr. Lynda Bonewald, University of Missouri–Kansas City, MO, USA). Secondly, the murine MC3T3 cell line (ATCC, Manassas, VA) which has pre-osteoblastic characteristics and finally, the C3H10T1/2 cell line (ATCC, Manassas, VA) which is a murine mesenchymal progenitor. MLO-Y4 cells were cultured on rat tail collagen (BD Biosciences, Bedford, MA) coated cell culture plastic with α-Modified Eagle's Media (α-MEM) supplemented with 5% calf serum (CS), 5% fetal bovine serum (FBS), 1% L-Glutamine, and 2% penicillin–streptomycin (P/S). MC3T3 cells were cultured in α-MEM supplemented with 10% FBS, 1% L-Glutamine and 2% P/S. C3H10T1/2 cells were cultured in D-MEM low glucose supplemented with 10% FBS, 1% L-Glutamine and 2% P/S (All media supplements from Sigma–Aldrich unless otherwise stated).

2.2. Cellular mechanical stimulation

The generation of dynamic fluid flow was achieved through the utilisation of a rocking platform which oscillated at a frequency of 0.5 Hz and with a tilt angle of 7° consistent with previously published work [20,25]. This system is used in order to generate large amounts of conditioned media when compared to other experimental apparatus such as the parallel flow plate chamber [26]. MLO-Y4 cells were cultured in T-175 flasks with a total of 10 ml media in each flask. Those undergoing mechanical stimulation were placed on the platform for a 24hr period. Mechanical stimulation of MC3T3 cells was also carried out as described above.

2.3. Experimental setup

For each experimental assay described below, MLO-Y4 cells were seeded at a density of 1,200cells/cm2 on type-1 collagen (0.15 mg/ml, BD) coated T-175 flasks (Sarstedt, Nuembrecht, Germany) and cultured in normal growth media under standard conditions (37C, 5% carbon dioxide). 72hr following seeding, cells were washed, the media were changed and the cells were cultured statically (No Flow) or mechanically stimulated (Flow) for an additional 24hr. After this period the osteocyte conditioned media were collected. A media control, lacking any cellular exposure, was also generated (Media Control). All media samples were centrifuged at 1,200 rpm for 5 min to remove any cellular debris from the conditioned media. Experiments were repeated using MC3T3 cells as above. For MC3T3 experiments, T-175 flasks were not coated with collagen and the media control consisted of MC3T3 growth media.

2.4. Effect of mechanically stimulated cell media upon migration

Cellular chemotaxis in response to conditioned media collected from mechanically stimulated cells was assessed by utilising a Boyden chamber technique. Well inserts were placed into a 12-well plate and the cells to be studied (C3H10T1/2 or MC3T3) were cultured on such inserts for 2 h in their normal appropriate media. After this time, the inserts were removed and placed into wells containing MLO-Y4 conditioned media from each group (Flow, No Flow and Media Control) for a period of 18hr. Following this incubation period, the inserts were removed and fixed with 10% formalin solution before staining with haematoxylin. Light microscopy (Nikon Eclipse 90i, Nikon), using NIS Element software was then used to calculate the number of migrated cells.

2.5. Effect of mechanically stimulated cell media upon proliferation

To assess changes in proliferation in response to conditioned media collected from mechanically stimulated cells, cells to be studied (C3H10T1/2 or MC3T3) were seeded at a density of 31,000cells/cm2. Cells were cultured for a 24hr period, washed 3x with PBS and treated with conditioned media from the Flow, No Flow, or Media Control groups. After a further period of 72hrs the cells were centrifuged at 1,200 rpm for 5 min, media aspirated and lysis buffer (TritonTM X-1oo, Sigma–Aldrich, USA) added to each well. Following three freeze-thaw cycles the DNA concentration in each well was calculated using the PicoGreen assay (Invitrogen, USA).

2.6. Effect of mechanically stimulated cell media upon osteogenesis

Osteogenesis was determined using C3H10T1/2 cells seeded at a density of 31,000cells/cm2. Cells were cultured for a 24hr period, washed 3x with PBS and incubated with conditioned media from the Flow, No Flow, or Media Control groups. An additional positive control study group was prepared and incubated with standard osteocyte media with added osteogenic supplements - Dexamethasone 100 nM, Ascorbic acid 2-P 50 μM, and β-

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