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

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ABSTRACT

We have investigated the effect of changes in the gravity vector on osteoblast behaviour, using the clinostat set at 8 rpm. Two sources of osteoblasts were used: secondary cultures of fetal rat bone cells, 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 measurement of optical density at 570 nm (OD). Alkaline phosphatase activity was detected by standard cytochemical methods. Dividing cells were localised by labelling dividing nuclei with Bromodeoxyuridine (BrdU), detected by immunofluorescence. Cell culture was initiated at densities between 1-4x10^4 cells ml^{-1}. Growth rates in all cultures during the first 48 hours exposure to clinostat rotation were less than in stationary controls. 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, but after culture in the clinostat the enzyme was present sporadically, and the cells were cuboid. There was also no BrdU uptake in nuclei, but it was present in cell cytoplasms. We conclude that the clinostat decreases cell numbers and cell division. Both cell shape and the distribution of alkaline phosphatase activity in calvarial cell cultures were also affected. This implies that changes in the gravity vector can affect osteoblasts directly, without interaction with other cell types.

INTRODUCTION

The skeleton is a complex system, which is particularly responsive to changes in gravity. During long space flight, astronauts lose significant amounts of calcium from bone /1/. Bone formation and resorption are each brought about by specialised cells; osteoblasts form bone and it is resorbed by osteoclasts. Normally, their function is in equilibrium, but under microgravity we believe that this may be disturbed. It is unclear if this is from increased osteoclastic resorption, decreased bone formation or a combination of the two. We have concentrated on responses of osteoblasts to microgravity, using the analogous conditions provided by the clinostat, as previously demonstrated /2/. Osteoblasts may be characterised by several phenotypes related to bone formation, for example alkaline phosphatase activity, which is used as a classical marker for osteoblasts /3/. Here we aimed to use a clinostat, with minimum alteration to usual cell culture techniques to investigate the effects of changes in the gravity vector on differentiation, alkaline phosphatase activity, cell numbers and cell division in two types of osteoblast: (a) embryonic rat calvaria cells (a heterogeneous culture), and (b) rat osteosarcoma line (ROS) 17/2.8 (homogeneous line of partially differentiated osteoblast like cells /4/).

MATERIALS AND METHODS

Cell Culture

The clinostat was designed for use with plant and fungal tissues /2/, but was modified for mammalian cell culture as follows: (a) the plate carrier now supports up to four multiwell tissue culture plates during rotation (Fig. 1); (b) as aqueous medium was used for bone cell cultures, the plates (experimental and stationary controls) were sealed with sterilised self-adhesive gas permeable mylar tape [Dynatech Laboratories, Inc., Virginia, U.S.A.]. As shown in fig.1 the plastic carrier was designed for two 12 place multiwell plates, fitted at 90° to the rotational axis of the clinostat. Cells were always inoculated into wells on either side of this axis.

Osteoblast Proliferation

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 each well to seal the edges the film was in contact with the fluid surface). In order to ensure cultures were always in the log phase, they were initiated at seeding densities between 1-4x10^4 cells ml^{-1}. Preliminary experiments showed that high clinostat rotation speed of 25 rpm produced inconsistent changes in cell growth, but lower speeds had highly reproducible effects (data not shown). Here, we exposed cells to clinostat rotation of 8 rpm at 37°C, 5% CO_2. Controls were set up in the same conditions, but without rotation. Cell number was determined by incubation with 3-(4,dimethyl-2yl)- 2,5 diphenyl) tetrazolium

bromide (MTT) for 6 hours. After solubilisation with 200 µl dimethyl sulphoxide (DMSO), optical density (OD) was measured at 570 nm with an ELISA plate reader.

Cytochemical Reaction of Alkaline Phosphatase Activity

Bone cells derived from primary cultures of fetal rat calvariae were grown on Thermanox discs, placed in wells of 24 place multiwells, (initial seeding density, $4x10^4$ cells ml^{-1} per well). After the cells had adhered to the discs they were covered with a thin layer of collagen gel (Vitrogen 100, Celtrix Laboratories, Palo Alto, California), final concentration of 3 mg per ml made up according to manufacturers instructions, to ensure that the discs were always affixed to the bottom of the wells during rotation. After the collagen had solidified, the wells were completely filled with 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 37°C in a 5% CO_2 incubator. Controls were set up as described above. After 48 hours, samples from stationary controls and clinostat-treated experimental samples were tested for alkaline phosphatase activity by standard cytochemistry /4/.

Detection of Cell Division Using Labelled BrdU

Fetal rat calvarial secondary cultures were seeded onto glass coverslips, to prevent high background fluorescence, in 24 place multiwell plates. They were covered with collagen (see above), and were cultured under clinostat conditions for 4 days and then removed and cultured under control conditions for a further 4 days. The calvarial and ROS clinostat cultures were incubated with 1/500 dilution of Bromodeoxyuridine (BrdU, DAKO) which labelled dividing nuclei. They were then incubated with antibodies to BrdU raised in mouse (diluted 1/20) for 16 hours at 4°C, followed by incubation with antibodies to mouse IgG, raised in sheep, conjugated to streptavidin-Texas Red (diluted 1/200), for 1 hour at room temperature. Controls were incubated with medium instead of BrdU, but with the secondary and tertiary antibodies.

RESULTS

MTT and Cell Number

At all initial seeding densities, control ROS and calvarial cells at least doubled in number after 48 hours, reaching a plateau, after 3 days, but for cells cultured in the clinostat, the growth rate during the first 48 hours was lower, but approached that of controls when they were removed from the clinostat (Fig 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. 3, left). 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. 3, right).

Cell Division

BrdU could be detected in dividing nuclei of calvarial control cultures by immunofluorescence (Fig. 4, a). In contrast, after 4 days of clinostat culture the nuclei were unstained, but there was immunofluorescence in cytoplasms (Fig. 4, b). After removal from the clinostat and a further 4 days in normal conditions, BrdU could be detected in some nuclei, which were only partially stained (Fig. 4, c). In immune controls, i.e. cells not incubated with BrdU, nuclei were negative, there was faint immunofluorescence throughout the cytoplasm, which was less than in the cells exposed to the clinostat and in contrast, was poorly localised. (Fig. 4, d).

CONCLUSIONS

The work presented here represents a series of preliminary experiments designed to establish a simple methodology which can be applied effectively to a range of cultured mammalian cells with minimum change in established tissue culture technique. Although further controls need to be incorporated into these experimental series these results are extremely encouraging. It is possible that our observations were due to a rocking rather than a rotational movement of the clinostat, and this is the subject for further experiments. 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 some experiments the cells were covered with a collagen gel. Thus we conclude the clinostat conditions used in this study causes decreased cell number 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 the skeleton.

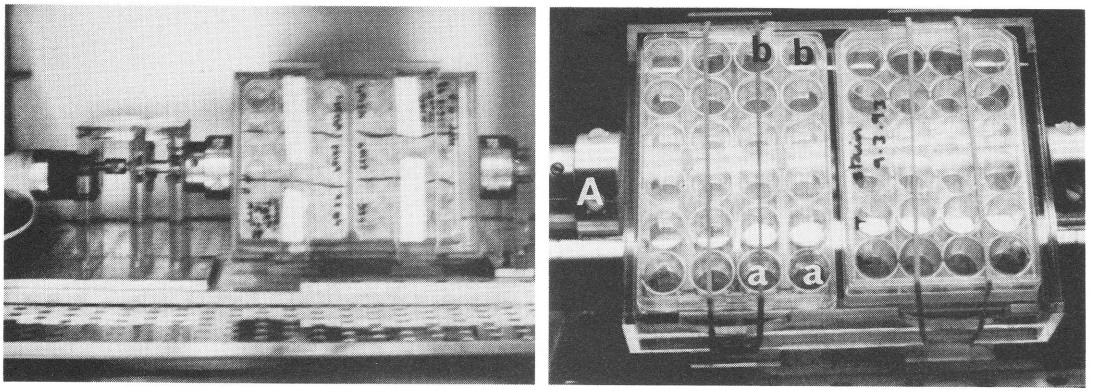

Fig. 1. The clinostat rig fitted with 24-well multiwell tissue culture plates. The left panel shows *in situ* in the incubator and the right panel shows the modified specimen carrier with its multiwell tissue culture plates. [A] indicates the axis of rotation and (a) and (b) are the rows into which cells were inoculated.

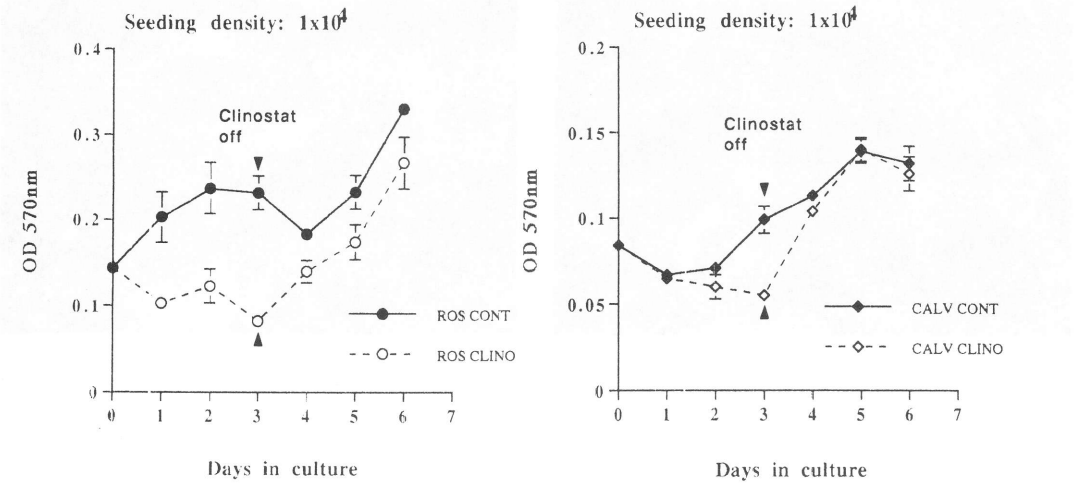

Fig. 2. ROS cell line (left hand panel) and fetal rat calvarial cell cultures (right hand panel) with an initial seeding density of $1x10^4$ cells ml^{-1}. For both cultures, growth rate in the clinostat was less than controls. After a further 3 days in normal conditions, cell numbers in these cultures increased to controls level. Each point is the mean of triplicate wells, vertical bars are SDs.

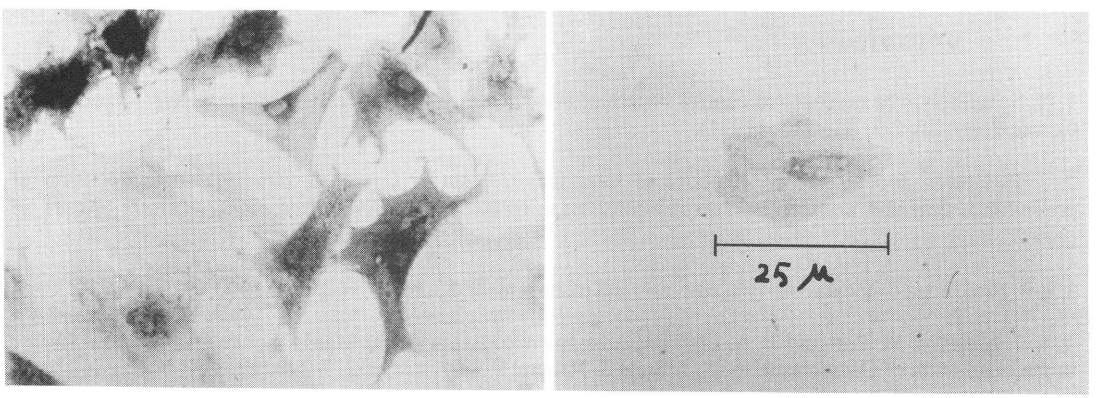

Fig. 3. Cells from fetal rat calvarial cultures stained for alkaline phosphatase activity. Left hand panel: control cultures showing uniform staining of cells which long processes. Right hand panel: a clinostat culture grown for 48 hours in the clinostat. Note the lighter staining and cuboidal cell shapes.

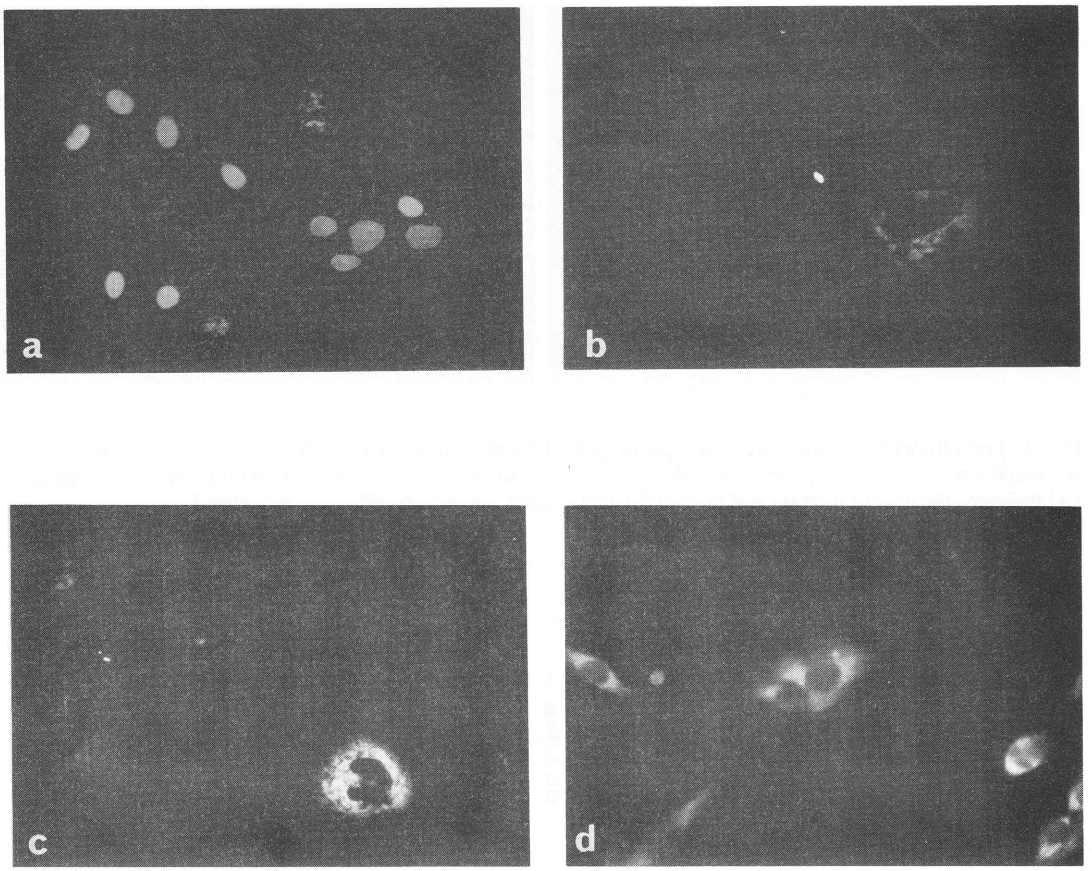

Fig. 4. The dividing nuclei of calvarial control cultures could be detected by BrdU (a). After 4 days in the clinostat, nuclei were unstained, but there was cytoplasmic immunofluorescence (b). After removal from the clinostat a further 4 days, BrdU could be detected in some nuclei (c). Immune controls are shown in (d).

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