Mechanical manipulation of bone and cartilage cells with 'optical tweezers'

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Abstract The single beam optical gradient trap (optical tweezers) uses a single beam of laser light to non-invasively manipulate microscopic particles. Optical tweezers exerting a force of approximately 7 pN were applied to single bone and cartilage derived cells in culture and changes in intracellular calcium levels were observed using Fluo-3 labelling. Human derived osteoblasts responded to optical tweezers with an immediate increase in $[Ca^{2+}]_i$ that was inhibited by the addition of a calcium channel blocker nifedipine. Force applied to different regions of cells resulted in a variable response. $[Ca^{2+}]_i$ elevation in response to load was lower in rat femur derived osteoblasts, and not apparent in primary chondrocytes and the osteocytic cell line (MLO Y4).

Key words: Bone; Cartilage; Calcium; Optical tweezers; Force

1. Introduction

It is widely accepted that bone models its mass and structure in response to mechanical strain. Exposure of bone to in vivo loading has been shown to result in periosteal osteoblast activation and bone formation [1–3]. However, the mechanism by which mechanical strain is converted into a biochemical signal is not fully understood. Previous work by other groups and ourselves has highlighted the possible role for calcium in mediating the response of osteoblasts to mechanical and hormonal stimuli [4–6]. Measurement of real time calcium changes in bone and cartilage derived cells in response to mechanical load has been possible via the development of both confocal microscopy and fluorescent linked calcium ester dyes [7,8].

Methods of mechanical strain application in vitro have been numerous producing mechanical loads of varying magnitude, frequency and duration [9–13]. A novel method involves 'optical tweezers'. Optical tweezers are a single beam optical gradient trap that permits non-invasive and non-destructive manipulation of cells [14–17] and have been used to determine the effect of forces in many biological systems such as the unbinding of myosin molecules [18] and the motility of spermatozoa [19]. In the optical tweezer, a laser beam of a wavelength not absorbed by biological matter produces photons which are refracted by the cell, resulting in a quantifiable mechanical force. In this way we can experimentally apply loads to single bone and cartilage derived cells in culture and monitor intracellular calcium responses.

Here we present data that highlight a variation in intracellular calcium levels to a force applied via optical tweezers on individual connective tissue cells. These results suggest a possible role for optical tweezers as a non-invasive mechanical strain mechanism for single cells in cultures, and demonstrate that our previous studies on rat osteoblasts also extend to human osteoblasts with a role for L-type calcium channels in mechanical load transduction pathways [20].

2. Materials and methods

2.1. Cell culture

Human osteoblast cells were derived from trabecular bone biopsies obtained from human tibial fractures. Patients undergoing surgery gave written informed consent for the study which was approved by the Research Ethics Committee, North Staffordshire Health Authority. Muscle, cartilage and blood were removed by saline washes from biopsies that were subsequently dissected. Bone fragments were placed into culture flasks containing α-MEM, 10% foetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin solution. Proliferating cells from the bone fragments were cultured to confluency in a 5% CO_2 incubator at 37°C (approximately 3 weeks). Rat long bone derived osteoblasts were cultured as described by Pitsillides et al. in a similar method to that of human osteoblast cells [21].

Primary chondrocytes were isolated from human articular cartilage removed during knee operations. Briefly, cartilage samples were cleaned and dissected into small fragments. The cartilage fragments were incubated with 1% pronase for 1 h and then overnight with 100 U/ml collagenase to release chondrocyte cells. Chondrocyte cells were then incubated in DMEM containing 10% FCS, 3% antibiotics and 2% L-glutamine in a 37°C, 5% CO_2 incubator until confluency. The osteocytic cell line MLO Y4 was cultured on collagen coated flasks (0.15 mg/ml diluted in 0.02 N acetic acid) containing α-MEM, 5% bovine calf serum, 5% FCS and 1% antibiotics [22]. All cell types were reseeded onto glass manipulation chamber for experimental use.

2.2. Optical tweezer system

The optical tweezer system described in this paper is the Cell Robotics Workstation (Cell Robotics Inc., Albuquerque, NM, USA). The optical trap is generated from a MOPA diode laser operating at 980 nm wavelength with a Gaussian profile beam. Cells are visualised using a Zeiss Axiovert 135 microscope using a Fluar 40X oil immersion with a numerical aperture of 1.3 objective (Carl Zeiss, Oberkochen, Germany). The beam can be attenuated from 0 to 99% of maximum intensity, varying the force exerted on the specimen, using a computer controlled attenuator and shutter.

2.3. Mechanical manipulation of cells with optical tweezers

All cells were loaded with Fluo-3 by incubation with Fluo-3 AM (10 μM) for 1 h at 37°C in a 5% CO_2 incubator. Solubilisation of the Fluo ester was aided with the addition of the detergent, pleuronic F125. A laser trap was dragged across individual cells exerting a force

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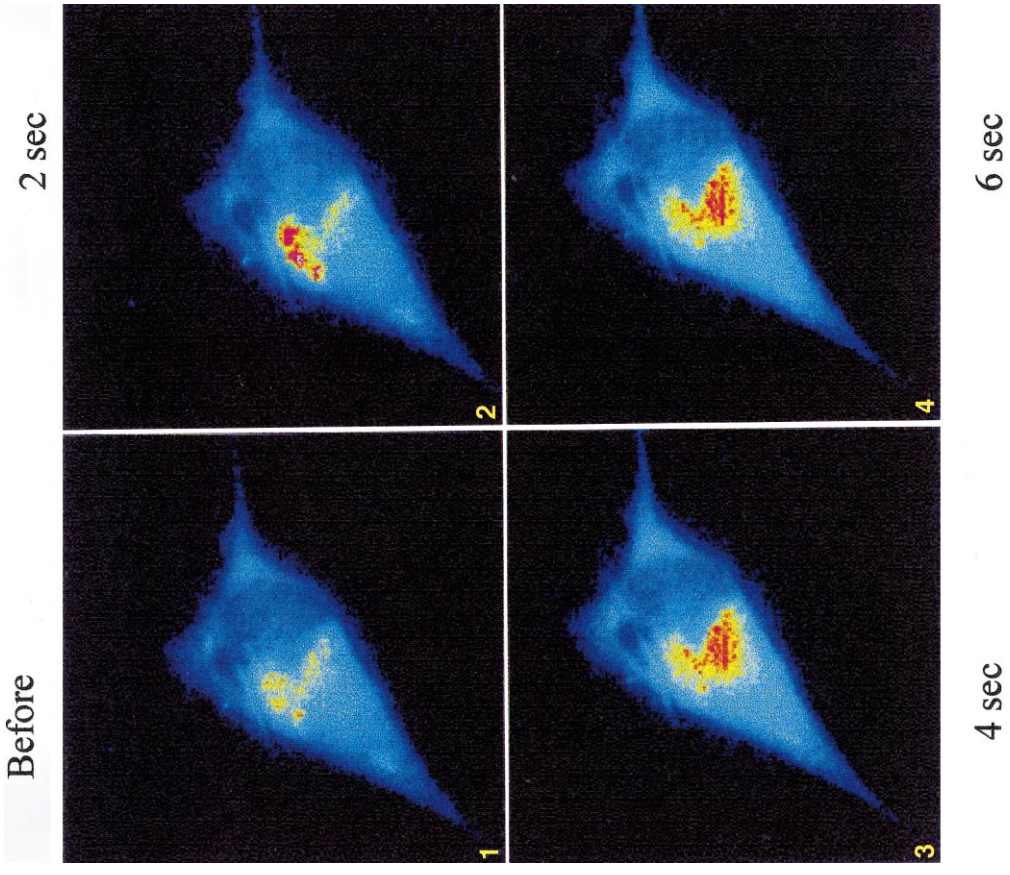

Fig. 1. Fluorescent images of a single human primary osteoblast captured on a confocal microscope before, 2, 4 and 6 s after application of a mechanical strain via optical tweezers. As described in Section 2, cells were loaded with the fluorescent linked calcium indicating dye Fluo-3 AM and a strain of 7 pN was applied to the cell. Colour describes fluorescent intensity, red = highest intensity = highest level of calcium, blue = low intensity = lowest level of calcium.

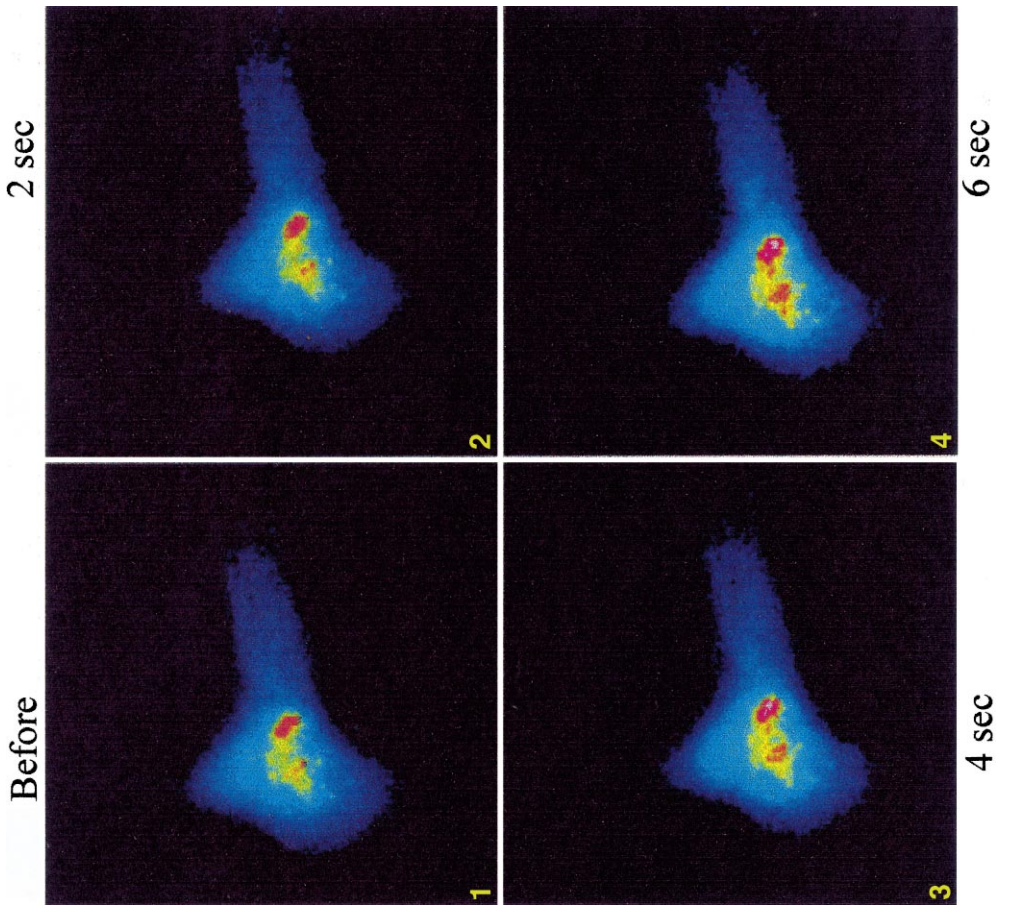

Fig. 3. Fluorescent images of a single human primary osteoblast adjoined to an adjacent osteoblast with a cellular process captured on a confocal microscope before, 2, 4 and 6 s after application of a mechanical strain via optical tweezers. As described in Section 2, cells were loaded with the fluorescent linked calcium indicating dye Fluo-3 AM and a strain of 7 pN was applied to the process adjoining the two cells. Colour describes fluorescent intensity, red = highest intensity = highest level of calcium, blue = low intensity = lowest level of calcium.

of approximately 7 pN (in addition, with human osteoblasts, the laser was also dragged across processes adjoining two adjacent osteoblasts). This force was measured using the calibration procedure of the software. Fluorescent images were observed for the immediate 10 s post load and changes in fluorescent intensity within the cells were recorded and analysed using a CCD camera module, an Argus 20 image processor (Hamamatsu Photonics GmbH, Herrsching, Germany) and the Optimas 6 image analysis system (Optimas Corp., Bothell, WA, USA). With all cell types, control experiments were performed which included passing the laser parallel to the cell (to exclude possible fluctuations triggered by laser light outside the laser trap) and passing the laser across the cell without manipulation to determine any natural intracellular calcium fluctuations in the cell or fluctuations triggered from the excitation light.

2.4. Involvement of L-type calcium channels in load induced calcium changes

Human osteoblasts were incubated with 10 μM nifedipine (dissolved in ethanol) prior to application of force via the optical tweezers. Post load images were collected and analysed as described in earlier methods.

3. Results

3.1. Manipulation of human osteoblasts with optical tweezers

Application of a mechanical strain of approximately 7 pN to human osteoblasts resulted in an elevation in fluorescence intensity of 24.4% ± 3.9 ($n = 12$) in the cell within 1 s suggesting an instant rise in intracellular calcium levels (Fig. 1). Further increases in fluorescent intensity were observed over 4 s after the laser crossed the cell (Fig. 2). Thereafter, the fluorescent intensity levels returned towards prestimulation levels following laser stimulation.

The addition of nifedipine, an L-type calcium channel antagonist, prior to application of strain inhibited the load induced intracellular calcium response (Fig. 2). The presence of nifedipine reduced the change in fluorescent intensity 1 s post stimulation to 4% ± 0.3 ($n = 6$).

In all experiments, the laser beam was directed through the body of a single cell. To determine whether the orientation of the laser beam was of importance, we directed the optical laser beam through a cellular process adjoining two adjacent osteoblast cells which resulted in no significant alteration in fluorescent intensity levels in the stimulated cells or surrounding cells (Fig. 3).

3.2. Manipulation of other cells with optical tweezers

All results are summarised in Fig. 4. Comparisons in responsiveness were determined between different connective tissue cell types. Application of load by optical tweezers to rat femur derived osteoblasts resulted in an increase in fluorescent intensity levels of 12% ± 4.3 within 1 s ($n = 12$). A further increase to 19% ± 2.2 was observed 4 s post load (Fig. 4).

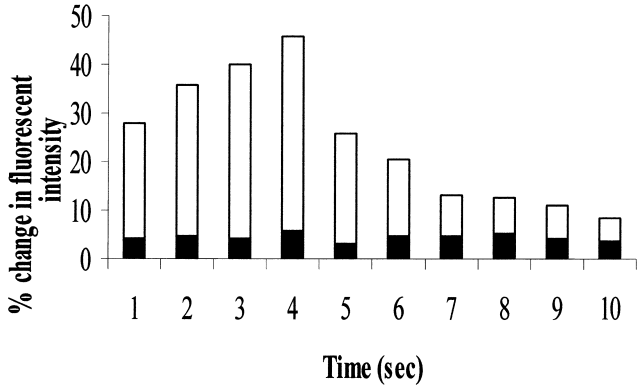

Fig. 2. Time course of load induced increase in $[Ca^{2+}]_i$ dependent fluorescence of a typical osteoblast with and without the presence of the L-type calcium channel blocker nifedipine. Post strain increases of fluorescent intensity are expressed as % increase over resting levels. Load applied at 0 s caused an immediate increase in fluorescent intensity. □, load; ■, load with nifedipine.

Human chondrocytes were subjected to similar loading levels with small fluorescent intensity changes observed post load. A force of approximately 7 pN resulted in an increase of 1.25% ± 1.6 ($n = 12$) after 1 s that had increased to 1.4% ± 0.2 after 4 s (Fig. 4). Similarly, small changes of 4.3% ± 0.3 (1 s post load) and 2.4% ± 1.2 (4 s post load) were observed in MLO Y4 cells ($n = 12$).

4. Discussion

A variety of in vivo and in vitro studies over the past two decades have identified a load induced increase in bone formation, and the pathways involved in converting this mechanical strain into a biochemical signal are currently being investigated [1,2]. In vitro studies have given an insight into the responses of bone to mechanical strains at the cellular level but most loading regimes and apparatus only allow strain to be distributed across a population of cells and not to individual cells [9,13,23,24].

Optical tweezers have been used to determine the effect of forces in many biological systems and we have developed this

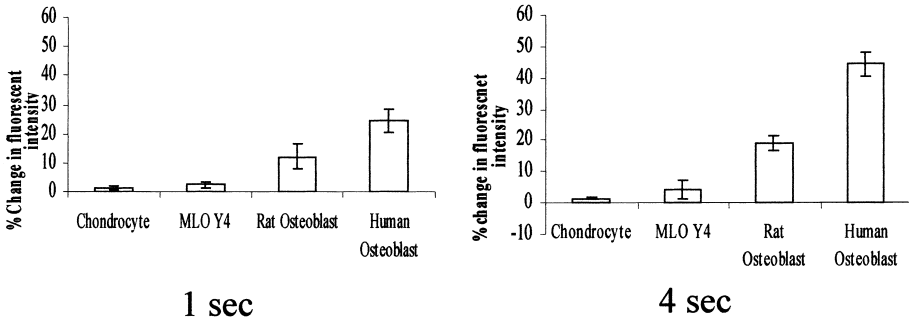

Fig. 4. A summary of the effect of mechanical strain by optical tweezers on bone and cartilage derived cells. Fluorescent intensity levels (% increases in comparison to prestimulation levels) for each cell type, human primary osteoblast, rat primary osteoblast, human primary chondrocyte and osteocyte cell line MLO Y4 are described at 1 and 4 s post stimulation ($n = 12$, mean ± S.E.M.).

system to include forces on bone and cartilage cells. Application of a force of 7 pN to isolated human primary osteoblasts resulted in an increase in fluorescent intensity levels indicating an increase in intracellular calcium. This load induced calcium increase was reduced in the presence of the L-type calcium channel antagonist nifedipine, suggesting a role for L-type calcium channels in the response of bone cells to mechanical strains. Other in vitro studies in bone cells have been described which support this observation [25–28]. Jones and colleagues [25] recorded an increase in $[Ca^{2+}]_i$ in osteoblasts 100 ms after application of a 800 μStr (1 strain is a change in length of 100%). It was suggested that the rise in calcium was preceded by an increase in IP_3 production. Furthermore, Vadiakis and Banes [26] described increases in Ca^{2+} in the rat osteosarcoma 17/2.8 cell line in response to cyclic strain. This load induced increase in calcium was diminished by verapamil, a blocker of voltage operated calcium channels.

It is difficult to compare the level of strain produced by the optical tweezer system with those reported in other studies as most strains are described in the arbitrary units, μStr. However, in our preliminary studies, pN strain levels are applied to cells, which are within recognised physiological strain levels.

Previous mechanotransduction studies have utilised a wide range of loading regimes [13,29,30]. Our study involved a single pulse of strain of duration 3 s (it takes approximately 3 s for optical tweezers to pass over the cell). We have described calcium changes in Section 3 at two time points, 1 s and 4 s, to give both immediate response to tweezers and response observed after the strain is applied.

In all experiments, optical tweezers crossed the body of the cell or parallel to the cell as observed in control experiments. To determine the role of optical tweezer orientation in load induced calcium changes in bone cells, the laser was directed through cellular processes adjoining two adjacent osteoblasts. This resulted in no increase in fluorescent intensity indicating that for the cells to respond to strain at the force level applied, the strain must be directed through the body of the cell. This raises interesting questions regarding the location of strain sensors within the cell.

Application of strains to rat derived primary osteoblasts resulted in an increase in fluorescent intensity levels. These cells were not as load responsive as the human osteoblasts. Both primary chondrocytes and the osteocytic cell line MLO Y4 demonstrated minimal responses to strain with optical tweezers. From these data it cannot be concluded that these cells are not mechanosensitive, but within this straining system, chondrocytes and osteocytes do not produce any load induced intracellular calcium fluxes. Furthermore, to date we have not been able to determine any calcium channel activity in this osteocyte cell line suggesting any load induced changes observed in osteocytes may not involve calcium channels.

In summary, the optical tweezer system allows us to apply mechanical strains, whilst controlling the size and orientation of the strain, to individual connective tissue cells and monitor any load induced intracellular calcium changes. This preliminary study highlights a variation in calcium response to a force applied via optical tweezers on individual connective cells.

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