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Microgravity-induced apoptosis in cultured glial cells

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SUMMARY

Apoptosis is a form of naturally occurring cell death that plays fundamental roles during embryonic developement. In adults, it neatly disposes of cells damaged by injuries provoked by external causes such as UV radiation, ionisation and heat shock. Alteration of the gravity vector may be one of the external apoptosis inducers. Neurophysiological impairment signs were seen during space flights in astronauts, but very few studies were carried out on the nervous system and none at the cellular level. In this study, we submitted cultured C_6 glioma cells to microgravity (0xg) of varying duration, obtained by clinorotation in a Fokker three-dimensional clinostat for 15min, 30min, 1h, 20h or 32h. After 30min at 0xg, numerous nuclei underwent the classical morphological alterations (chromatin condensation, nuclear fragmentation, apoptotic bodies) that lead to the programmed cell death. After 30min at 0xg, immunostaining for the enzyme caspase-7 was present in the cytoplasm of many cells concurrently with DNA fragmentation identified by the TUNEL method. At 32h, the number of apoptotic nuclei was much reduced indicating the ability of glial cells to adapt to altered gravity.

INTRODUCTION

Apoptosis, also called programmed cell death, is a multistep process characterised by cell shrinkage, chromatin condensation, DNA dissassemblation, nuclear fragmentation, and packaging of the nuclear fragments into apoptotic cell bodies (Wyllie *et al*., 1980): all these events lead to the death of the cell. Apoptosis is needed during embryonic development to shape organs and tissues, but it may occur to remove, in a neat, orderly way, damaged cells in the adult. Extreme insults, such as ionising or UV radiation, heat shock or hypoxia, as well as environmental stresses such as hyperosmolarity (Pellicciari *et al*., 2000), may activate signals that induce apoptosis. Gravitational force alteration may also be one of those triggering inputs. Apoptosis was observed in lymphocytes (Jurkat) during spaceflight (Lewis *et al*., 1998; Cubano and Lewis, 2000). Gravity vector changes, in fact, have been reported to damage the cytoskeleton of lymphocytes (Gmunder *et al*., 1990; Cogoli-Greuter *et al*., 1994; Lewis *et al*., 1998; Hashemi *et al*., 1999; Walther *et al*., 1999) and of HL-60 promyelocytes in culture (Piepmeier *et al*., 1997), both during space flight and in simulated microgravity on ground-conducted experiments. In previous experiments, we observed severe damages to the cytoskeleton of cultured glial cells submitted

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on-ground to simulated microgravity (Uva *et al*., 2000; Uva *et al*., 2001a,b); nuclear alterations were also observed (Uva *et al*., 2001c). Glial cells are the most numerous type of cells in the mammalian nervous system. Neuroglia interact with neurons in a complex way; besides being fundamental for nutrition, they are also fundamental, for neurogenesis, neural regeneration and signal transmission. Damage at the glial cell level leads to impairment of the entire nervous system. Neurophysiological problems were reported to occur in astronauts during space flights (Krasnov, 1994), but very few studies were performed on the nervous system and none at the cellular level.

The aim of this study was to investigate if weightlessness, which has been proved to cause cytoskeletal alterations, may induce programmed cell death in glial cells. We analysed the morphological evidences of apoptosis in cultured C_6 glioma cells, submitted on-ground to simulated microgravity, using the nuclear stains 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) and propidium iodide. Simulated microgravity was obtained using the so called "clinostat", a random positioning machine that simulates some of the physical effects of space flight by providing a vector averaged reduction of the apparent gravity without generating significant shear forces (Gmunder *et al*., 1990). DNA fragmentation *in situ* was identified by a method based on the incorporation of biotinylated nucleotides, mediated by a terminal deoxynucleotidyltransferase (TdT), into the 3' ends of DNA fragments [terminal dUTP nick end labelling (TUNEL)] (Gavriely *et al*., 1992). The presence of caspases (cysteinyl aspartate specific proteases), a family of cysteine proteases whose activation causes death by apoptosis (Alnemry *et al*., 1996), was determined by investigating the presence of caspase-7; this enzyme is thought to coordinate the execution phase of apoptosis by cleaving multiple structural and repair proteins (Slee *et al*., 2001) and is expressed in glioma cell lines (Glaser and Weller, 2001).

MATERIALS AND METHODS

Cell and culture procedure

C_6 glioma cells (kindly provided by prof. C. Pellicciari, University of Pavia, Italy), a cell line derived from rat brain tumor, were grown in D-MEM medium (Sigma, St. Louis, MO) with the addition of 10% foetal bovine serum,1% gentamycin and 1% L-glutamine (Celbio, Milano, Italy).

For the experiments, cells were placed in monolayer cultures in flasks on slides. The flasks on slides were positioned in a random positioning machine (Fokker three-dimensional clinostat kindly provided by Prof. P. Pippia, University of Sassari, Italy) and kept under continuous rotation at 60 rpm for 15min, 30min, 1h, 20h and 32h (simulated microgravity, 0xg). Ground (1xg) controls, kept in an identical machine and treated in parallel, were placed onto the supporting frame of the machine to subject the cells to the same vibrations. At the end of each experiment, the flasks were filled with phosphate buffered saline (PBS) containing 4% paraformaldehyde.

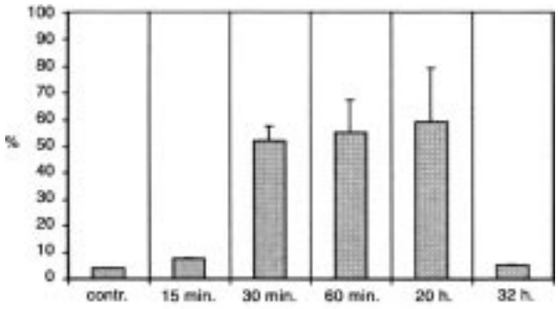
Staining procedure and immunohistochemistry

The slides were removed from the flasks and submitted to staining procedures and immunohistochemistry. Slides with cultured cells submitted to different duration 0xg conditions underwent the indirect immunofluorescence technique (Coons *et al*., 1955). After permeabilisation with 0.1% Triton X-100 (Sigma) and exposure to normal goat serum (diluted 1:50; Sigma) at 20°C in a moist chamber, the cells were incubated overnight at 4°C with the antiserum Ab-caspase-7 (Purified, Active), raised in rabbits (diluted 1:200, Alexis, Biochemical, San Diego, USA). After washing in 0.01 M PBS, pH 7.4, cells were incubated for 30 min in a moist chamber at 20°C with fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit IgG raised in goat (diluted 1:100, Sigma). The slides were rinsed in PBS and mounted with gel-mount (Biomeda Corp., Foster City, CA). The specificity of the immunostaining was verified by omitting one of the steps of the immunohistochemical procedure, or by replacing the primary antiserum with non-immune rabbit serum or PBS. DNA fragmentation was visualised with TUNEL (*in situ* cell death fluorescein detection kit, Roche Diagnostic Co., Indianapolis, IN., USA)

The nuclear alterations were observed with a conventional epifluorescence microscope (Olympus). Nuclei were stained for two hours with 1ml PBS containing 5mg/ml propidium iodide, !00 U/ml RNase or 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI).

Percentage of altered cells was counted in each experiments from control and treated cultures. Data are expressed as mean and standard deviations of the percent of cells calculated from three randomly chosen fields in two slide preparations for each sample.

Table I
Percentage of altered nuclei to simulated microgravity at different exposure times (means ± SD)



RESULTS

We monitored alterations occurring in the C_6 cultured glioma cells submitted to simulated microgravity for 15min, 30min 1h, 20h and 32h. From 15min to 20h at simulated 0xg, the percentage of altered nuclei increased, while in the controls it remained as low as 4.01%. After 32h at 0xg, the percentage of the altered cells was back in the range of 5%, as was that of the control cultured cells (Table I), but the density of the cell populations was much lower than that of the controls (20%).

After 30min, 1h and 20h at 0xg, the chromatin was condensed in patches in altered nuclei (Fig. 1b,c) and binucleated cells were frequently seen (Fig. 1d). Nuclear blebbing was numerous (Fig. 2d), micronuclei were present in the cytoplasm, and cellular shrinkage and nuclear fragmentation were found in almost all the altered cells after 20h at 0xg (Fig. 2b,c).

A TUNEL-positive DNA fragmentation pattern was easily identified in nuclei starting at 30min at 0xg (Fig. 3b). In binucleated cells, the nuclei were TUNEL-positive as were the micronuclei and the nuclear blebs (Fig. 3d,f).

Immunostaning for caspase-7 was identified in the cytoplasm of many cells after 30min at simulated 0xg; the immunostaining was stronger in cells with nuclei showing highly condensed chromatin (Fig. 4a,b).

TUNEL-positivity and immunoreaction for

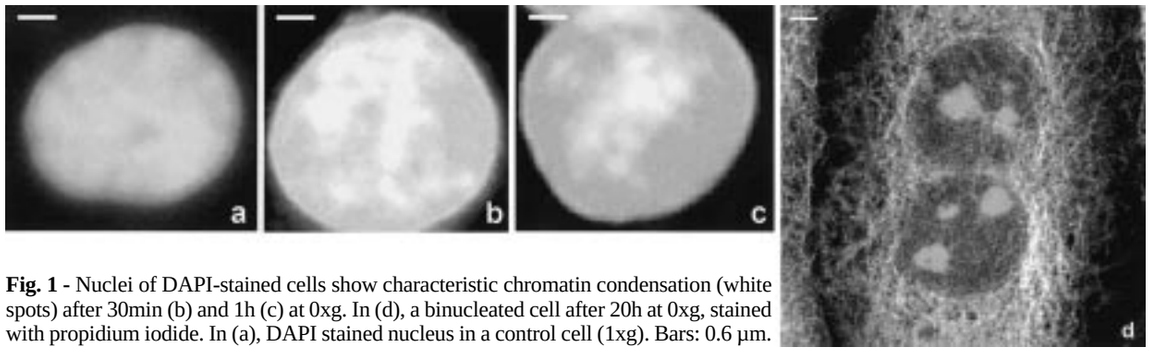


Fig. 1 - Nuclei of DAPI-stained cells show characteristic chromatin condensation (white spots) after 30min (b) and 1h (c) at 0xg. In (d), a binucleated cell after 20h at 0xg, stained with propidium iodide. In (a), DAPI stained nucleus in a control cell (1xg). Bars: 0.6 µm.

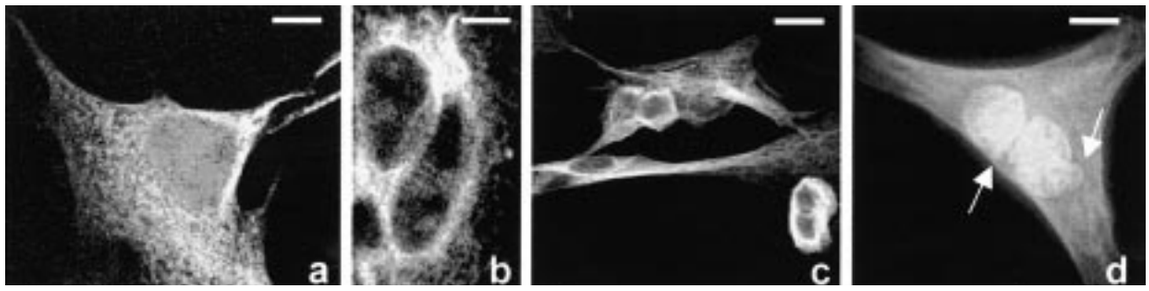


Fig. 2 - (a) Control normal cell, 1xg; (b) apoptotic cell with fragmented nucleus after 20h of simulated microgravity; (c) cell shrinkage after 20h at 0xg; (d) nuclear blebs (arrows) after 30min at simulated 0xg. Nuclei were stained with propidium iodide (a,b.c) or DAPI (d). Microtubules were immnostained with Ab-a tubulin. Bars: 2 µm (a); 1.5 µm (b); 0.5 µm (c); 2.2 µm (d).

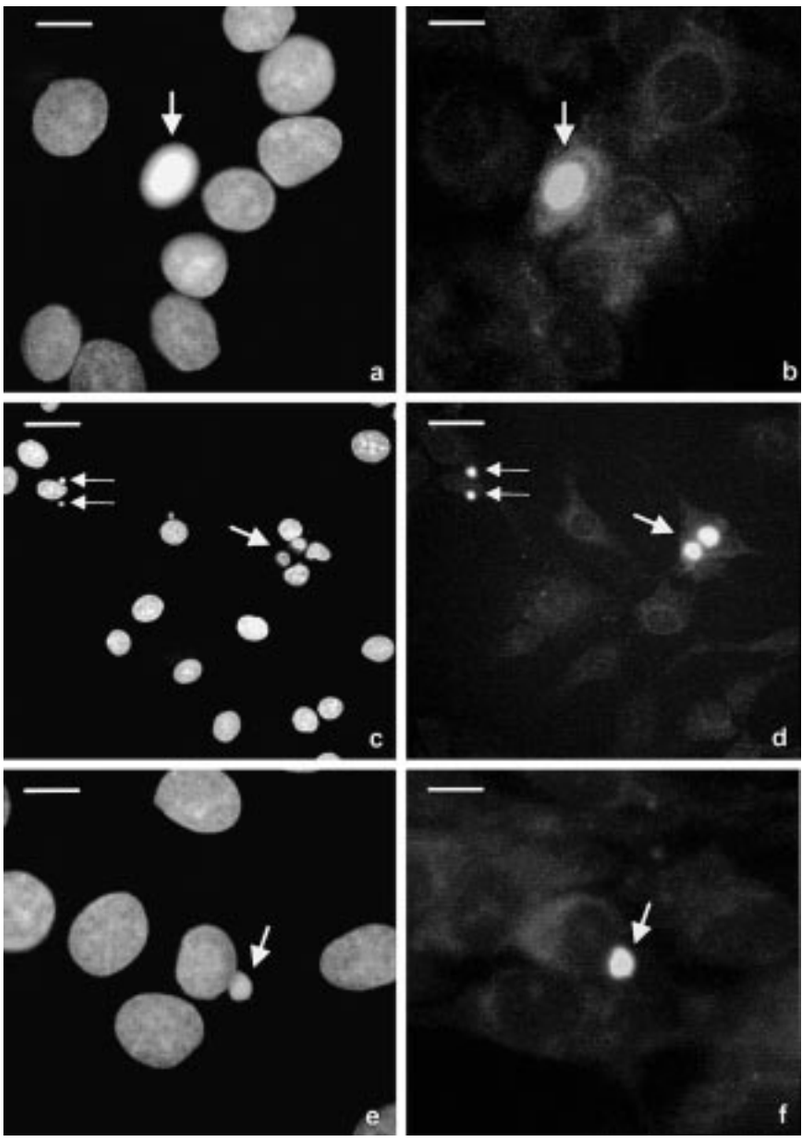


Fig. 3 - Cells after 1h at simulated microgravity. a,c,e: DAPI stained nuclei; b,d,f: TUNEL positivity in the same cells as a,c,e. The intense reaction in (b) corresponds to a nucleus with highly condensed chromatin (a) (arrows). TUNEL stained binucleated nuclei and micronuclei (arrows) in (d) correspond to the DAPI-stained cells in (c). TUNEL-positive nuclear bleb in (f) corresponds to a bleb with condensed chromatin in (e) (arrows). Bars: 2.8 µm (a,b); 8 µm (c,d); 2.8 µm (e,f).

caspase-7 were much less evident in the control cultured cells positioned onto the support frame of the clinostat. Immunohistochemical controls yielded negative results.

DISCUSSION

The present study shows that a time-dependent microgravity-related apoptosis occurs in cultured glial cells submitted to simulated microgravity (0xg) obtained by clinorotation using a Fokker three-dimensional clinostat. The presence of typical events such as cell shrinkage, chromatin condensation, nuclear fragmentation, micronuclei formation and apoptotic bodies suggest that microgravity-induced apoptosis occurs through the well-known series of morphological events. Apoptosis was identified in Jurkat cells flown in the Space Shuttle (Cubano and Lewis, 2000) on

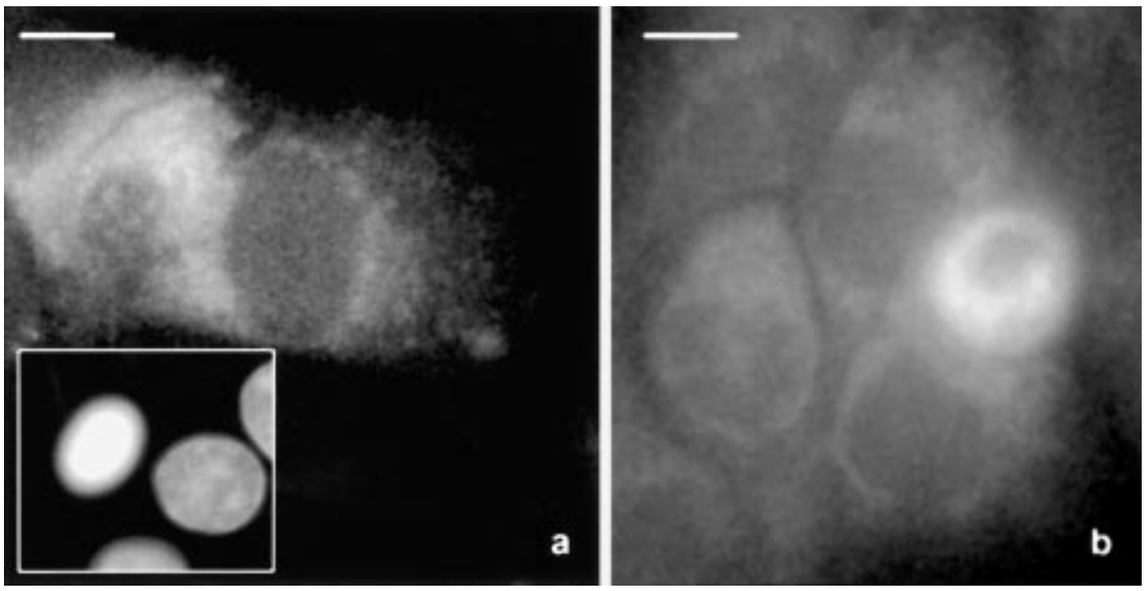


Fig. 4 - Caspase-7 immunostaining in apoptotic cells after 1h (a) and 30 min (b) of simulated microgravity. In (a), the insert shows, in the same field, a DAPI-stained nucleus with condensed chromatin. Bars: 2.1 µm (a); 2.4 µm (b).

the basis of nuclear morphology and the presence of the cell death factor sFAS/APO-1. In those experiments, apoptosis was microgravity-dependent and time-dependent, reaching the maximum density of apoptotic nuclei a 4h.

In our experiments, apoptotic nuclei began to be observed after 30min at 0xg, increased until 20h and then were almost absent at 32h. The presence of caspase-7, one of the major contributors to the enymatic cascade leading to apoptosis, was detected in the cytoplasm starting at 30min at 0xg. Caspase-7 cleaves many of the same substrates as caspase-3, including poly (ADP-ribose) polymerase (PARP) (Germain *et al*., 1999) and the structural components of the nuclear lamina, a rigid structure underlying the nuclear membrane and is involved in chromatin organisation. The lamina is formed by proteins called lamins. By disrupting lamins, caspases cause lamina to collapse and chromatin to condense (for a review see Thornberry and Lazebnik, 1998). DNA fragmentation, evidenced by the TUNEL method, is also present after 30min at 0xg.

All these data indicate that cells were affected by weightlessness within minutes, during which time apoptosis was induced. It has been reported that apoptosis affects the cytoskeleton, particularly actin microfilaments (Spano *et al*., 2000). However, one cannot exclude that things might go the other way round: an alteration in the cytoskeleton might be one of the inducers of cell death. Interaction between central spindle microtubules and cortical actin filaments leads to cytokinesis. When either of these structures is perturbed, cell division is suppressed (Gatti *et al*., 2000); binucleated cells may frequently be formed and cell death may be induced. According to this view, cytoskeletal changes observed in lymphocytes and glial cells after weightlessness might be the endogenous stimulus triggering apoptosis.

Long-term experiments showed that cells are able to adapt to changes in the gravitational field, and to reorganise their cytoskeleton (Uva *et al*., 2000, 2001a,b). In the present study, we observed that the number of apoptotic nuclei dramatically decreased from 20h to 32h: once more this underlines the high plasticity of glial cells and suggests the reasonable hypothesis that the absence of gravity may induce only transient alterations in the glial cells.

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