EFFECT OF CLINOSTAT ROTATION ON DIFFERENTIATION OF EMBRYONIC BONE *IN VITRO*

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Abstract

Using an 8 r.p.m. clinostat as a laboratory analogue of microgravity we have investigated the effect of changes in the gravity vector on osteoblast behaviour. Two sources of osteoblasts were used: secondary cultures of rat bone cells derived from explanted fetal rat calvaria, and the rat osteosarcoma line 17/2.8 (ROS). Cell number was determined by incubation with 3-(4,dimethyl-2yl)-2,3 diphenyl) tetrazolium bromide (MTT), and alkaline phosphatase activity was detected by standard cytochemical methods. ROS and calvarial growth rates during the first 48 hours exposure to clinostat rotation were less than in stationary controls (e.g. OD of ROS cell cultures initiated at 4×10^4 cells ml^{-1} increased from 0.12 to 0.28 in stationary controls but only to 0.18 in clinostat cultures). After 3 days, ROS cell numbers were 35% lower, and calvarial cells 39% lower than their respective controls. Alkaline phosphatase activity in calvarial control cultures was uniformly present in characteristically polygonal cells between which intercommunicating cytoplasmic processes were abundant. In clinostat cultures, the enzyme was present sporadically, and the cells were cuboid and with few processes. We conclude that the clinostat analogue of microgravity decreases cell numbers and alters both cell shape and the distribution of alkaline phosphatase activity in calvarial cell cultures and the ROS cell line. This implies that changes in the gravity vector can affect osteoblasts directly, without interaction with other cell types.

1. Introduction

Bone is a part of the complex mechanical skeletal system, which is particularly responsive to changes in gravity. During long space flight, astronauts lose significant amounts of calcium from bone. Bone is formed through the action of special cells called osteoblasts, and resorbed by a special cells called osteoclasts. Normally, the activity and differentiation are in equilibrium, but under microgravity this balance is disturbed. It is unclear whether this results from increased osteoclastic resorption, decreased bone formation or a combination of the two. We have attempted to concentrate on responses of osteoblast differentiation to microgravity, using the conditions analogous to microgravity which are provided by the clinostat. Osteoblasts are mainly characterized by two phenotypes. First, they are responsible of bone matrix mineralization, secondly they have abundant alkaline phosphatase enzyme activity, this being a classical marker of osteoblasts.

Our aims have been to use a clinostat to investigate, with minimum alteration to the usual cell culture techniques, the effect(s) of changes in the gravity vector on differentiation, alkaline phosphatase activity and cell numbers in two types of osteoblast: (a) embryonic rat calvaria cultures, and (b) rat osteosarcoma line 17/2.8 (ROS).

The clinostat was designed for use with plant and fungal tissues (constructional details in Hatton & Moore, 1992). It was modified for cultures of mammalian cells as follows : (a) the plate carrier was modified to support up to four multiwell tissue culture plates during rotation (Fig. 1); (b) as aqueous medium was used for bone tissue cultures, the plates

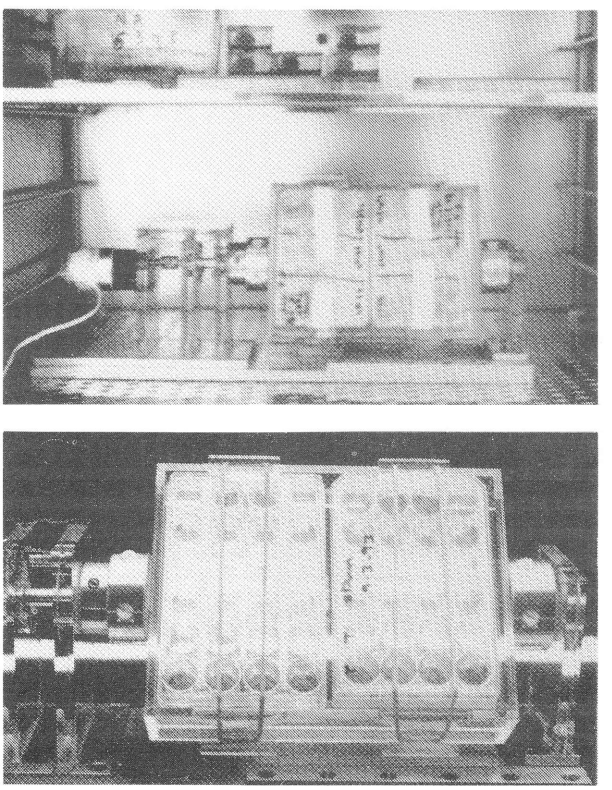

Fig. 1. The clinostat rig fitted with a 24-well multiwell tissue culture plate. The top panel shows the rig *in situ* in the incubator and the bottom panel shows the modified specimen carrier with its multiwell tissue culture plates.

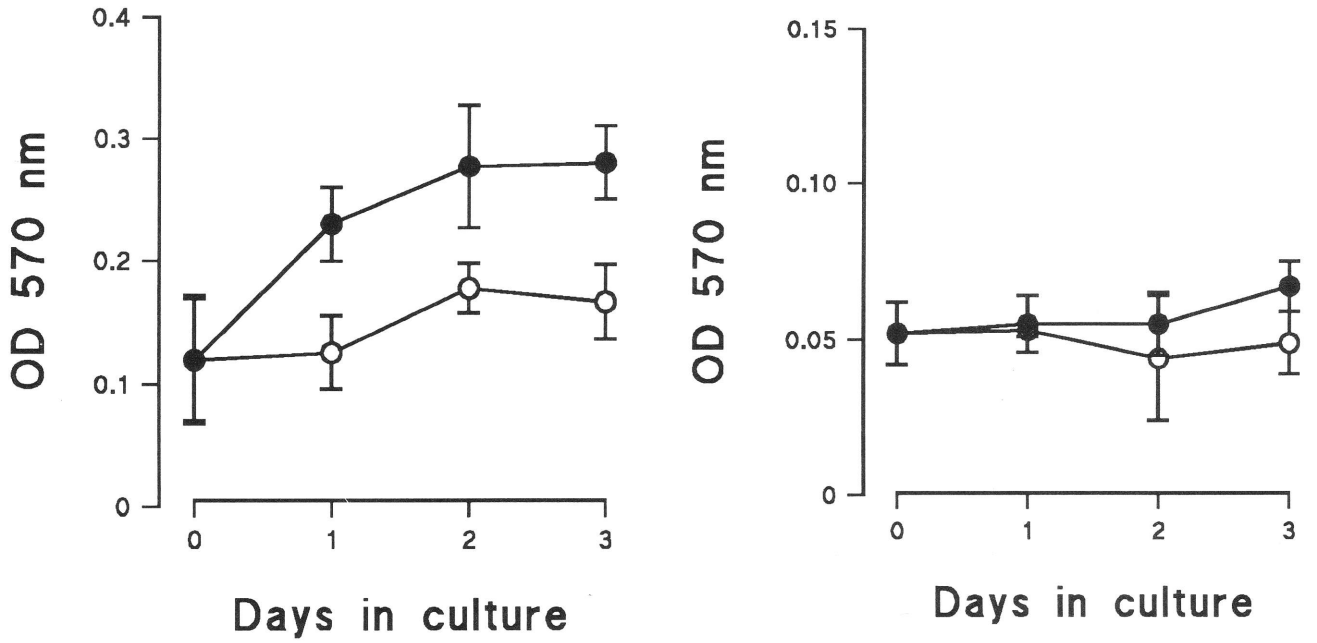

Fig. 2. Population changes (in terms of MTT staining) over a three day period in a ROS cell line (left hand panel) and fetal rat calvarial cell cultures (right hand panel) with an initial seeding density of $4x10^4$ cells ml^{-1}. For both cell types, a decrease in growth rate seems to be evident in clinostat cultures compared with the control cultures. Closed circles are stationary controls, open circles are clinostat cultures. Error bars indicate standard deviation.

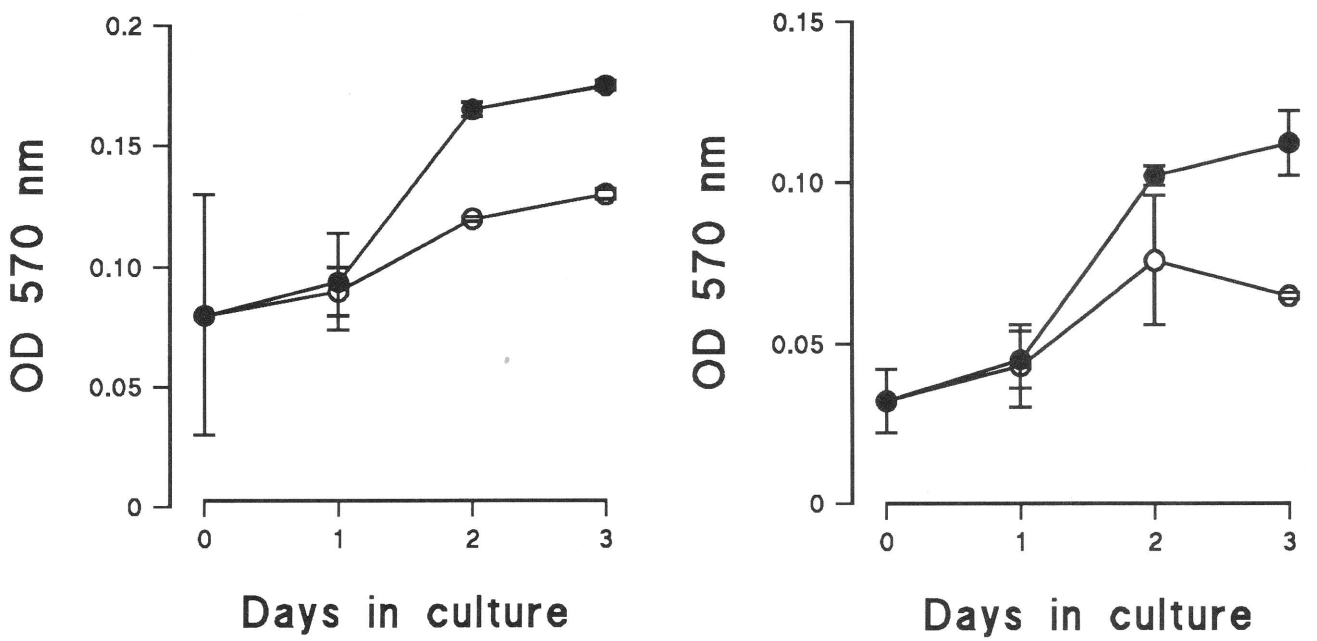

Fig. 3. Population changes (in terms of MTT staining) over a three day period in a ROS cell line (left hand panel) and fetal rat calvarial cell cultures (right hand panel) with an initial seeding density of $2x10^4$ cells ml^{-1}. For both cell types, a decrease in growth rate seems to be evident in clinostat cultures compared with the control cultures. Closed circles are stationary controls, open circles are clinostat cultures. Error bars indicate standard deviation.

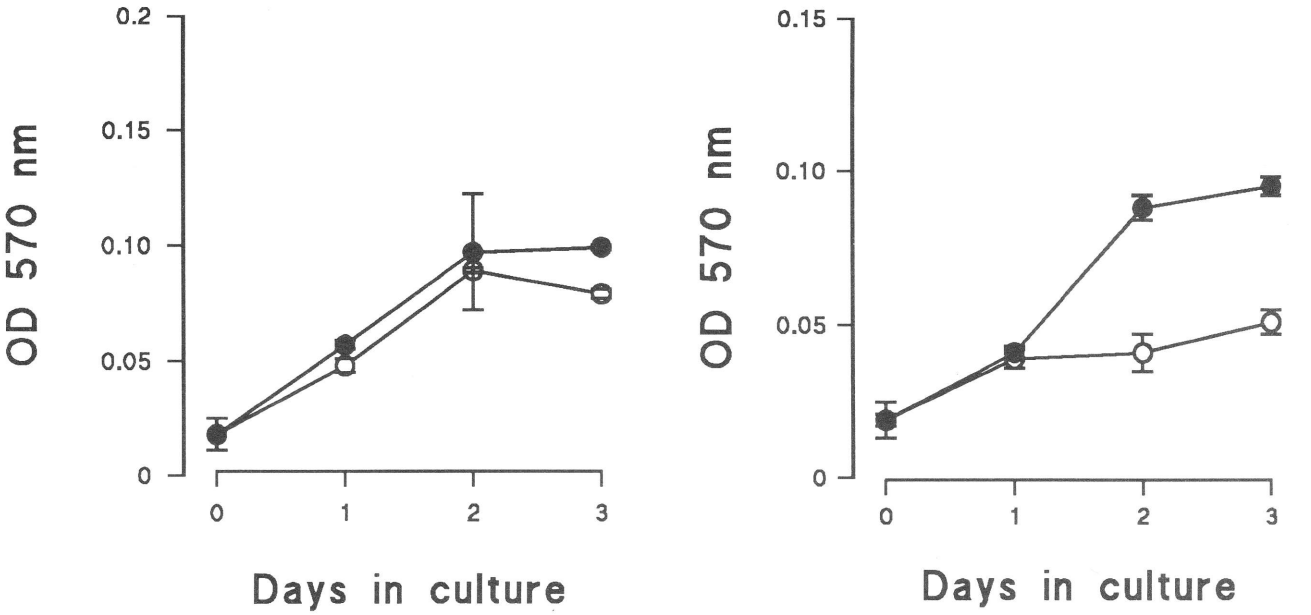

Fig. 4. Population changes (in terms of MTT staining) over a three day period in a ROS cell line (left hand panel) and fetal rat calvarial cell cultures (right hand panel) with an initial seeding density of $1x10^4$ cells ml^{-1}. For both cell types, a decrease in growth rate seems to be evident in clinostat cultures compared with the control cultures. Closed circles are stationary controls, open circles are clinostat cultures. Error bars indicate standard deviation.

(experimental and stationary controls) were sealed with sterilised self-adhesive gas permeable mylar tape ['Plate Sealers', Dynatech Laboratories, Inc., Chantilly, Virginia, U.S.A.].

Because of the size and design of the tissue culture plates, most of the wells were parallel to, rather than along, the rotational axis of the clinostat. As experimental subjects are moved away from the centre of rotation, the circular displacement of any sedimenting (or floating) objects within them increases, and this may influence the response made by the subject. In the experiments reported here, any such effect will be reflected in the degree of dispersion of data obtained from replicate wells. Experiments are planned for the future to determine the exact relationship between position relative to the axis and degree of response since this may be used in an analytical sense to establish physical characteristics of the intracellular objects involved in gravity sensing.

2. Materials and Methods

2.1. Osteoblast differentiation using MTT

ROS cell line 17/2.8 and secondary cultures of rat bone cells, derived from explanted fetal rat calvariae, were plated in 96 well plates at up to 250 µl per well (the wells were not filled, but when the mylar sealing tape was pressed onto the well to seal the edges the film contacted the fluid surface and stabilised it within the well). Cultures were initiated at seeding densities between $1\text{-}4 \times 10^4$ cells ml^{-1}. After sealing, they were exposed to clinostat rotation at 8 rpm in a 37°C, 5% CO_2 incubator. Cell number was determined by incubation with 3-(4,dimethyl-2yl)-2,5 diphenyl) tetrazolium bromide (MTT) for 6 hours. This reagent stains the mitochondria. Excess MTT solution was removed, then 200 µl dimethyl sulphoxide (DMSO) was added to each well and optical density (OD) measured at 570 nm using an ELISA plate reader.

2.2. Cytochemical detection of alkaline phosphatase activity

Fetal calvarial bone secondary cultures were plated in 24 multiwell plates embedded in collagen gel upon thermonox disks. To prepare the cells, they were allowed to attach to the disks during an overnight incubation in minimum essential medium (MEM) supplemented with 10% fetal calf serum, then excess medium was drained off and replaced with 500 µl of a solution of collagen [0.9 ml 7.5% sodium bicarbonate (Flow Laboratories, Irvine, Scotland) + 1 ml 10x MEM + 8.1 ml collagen solution ('Vitrogen 100', Celtrix Laboratories, Palo Alto, California)]. The gel was solidified by incubation at 37° for 20-30 minutes, then the wells were completely filled with 2.5 ml of MEM supplemented with 10% fetal calf serum, and covered with mylar sealing tape. Plates were then rotated on the clinostat

at 8 r.p.m. and incubated in a 37°, 5% CO_2 incubator. Control experiments were set up in the same conditions, but without rotation. After 48 hours, samples from stationary controls and clinostat-treated experimental samples were tested for alkaline phosphatase activity using standard cytochemical methods.

3. Results

3.1. MTT and cell number

Daily sampling of cells over 3 days, showed that control ROS and calvarial cells at least doubled in number after 48 hours, reaching a plateau, at all initial seeding densities tested, after 3 days, with the rate of increase being inversely proportional to initial seeding density (Figs 2-4). In both ROS and calvarial cell cultures the growth rate during the first 48 hours exposure to clinostat rotation was less than that of the corresponding controls. For example, with initial seeding density of $4x10^4$ cells ml^{-1}, ROS cells increased from 0.12 to 0.28 OD in controls, but to only 0.18 OD in clinostat cultures (Fig. 2). After three days, ROS cell number was 35%, and calvarial cell number 39% lower than their respective controls.

3.2. Alkaline phosphatase activity

Alkaline phosphatase activity in calvarial cultures was uniformly present in characteristic polygonally shaped cells in control cultures, in which intercommunicating cytoplasmic processes were abundant (Fig. 5, top). In contrast, in clinostat cultures the enzyme staining was variable, but generally lighter than in controls, but whatever their staining intensity, the cells were cuboid in shape with few processes (Fig. 5 middle and bottom).

4. Conclusions

Here we present preliminary experiments designed to establish a simple methodology applicable to a range of cultured mammalian cells with minimum change in established technique. Although we intend to adapt the clinostat rig so that it rocks the cultures without rotating them, to examine the possibility that agitation of the culture, rather than rotation, is influencing cell growth, we believe this to be an unlikely explanation. In all experiments the liquid medium was stabilised with a covering of mylar film, thus excluding the possibility of a moving air-liquid interface, and in one series of experiments the cells were embedded within a collagen gel.

Thus we conclude that it is highly probable that clinostat treatment causes a decrease in cell numbers and altered cell differentiation in both ROS and calvarial cultures. This implies that change in the gravity vector can affect osteoblasts directly, without interaction with other cell types in skeletal tissues.

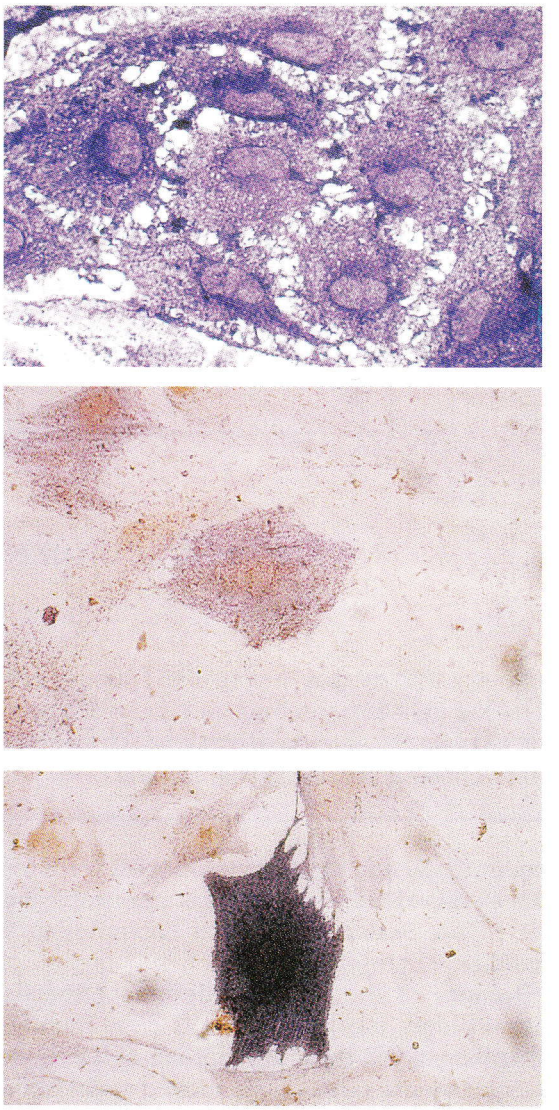

Fig. 5. Cells from fetal rat calvarial cultures stained for alkaline phosphatase activity. Top panel: a control culture grown under normal conditions showing heavy, uniform staining of cells which have long processes; middle panel: four cells from a culture (initial seeding density $5x10^4$ cells m^{-1}) grown for 48 hours on the clinostat. Note lighter staining than the controls and a more angular ('cuboid') cell shape with few processes; bottom panel: although some cells in clinostat treated cultures stain heavily, they are still cuboidal with few processes.

If change in the gravity vector alters cell shape and decreases the alkaline phosphatase activity of calvarial cultures it may also affect other cytochemical functions. In particular, the loss of intercommunicating cytoplasmic processes may cause loss in communication between cells.

5. Reference

Hatton, J. P. & Moore, D. (1992). Kinetics of stem gravitropism in *Coprinus cinereus*: determination of presentation time and 'dosage-response' relationships using clinostats. *FEMS Microbiology Letters* **100**, 81-86.