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RANKL and OPG activity is regulated by injury size in networks of osteocyte-like cells

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ABSTRACT

Bone remodelling is an intricate process encompassing numerous paracrine and autocrine biochemical pathways and mechanical mechanisms. It is responsible for maintaining bone homeostasis, structural integrity and function. The RANKL-RANK-OPG cytokine system is one of the principal mediators in the maintenance of bone cell function and activation of bone remodelling by the Basic Multicellular Unit (BMU) which carries out remodelling. Theories surrounding the initiation of bone remodelling include mechanical loading, fluid flow and microdamage as potential stimuli. This study focused on microdamage. In an *in vitro* simulated bone environment, gel embedded MLO-Y4 cell networks were subjected to damage in the form of planar, crack-like defects of constant area and varying thickness. The biochemical response was determined by ELISA and luciferase assay. The results showed that RANKL release increased and OPG decreased in a manner which depended on injury size (i.e. thickness) and time following application of injury. The effect of microdamage on cell viability and apoptosis was also evaluated. This work demonstrates that injury alone, in the absence of imposed strain or fluid flow, is sufficient to initiate changes in cytokine concentrations of the type which are known to stimulate bone remodeling.

Introduction

Bone is a dynamic material which is continuously being remodelled in order to maintain calcium homeostasis and to preserve volume. Bone remodelling is controlled by the basic multicellular unit (BMU), which requires a tightly coordinated grouping of osteocytes, osteoblasts and osteoclasts. The contributing elements in the functioning of bone homeostasis are regulated hierarchically through a series of cell signals, cross talk and cascades, essentially focused on members of the tumour necrosis factor superfamily—receptor activator of NFκ B Ligand (RANKL) and its receptors, receptor activator of NFκ B (RANK) and osteoprotegrin (OPG) [1,2]. These, along with other factors, such as ephrins and interleukins, are central mediators of differentiation, proliferation and inhibition of osteoclasts, and are pivotal in the bone remodelling process [3,4].

Osteocytes, which constitute 90–95% of all bone cells, being present in quantities of 12,000–25,000 cells per mm^3, form intricate cell process networks *via* cellular processes (typically 50–100 processes per cell) which penetrate canaliculi in the bone matrix [5–7]. Osteocytes are believed to be capable of mechanotransduction, allowing them to detect alterations in matrix strain and canalicular fluid flow [8].

Microdamage in bone is a naturally occurring phenomenon resulting from daily cyclic loading, which can manifest itself as ellipsoidal cracks, normally ranging from 50 to 400 μm in width, measured transverse to the bone's axis. A build up of microcracks in bone is a factor in reduced strength [9]. Force and loading on a bone has been found to elicit DNA damage in osteocytes proportional to the force applied [10]. It has long been thought that microdamage in bone is one of the principal mediators of bone remodelling and in the initiation of the BMU, which then functions in removing damaged or compromised bone surrounding [11–13]. It has been found that the birth rate and longevity of the BMU is directly proportional to the frequency and intensity of microcracks and that mechanical stimulus is a major contributing factor to the dedifferentiation process of osteocytes into osteoblasts [14].

It has been established that the RANKL-RANK-OPG signaling pathway is greatly involved in the activation of bone remodelling by the BMU. RANKL is a primary mediator in the activation and differentiation of preosteoclasts into osteoclasts, and so functions in stimulating and sustaining bone remodelling [15,16]. RANKL has been shown to be induced by numerous factors, including parathyroid hormone tumour necrosis factor alpha (TNF), TGF α, 1, 25 dihydroxyvitamin D3, prostaglandin E_2 (PGE_2), interleukin 1 (IL-1), interleukin 6 (IL-6) and interleukin 11 (IL-11) [17–19]. Osteoclast activation occurs as a result of RANKL binding to the RANK receptor on the cell surface of preosteoclasts and mature osteoclasts [1,20–22].

RANKL is antagonised by OPG, a member of the TNF superfamily, which functions by binding to and sequestering RANKL, impeding the

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resorption of bone [1,23,24]. OPG is released from the osteoblast lineage of cells on receipt of signals such as oestrogen, BMPs and TGF-β [21]. OPG blocks receptor function of RANKL through alteration of orientation and by blocking cytoplasmic interactions and NFκ B activation [25].

There is strong evidence to suggest that remodeling is targeted to regions containing microcracks [11] implying that the osteocyte network is capable of detecting them. As yet, the mechanism of detection is unclear, though some workers have suggested that this could be done via the cells' mechanotransduction capabilities [26]. We have proposed an alternative mechanism, by which cellular processes spanning the crack could be ruptured by a shearing mechanism similar to the action of a pair of scissors [27–29].

To understand the bone remodelling response to microdamage, a microenvironment using an osteocyte cell line (MLO-Y4) seeded in collagen–matrigel constructs was developed. MLO-Y4 cells have been shown to have many characteristics of osteocytes, predominantly, similar phenotype in a stellate morphology, and the ability to produce large amounts of osteocalcin and type 1 collagen. MLO-Y4 cells were cultured in collagen/matrigel composite gels which facilitated the formation of cell–cell process networks similar to those seen *in vivo*. Crack-like planar defects were created in the gel embedded cells, and the biochemical response quantified by ELISA and luciferase assay. The objective of this study was to determine the effect of microdamage on RANKL and OPG release and to quantify this effect as a function of the severity of the damage applied.

Methods

Cell culture

MLO-Y4 cells were maintained in α Modified Eagles medium (Biosera) supplemented with 5% fetal bovine serum (Biosera), 5% iron supplemented calf serum and 1% antibiotics (penicillin/ streptomycin) (Sigma Aldrich). Cells were cultured in collagen coated flasks (0.15 mg/mL rat tail collagen type 1) at 37 °C at 5% CO_2. They were passaged every 2–3 days at 80% confluency.

Three dimensional MLO-Y4 culture

In order to simulate a 3D *in vitro* bone micro-environment, MLO-Y4 cells were embedded in a 3D rat tail collagen type I/Matrigel construct (BD Biosciences). Constructs were prepared by the addition of 1 volume of Matrigel basement membrane matrix to 1 volume of a collagen solution comprised of 58% Collagen, 26% 5× DMEM (Sigma Aldrich), 2.5% FBS, 2.5% CS, 5% Sodium Hydroxide (Sigma Aldrich) and 5% cell suspension. Such 3D culture ensures cell process formation from osteocytes. The collagen / Matrigel constructs containing 1×10^6 cells/mL of gel were cultured in 24 well plates and incubated for 1 h prior to addition of normal growth medium [30]. They were cultured for 5 days prior to experimentation to facilitate cell process network formation.

Microinjury study of 3D gel embedded MLO-Y4 cells

In order to apply local damage to the gel embedded MLO-Y4 cells, gels were subjected to microdamage of a set length and width and variable thickness using acupuncture needles (Harmony Medical), of four different diameters: 160, 300, 400 and 800 μm. A single planar defect was created in each culture well by inserting a needle vertically into the centre of the hydrogel (see Fig. 1A) and drawing it through at a right angle, creating a defect with dimensions of 7 mm in length × 5 mm in depth. Negative controls were uninjured cells and hydrogels excluding cells. Gels were incubated using OptiMEM (Invitrogen) serum-free media. Samples were taken at 24-h intervals over a 72-h time period. RANKL and OPG were quantified by means of single sited specific ELISA (Mouse RANKL and OPG ELISA, R and D systems). $n=3$ cultures were assayed at each injury level, in addition to non-injured controls.

Plasmids

A RANKL promoter construct developed by O Brien et al. [31] was used, which contains a luciferase gene reporter downstream of the RANKL promoter, in addition to a neomycin resistance gene to ensure growth of a transfected cell population. Expression of luciferase downstream of the RANKL promoter is indicative of RANKL gene expression in the cells. This then allows for quantification of RANKL promoter activity which correlates to RANKL production. In addition to quantifying the production of RANKL from live cells and allowing the comparison between the release of cell bound RANKL from MLO-Y4 cells and RANKL gene expression, the use of the promoter construct may also indicate that some cells remain viable at the point of injury. The RANKL – luciferase plasmid was prepared in 50 μl TRIS/EDTA. One Shot chemically competent E coli cells (Invitrogen) were transformed with 5 μl RANKL ligation reaction and grown overnight in 2 mL LB Agar containing 100 mg/mL kanamycin (Sigma Aldrich). Plasmid cultures were isolated and grown overnight in LB broth preceding plasmid purification using Qiagen Miniprep. A 250 mL overnight culture of transformed cells was cultured under shaking conditions (225 rpm) and the plasmid purified by means of Qiagen Maxiprep kit. Plasmid DNA was quantified by spectroscopy (Mason Nanodrop 1000).

Transfection of GFP plasmid

MLO-Y4 cells were grown at a density of 1×10^5 cells in a 24 well plate 24 h prior to experimentation. Transfection was optimised by means of 1 μg/μl green fluorescent protein (GFP) positive plasmid and were conducted using Lipofectamine 2000 (Invitrogen). Three ratios of plasmid: lipofectamine were used to establish the best means of transfection efficiency; 1:1, 1:2 and 2:1. Plasmid DNA and lipofectamine was diluted accordingly with an appropriate quantity of optiMEM (50 μl: 50 μl lipofectamine complex: plasmid complex) and applied to cells. Following 6 h of incubation, the plasmid–lipofectamine solution was replaced with MLO-Y4 culture medium and incubated for 48 h. Cells were analysed by means of fluorescent microscopy to establish the optimum transfection conditions and lipid/pDNA transfection ratios showing the greatest quantity of GFP positive cells (data not shown).

Transfection of MLO-Y4 cells with RANKL promoter plasmid

MLO-Y4 cells were grown at a density of 1×10^5 cells in a 24-well plate 24 h prior to experimentation, and cells were transfected with a plasmid containing the reporter gene luciferase under the control of the RANKL promoter. Transfections were conducted using a 2:1 ratio RANKL promoter + luciferase plasmid and lipofectamine at a concentration of 1 μg/μl plasmid. Plasmid DNA and lipofectamine was diluted accordingly with an appropriate quantity of optiMEM (50 μl: 50 μl lipofectamine complex: plasmid complex) and applied to cells. Following 6-h incubations, the plasmid–lipofectamine solution was replaced with MLO-Y4 culture medium and incubated for 48 h. Luciferase activity was confirmed by means of a luciferase assay.

Determination of RANKL promoter activity: luciferase assay

Luciferase activities were measured using a luciferase reporter assay kit (Promega) followed by quantification using spectroscopy. Culture medium was removed and cells were rinsed with dPBS. Four hundred-microliter lysis reagent was added to cells followed by a 15-min incubation. The lysis buffer was placed in a tube and incubated briefly. 20 μl of cell lysate was then added to 100 μl luciferase assay reagent and the luminescence measured with the Victor 3 Wallac 1420 multilabel centre.