CELL STRUCTURE AND FUNCTION 20: 369–375 (1995)

Shape Changes of Osteoblastic Cells Under Gravitational Variations during Parabolic Flight —Relationship with PGE_2 Synthesis—

Alain Guignandon*, Laurence Vico, Christian Alexandre, and Marie-Hélène Lafage-Proust

Laboratoire de Biologie du Tissue Osseux —GIP "EXERCICE"— 15 rue Ambroise PARE, Faculté de Médecine, 42023 Saint-Étienne Cedex 2, France

Key words: osteoblasts/parabolic flight/PGE_2/microgravity/hypergravity

ABSTRACT. **The relationship existing between cell morphology and cell metabolism, and the role of mechanical load in bone remodelling are well-known. In osteoblasts, PGE_2 mediates part of the response to mechanical stress and induce cell shape changes. We studied the influence of gravity variations on osteoblast morphology and its relationship with PGE_2 synthesis during a parabolic flight. ROS 17/2.8 osteosarcoma cells flew 15 or 30 parabolae. We measured cell area and shape factor after fluorescein staining with a semi-automatic image analyser and PGE_2 levels by RIA. Significant flight-induced shape changes consisted in a decrease in cell area and an increase in shape factor (cell irregularity), in some cells, as compared to ground controls. This heterogeneity in cell response might be explained by a cell-cycle sensitivity to mechanical stress. A 45 min pretreatment with indomethacin inhibited the flight-induced increase in cell irregularity whereas cell area remained decreased. PGE_2 levels were higher in flight than in ground controls. Linear regression analysis showed a significant negative relationship between cell area and PGE_2 synthesis. We concluded that ROS 17/2.8 are highly sensitive to gravitational variations and that PGE_2 is partly implicated in cell shape changes observed during parabolic flight. However, other mechanisms than PGE_2 synthesis condition ROS 17/2.8 morphology in response to mechanical changes.**

There is now a large body of evidence that mechanical loading plays a critical role in bone cell function and differentiation (1). However, the precise mechanisms that regulate the transduction of a mechanical signal into a biochemical signal remain unknown. Parabolic flight and space flight are some of the means we can use in order to analyze the impact of gravity on bone cell functions. During the Biocosmos X mission we had the opportunity to culture rat osteosarcoma cells ROS 17/2.8 for 6 days (2). These cells exhibited dramatic morphological changes observed with scanning electron microscopy. In the same culture chamber some cells became rounder and piled up, others retracted and presented long cytoplasmic extensions, and a third population remained spread out with similar shape to ground controls and in-flight centrifuge controls. These data suggested that even clonal cells might respond differently to microgravity. Moreover, this study showed that cell shape changes can be the expression of a response to gravitational changes. Interestingly, previous studies have established that cell shape can be responsible for variations in cell proliferation and gene expression (3). Prostaglandins are known to induce osteoblastic shape changes (4) and to mediate part of the response to mechanical load (5). The effect of mechanical load on PGE_2 synthesis have already been documented (6, 7). During a parabolic flight the cell mechanical environment switches from hypergravity (2g) to microgravity and constitutes a sequential mechanical stress. In this work, we analysed a structural parameter and a particular biochemical function, respectively by quantifying cell shape changes and by measuring PGE_2 synthesis and release. The aim of this work was first to determine if ROS 17/2.8 are sensitive to the parabolic flight conditions and, second, to try to establish the relationship between morphological changes and PGE_2 synthesis.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

The Parabolic Flight. The flight was performed during the 18th ESA campaign. The 2 h 30 minute-flight consisted in 2 groups of 15 parabolae. Each parabola lasted 3 minutes and included 30 sec at 2 g, 25 sec at 0 g and 30 sec at 2 g. Five minutes of 1 g flight separated each group of 3 parabolae.

The "Plunger-box". The hardware used for the flight consisted in eight manual "plunger-boxes" (C.C.M.® Netherlands), this cell culture compartment has been developed for space flight mission. It is composed of one or two culture chambers and contains different compartments, filled by medium, buffer and fixative (cf. Figure 1). The medium is forced

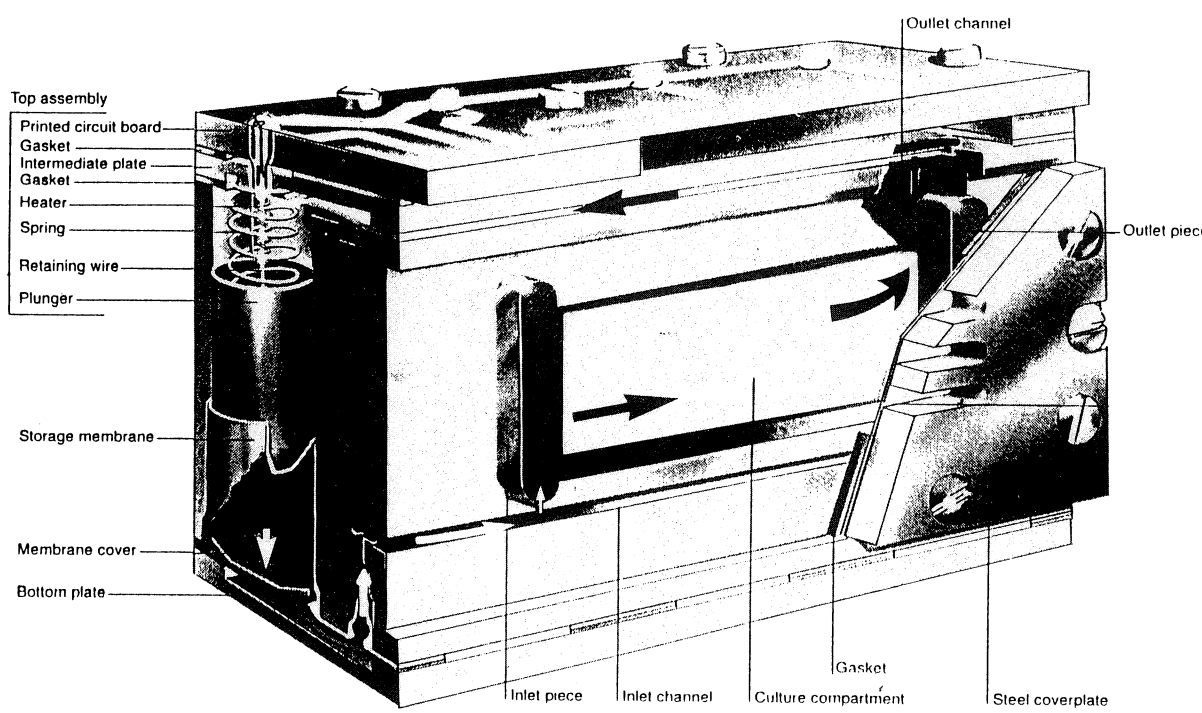
into the culture chamber by releasing a spring load plunger by sequential manual activations that allows medium, fixative and lysis buffer changes.

Cell culture. Cells used in this work were rat osteosarcoma cells (ROS 17/2.8), a well defined osteoblastic cell line. Culture medium was DMEM supplemented with 10% foetal calf serum, 2 mM L-glutamine and 1% of a 10000IU penicillin-10 mg/ml streptomycin solution. Since no gas exchange occurs in these closed culture chambers, the medium was buffered with $NaHCO_3$ 3.7 mg/L and 20 mM HEPES. In these conditions cell growth and alkaline phosphatase activity are close to those in normal O_2/CO_2 culture conditions. Cells were plated on glasscoverslips at a 5×10^4 cells/cm^2 density and cultured at 37°C for 36 hours. During transportation cells were cultured at 24°C in a portable incubator. Culture temperature was switched to 37°C 18 hours before the flight. Forty-five minutes before the first parabola cells were switched to serum free medium and, where mentioned, some cells were treated either with 1 mM PGE_2 or 2 mM indomethacin (Sigma, France). After the end of the 15th and the 30th parabolae, cells were washed with PBS and fixed with 2.5% glutaraldehyde or lyzed with 0.1% Triton-X100 buffer. After landing supernatants and lyzed cells were frozen. Some coverslips were then stained with 1% carboxyfluorescein (Molecular Probes, OR-USA) in 1% methanol and observed with a FITC-filter on an epifluorescence microscope. This fixation and staining procedures allowed us to visualise precisely even tiny morphological changes.

Morphological analysis. Cell shape was analyzed with a semi-automatic image analyzer (SAMBA-Alcatel®) on 150 cells per condition. The best parameters taking into account cell shape changes were area and shape factor (Perimeter $\times$ $100/4 \times \pi \times$ Area). Cell shape factor represents the degree of "stellar deformation" of the cell. In other words, the higher the shape factor, the less round the cell.

PGE_2 measurement. PGE_2 levels were measured in medium (PGE_2 release) and in lyzed cells (PGE_2 synthesis) with a competitive R.I.A. (RIA Pasteur, France). Protein content was measured using Coomassie blue method (8). Medium and cellular results are given in pg/mg protein. Each result corresponds to the mean of two independent culture chambers and each condition was measured in triplicate. A ground control experiment was performed mimicking similar culture conditions (Plunger-boxes integration, transport temperature and activation times) except gravitational variations.

Statistic. Means and s.d. were calculated for all the parameters. Groups differences were assesed by unpaired t-test, the significant levels was $P < 0.05$. An adhoc recognition of gaussian populations allowed us to describe distributions. Linear regression analysis estimated the relationship between mor-



Plungerbox-unit, partially in cross section.

Fig. 1. A schematic representation of a cell culture device (Plunger-box) made out of a single block poly-ether-sulfone. Arrows indicates the flow direction of medium, buffer or fixative stored in fluid reservoirs.

phology and PGE_2 levels (Stat 2005 software- Alcatel® TITN Answare- version 3.0).

RESULTS

After 15 as well as 30 parabolae cells appeared more irregular with cytoplasmic retractions and cell membrane ruffling associated with a decrease in cell-cell contacts as compared to ground controls which were flat and well spread with a smooth peripheral membrane (Figure 2). This was confirmed by image analysis. Cell area was significantly smaller and cell shape factor significantly higher in flight as compared to ground controls (186 ± 22 and 146 ± 16, $p < 0.001$) (Figure 3). The maximum reduction of cell area was already achieved by the end of the 15th parabola. We noticed that, during the flight, a part of the cells remained flat and well spread on glass suggesting that cellular response to gravitational stress was heterogeneous. To further characterise this heterogeneity we plotted the cell area distribution in ground control and in flight conditions after 30 parabolae (Figure 4). In ground controls we observed that cells were distributed in two populations: a "small cell" and a "big cell" population (1 : 68% and 2 : 32%, respectively, in Fig. 4A). In flight, the "small cell" population increased whereas a major decrease occurred in "the big cell" population (1 : 84.5% and 2 : 15.5%, respectively, in Fig. 4B) suggesting that cells with a larger area could be more sensitive to gravitational variations, or that area decrease was more visible on "big cells".

In order to better understand the effect of PGE_2 in the flight-induced cell responses, some cells were treated 45 min before the first parabola with either 2 mM indomethacin, which is a prostaglandin synthesis inhibitor, or PGE_2 1 mM. In flight conditions, after 30 parabolae, cells exhibited mixed types of morphologies including normal flat cells and retracted cells with decreased area as seen in Figure 5A and B. Quantitative analysis showed that, on ground, indomethacin induced a slight increase in cell area as compared to untreated controls (213 ± 23 vs 186 ± 22, $P < 0.05$). In flight conditions, indomethacin was able to inhibit the flight-induced increase in shape factor whereas the cell area remained unchanged as compared to untreated ground controls (Figure 5C). As seen in Figure 6, PGE_2 on ground (10^{-4} M to 10^{-8} M, for 180 minutes) was not able to completely mimic flight-induced cell shape changes. PGE_2 treatment induced cell membrane ruffling and a slight nonsignificant decrease in cell area as compared to untreated cells. In flight PGE_2 treatment induced cell lifting in such a way that only a few cells remained on the cover-

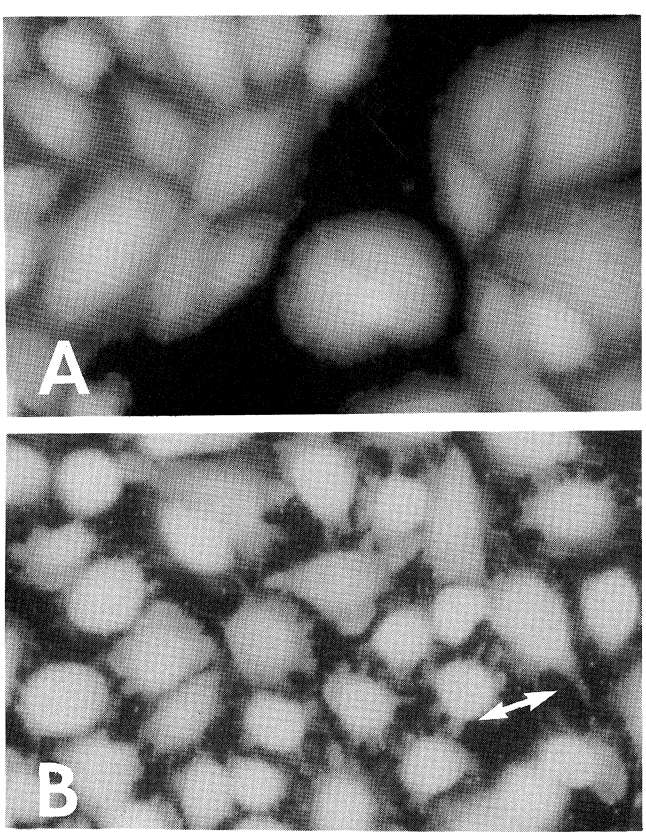


Fig. 2. Representative examples of ROS 17/1.8 deformation induced by 15 parabolae. Carboxyfluorescein staining observed in epifluorescence, (Mag: 40X). A: Ground control cells were cohesive and normally spread out. B: Most of the flight cells exhibited cytoplasm retraction and membrane ruffling (Arrow).

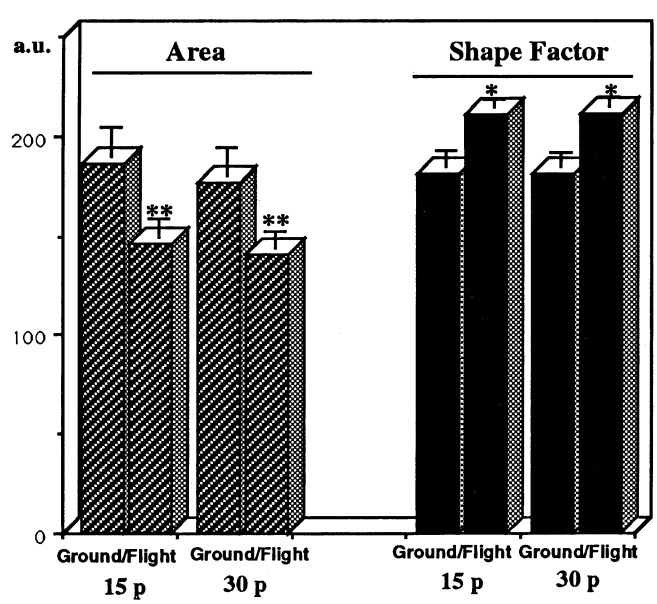
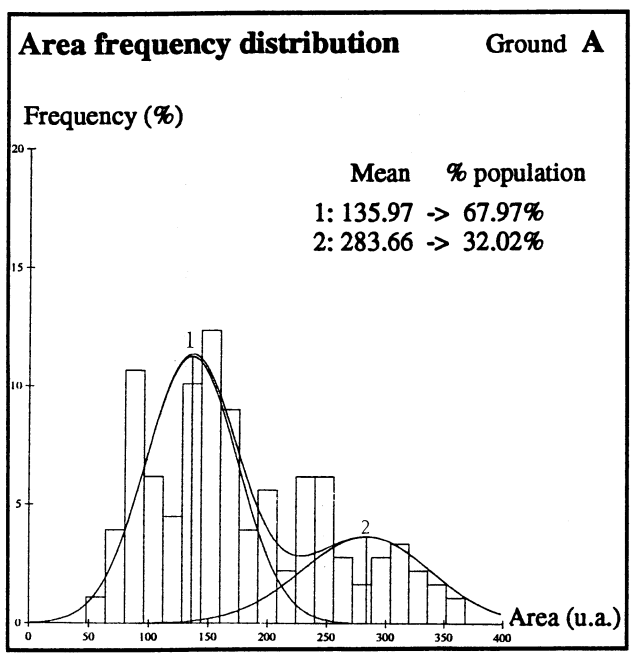


Fig. 3. Quantitative analysis of cell shape changes during parabolic flight after 15 (15p) or 30 (30p) parabolae as compared to ground controls. (*: $p < 0.05$ vs Ground controls; **: $p < 0.01$ vs Ground controls)

slip, suggesting that combined flight and PGE_2 effects were responsible for a major cell retraction leading to a loss of cell adhesion.

The cellular PGE_2 levels were 20-fold higher in the cells than in the medium (Figure 7). In flight PGE_2 levels in the supernatants were not significantly different from ground controls whereas intracellular levels in-



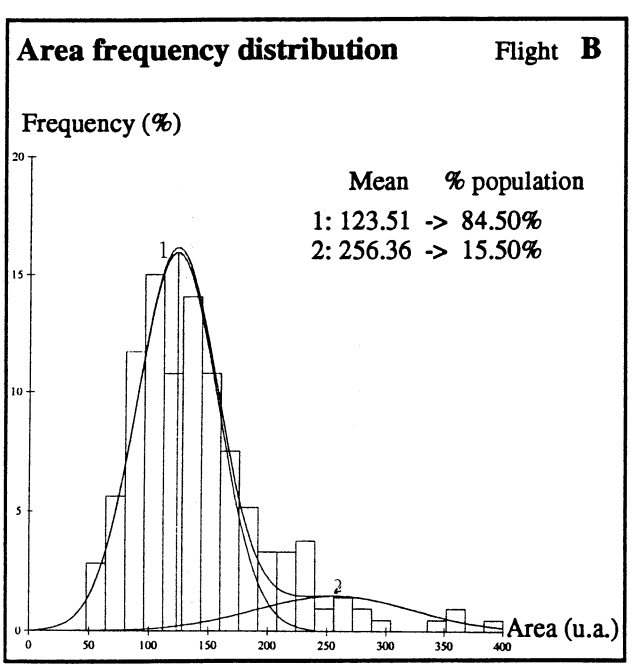


Fig. 4. Cell area distribution. A: Ground controls. B: Flight cells. Note the reduction in the size of the "big cell" population (2) during the flight.

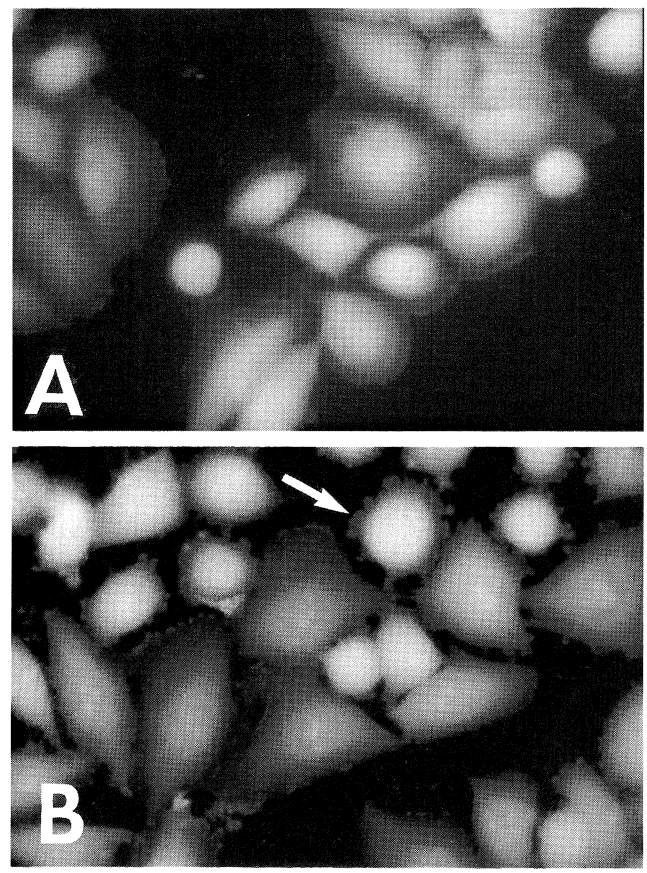


Fig. 5. Morphological aspects of indomethacin effects on ROS 17/2.8 cell shape. A: Ground controls. B: In flight after 30 parabolae. (Mag: 40X). Note that some cells exhibited flight-induced morphological changes (Arrow). C: Quantitative analysis of cell morphology. *: $p<0.01$ vs ground indomethacin treatment.

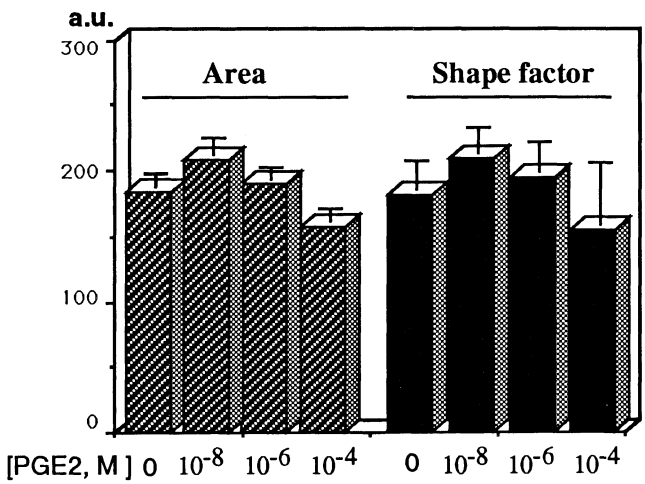


Fig. 6. Concentation dependant PGE_2 effects on cell shape on ground. PGE_2 reduces cell area for very high concentrations 10^{-4} M.

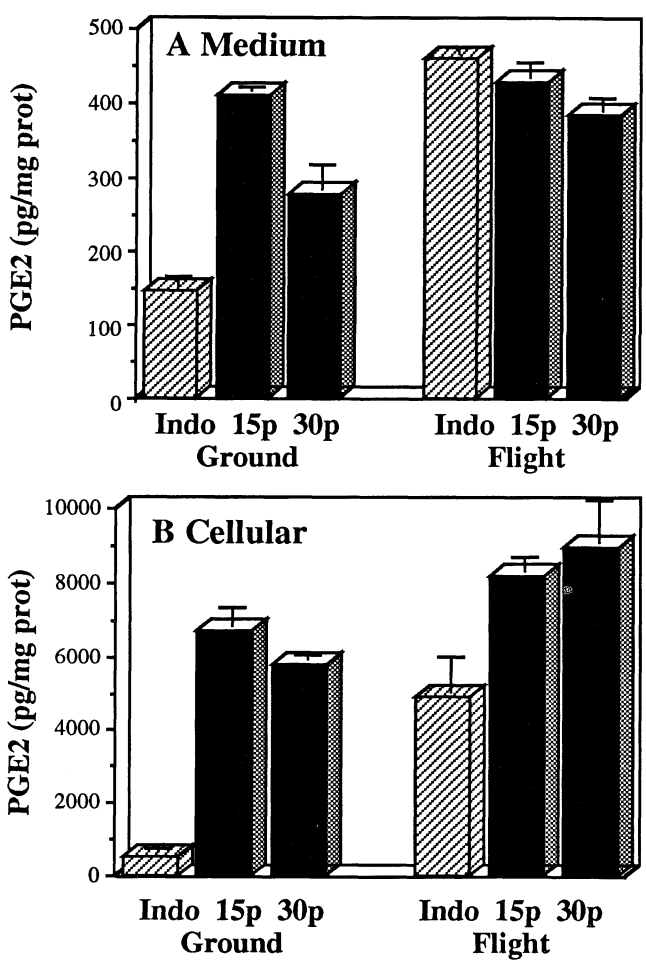


Fig. 7. Medium (A) and cell layer (B) PGE_2 levels measured with R.I.A. Note the differential indomethacin inhibition of PGE_2 levels on ground and in flight.

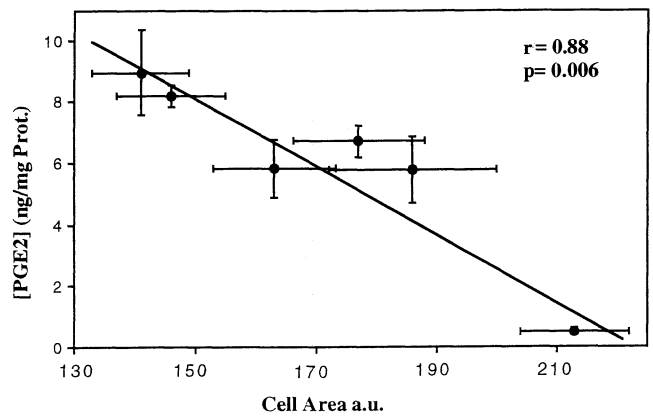


Fig. 8. Linear regression analysis between cell area (arbitrary units) and intracellular PGE_2 levels (pg/mg protein). Data plotted represent all the flight and ground controls conditions except PGE_2 treated cells; intracellularlevels of native PGE_2 were not measured.

creased by 20%, suggesting that gravitational stress increased PGE_2 synthesis. In ground controls a 45 min-pretreatment with indomethacin inhibited PGE_2 synthesis by 90% and PGE_2 medium levels were decreased by 40% as compared to untreated controls (513±96 vs 6,688±506 pg/mg protein and 409±6 vs 146±13 pg/mg protein, respectively). In flight, a similar 45 min-pretreatment with indomethacin only decreased intracellular PGE_2 levels by 40% and did not affect medium PGE_2 levels as compared to flight untreated controls. In the indomethacin-treated cells, PGE_2 synthesis was found to be 6-fold higher in flight cells than in ground controls. These data suggest that indomethacin-PGE_2 synthesis inhibition is less efficient in flight than on ground. The linear regression analysis showed a significant negative relationship between PGE_2 synthesis and cell area in every condition with $r=0.88$ and a less strong correlation with the shape factor ($r=0.72$) (Figure 8).

DISCUSSION

Osteoblasts are more sensitive to a large category of mechanical stressor (i.e., hypergravity, shear stress, hydrostatic pressure) than fibroblasts or endothelial cells (9) and ROS 17/2.8 are known to be sensitive to an increase in mechanical stress (10) or to microgravity (2). In this study we showed that sequential gravitational variations were able to induce cell shape changes which occurred early since the maximum of cell response was observed at the end of the 15th parabola (i.e. after 90 min of parabolic flight). The quantitative morphological analysis performed in this work allowed us to detect small modifications of cell shape; with similar morphometric measurement Haskin *et al.* (11) showed morphological alterations in MG-63 cells submitted to physio-

logical levels of hydrostatic pressure. Moreover, we showed that shape changes were observed in part of the cell population. It seems that only cell population named "2" in Fig. 3A is sensitive to the flight conditions; this population represents 30% of the measured cells. Interestingly, Civitelli *et al.* (12) showed that UMR-106 osteosarcoma cell response to PTH and PGE_2 could depend upon cell morphology, and Bizzarri *et al.* (13) showed that these findings could be related to differences in cell cycle. Since ROS 17/2.8 are clonal cells and were asynchronous at the time of the flight we hypothesize that the heterogeneity of ROS 17/2.8 response might be explained by a cell-cycle sensitivity to gravitational stress. Cell shape produce, a range of effects mediated by membrane integral proteins (integrins) and the cytoskeleton, which may be important in transducing mechanical deformation in biological response (14). Carvalho *et al.* (15) reported that immunolocalisation of vimentin, alpha-actinin and focal contact proteins and PKC showed a marked difference between strained and nonstrained cells. Tripathi *et al.* (16) and Jackson and Bellet (18) reported a relationship between cytoskeleton and cell cycle. During cell cycle progression from S to M, we and others (17) observed that cells became rounded. At that time, it has been shown that actin stress fibers across the cell disappeared and formed submembranous peripheral rings (18).

Previous studies have shown that, in bone tissue, PGE_2 is implicated in the early response to mechanical load (19). Unlike UMR-106, ROS 17/2.8 did not increase their AMPc levels after PGE_2 stimulation (20). This is consistent with the fact that they express only EP_1 receptor to prostaglandin which transduces signal through the intracellular calcium pathway (21). Moreover ROS 17/2.8 synthesize high levels of PGE_2 (22) and can therefore respond to stimuli with PGE_2 in an autocrine manner. In our experiment we found a flight-induced increase in PGE_2 in the cell layer. The major differences in PGE_2 levels between cells and medium could be related to a rapid degradation of PGE_2 in the medium (23) as suggested by Jones *et al.* (19). On the one hand, regression analysis found that there was a negative relationship between cell area, shape factor and intracellular levels of PGE_2. On the other hand, neither PGE_2 was able on ground to completely mimic flight-induced cell shape changes nor was indomethacin to completely inhibit them during the flight. We concluded that PGE_2 is partly implicated in cell shape changes induced by gravitational variations. However, PGE_2 was not responsible for cell area decrease, suggesting that other mechanisms may regulate ROS 17/2.8 responses to mechanical stress.

Our experiment showed that osteoblastic cells experienced persistent shape changes occuring at least before the 15th parabola and maintained through the 30th parabola. However, it is not known whether these morphological changes are due to microgravity itself, as seen in macrophages (24), to hypergravity itself, or related to the sequential variations from hypo- to hypergravity. Further experiments will be necessary in order to study a kinetic of osteoblast behaviour during gravitational stress.

REFERENCES

1. Rodan, G.A. 1991. Perspectives: mechanical loading, estrogen deficiency and the coupling of bone formation to bone formation. *J. Bone Miner. Res.*, **6**: 527–530.
2. Genty, C., Guignandon, A., Palle, S., Lafage, M.H., Vico, L., and Alexandre, C. 1995. Osteoblastic cells shape changes in microgravity. *Submitted.*
3. Aggeler, J. 1990. Cytoskeletal dynamics in rabbit synovial fibroblasts: II. Reformation of stress fibers in cells rounded by treatment with collagenase-inducing agents. *Cell Motil cytoskeleton.*, **16**: 121–132.
4. Raisz, L. and Martin, T. 1984. Prostaglandins in bone and mineral metabolism. In Bone and Mineral Research. vol. 3. W.A. Peck, editor. Elsevier, Amsterdam, **2**: 286–310.
5. Yeh, C. and Rodan, G.A. 1984. Tensile forces enhance prostaglandin E_2 synthesis in osteoblastic cells grown on collagen rubbons. *Calcif. Tissue Int.*, **36**: 67–71.
6. Reich, K.M. and Frangos, J.A. 1991. Effect of fluid flow on prostaglandin E_2 and inositol triphosphate levels in osteoblasts. *Am. J. Physiol.*, **261**: C428–C432.
7. Murray, D.W. and Rushton, N. 1990. The effect on bone cell prostaglandin E_2 release: a new experimental method. *Calcif. Tiss. Int.*, **47**: 35–39.
8. Spector, T. 1978. Refinement of the Coomassie blue method of protein quantification. *Annal. Biochem.*, **86**: 142–146.
9. Jones, D.B., Leivseth, G., Goodship, A., Sasse, L., and Van der Sloten, J. 1995. Osteoblast intracellular free calcium measurements in microgravity by ratio imaging and photometry. In Experiment results of ESA and CNES parabolic flight campaigns tenth anniversary of first ESA parabolic flight campaign. ed. ESA WPP-90. 363–380.
10. Kubota, T., Yamauchi, M., Onozaki, J., Sato, S., Suzuki, Y., and Sodek, J. 1993. Influence of an intermittent compressive force on matrix protein expression by ROS 17/2.8 cells, with selective stimulation of osteopontin. *Arch. Oral Biol.*, **38**(1): 23–31.
11. Haskin, C. and Cameron, I. 1993. Physiological levels of hydrostatic pressure alter morphology and organization of cytoskeletal and adhesion proteins in MG-63 osteosarcoma cells. *Biochem Cell Biol.*, **71**: 27–35.
12. Civitelli, R., Fujimori, A., Bernier, S., Warlow, P.M., Goltzman, D., Hruska, K.A., and Avioli, L.V. 1992. Heterogenous intracellular free calcium response to parathyroid hormone correlation with morphology and receptor distribution in osteogenic sarcoma cells. *Endocrinology,* **130**: 2392–2400.
13. Bizzarri, C. and Civitelli, R. 1994. Activation of Ca^{2+} message system by parathyroid hormone is dependent on the cell cycle. *Endocrinology,* **134**: 133–140.
14. Wang, N. and Ingber, D.E. 1994. Control of cytoskeletal mechanics by extracellular matrix, cell shape, and mechanical tension. *Biophys. J.*, **66**: 2181–2189.
15. Carvalho, R.S., Scott, J.E., Suga, D.M., and Yen, E.H.

1994. Stimulation of signal transduction pathways in osteoblasts by mechanical strain potentiated by parathyroid hormone. *J. Bone Miner. Res.,* **9(7)**: 999–1011.

16. Tripathi, S.C. 1989. A possible role of actin in the mechanical control of the cell-cycle. *Biol. Cell,* **67(3)**: 531–534.
17. Folkmann, J. and Moscona, A. 1978. The role of cell shape in growth control. *Nature,* **273**: 345–349.
18. Jackson, P. and Bellet, A.J. 1989. Relationship between organization of actin cytoskeleton and the cell-cycle in normal and adenovirus-infected rat cells. *J. Virol.,* **63(1)**: 311–319.
19. Jones, D.B., Nolte, H., Scholuebbers, J.G., Turner, E., and Veltel, D. 1991. Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials,* **12**: 101–110.
20. Partridge, N., Alcorn, D., Michelangeli, V., Ryan, G., and Martin, T.J. 1983. Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. *Cancer Res.,* **43**: 4308–4314.
21. Harada, S., Duong, L.T., Adam, M., Abramovitz, M., Funk, C.D., Rodan, S.B., and Rodan, G.A. 1994. Expression of prostaglandin E_2 receptors (EP1, EP2, EP3) in bone cells. *Abstract J. Bone Min. Res.,* **9**: supl. 1, S358, C46.
22. Rodan, S.B., Rodan, G.A., Simmons, H.A., Wolenga, R.W., Feinstein, M.B., and Raisz, L. 1981. Bone resorptive factor produced by osteosarcoma cells with osteoblastic features is PGE_2. *Biophys. Biochem. Res Com.,* **102**: 1358–1365.
23. Maclouf, J., Pradel, M., Pradelles, P., and Dray, F. 1976. 125 I derivatives of prostaglandins: a novel approach in prostaglandins analysis by radioimmunoassay. *Biochemica and Biophysica Acta,* **431**: 139–146.
24. Armstrong, J.W., Gerren, R.A., and Chapes, S.K. 1995. The effect of space flight and parabolic flight on macrophage hematopoiesis and function. *Exp Cell Res.,* **216**: 160–168.

(*Received for publication, June 5, 1995*
and in revised form, July 14, 1995)