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Effect of Simulated Microgravity on Murine Melanoma Cells: Altered Tumor Cell and Tumorigenicity

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Summary

Short-term or long-term space flight induces numerous biological stressors that affect several human physiological systems. The physical factors and their physiologic stressors cause several system dysfunctions. The incidence of tumor growth and carcinogenesis in microgravity is yet unknown. Hence, we investigated the effects of simulated microgravity on tumor growth and tumorigenicity using ground-based *in vitro* and *in vivo* models. Murine B16-F10 melanoma cells were cultured in a rotating wall vessel bioreactor (BIO), designed by NASA to simulate some aspects of microgravity, and a tissue culture flask (FL). We then measured cell growth, melanin production and apoptosis. After 48 hours of culture in FL and BIO, cells were inoculated subcutaneously in C57BL/6 mice syngeneic hosts for B16-F10 tumor cells. The growth of BIO cells decreased to 50% ($p<0.01$), and doubling time increased. Melanin production, a marker of differentiation, increased to 150% ($p<0.05$) in BIO. Flow cytometry analysis showed that there was increase in the percentage of apoptotic cells in the BIO when compared with that in the FL. When BIO cultured melanoma cells were inoculated subcutaneously in mice there was significant increase in tumorigenicity ($p<.001$) as compared to FL cells. Our results indicate that simulated microgravity may have altered the tumor cell characteristics and enhanced the invasive property. It is possible that the microgravity analog culture environment may have selected highly tumorigenic cells for survival despite the decreased overall growth in the microgravity analog.

Key words: Tumor growth, tumorigenicity, microgravity

Introduction

The spaceflight environment has many stressors that contribute to adverse effects on human physiology. Some of these effects may be the result of exposure to the physical environment of space flights (microgravity, cosmic radiation) or the result of psychoneuroendocrine changes due to physical and psychological stresses involved in space flight. The physical factors and their physiologic stress effects result in several dysfunctions. As a result, carcinogenic events may be influenced during prolonged spaceflight periods.

Since radiation induced cell-mutation has been well known, many investigators seemed to focus

on the influence of radiation as a serious cancer risk for space missions. The short-term or long-term exposure to radiation can be mutagenic and induce radiogenic cancers. Cosmic radiation consists of protons, alpha particles, heavier ions and a wide energy range. Neutrons form an important part of the secondary radiation spectrum as a result of interaction with the atmosphere and spacecraft material (Keifer et al. 1999). Many investigators have raised critical concerns related to carcinogenic and mutagenic effects in the space environment (Cucinotta and Wilson 1995, Stanford and Jones 1999, Setlow 1999, and Hamm et al. 1999). Incidence of cancer among commercial airline and military aircraft pilots and crew is high for certain types of cancers (Hammer et al. 2002, Boice et al. 2000, and Rafnsson et al. 2002). The most notable documented evidence is development of malignant melanoma in these commercial and military aircraft crews.

Although there is no direct evidence that immunosurveillance actually protects individuals from developing tumors, certain observations and clinical evidence support the validity of the role of immunosurveillance in cancer immunity. Lethal cancers can arise in immunocompetent individuals; however, a weakened immune system can place one at risk for developing tumors. Decrease in immunosurveillance, in astronauts and cosmonauts, has been reported during both long and short duration flights. Microgravity induces a decline in cellular response to mitogens and T cell counts with leukocyte counts being somewhat variable (Taylor 1993) in short-duration flights. Long-duration studies (1-2 months) performed by Russians on board the Mir space station have documented a 50% reduction in lymphoproliferation response to phytohemagglutinin (PHA) on the day after mission, as compared with the pre-flight response (Konstantinova et al. 1993). Other studies showed a decrease in graft-versus-host response to xenoantigens and mitogens-induced IL-2 production. We have shown similar immune dysfunctions in the ground-based *in vitro* microgravity analog BIO (Hales et al. 2002) e.g. a decline in T cell proliferation to PHA and IL-2 production.

On the other hand, microgravity alters properties of cells such as cytoskeleton, proliferation ability and gene expression in different types of cells. These observations raise questions about whether microgravity may influence tumor growth and carcinogenesis. Therefore, these studies were designed to test the hypothesis that simulated microgravity would have influence on tumor growth, properties and tumorigenesis. Hence, we have studied *in vitro* and *in vivo* characterization of B16-F10 melanoma cells.

Materials and Methods

Cell line. The B16-F10 murine melanoma cell line was purchased from American Type Culture Collection (Manassas, VA) and was maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100,000 U/L of penicillin, 50mg/L of streptomycin and 10% fetal bovine serum (FBS) at 37℃ with 5% CO_2 and 95% air. All culture media and supplements were purchased from GIBCO (Rockville, MD). The cells of passage (p) from p5 to p20 were used for the experiment.

Cell culture procedure. Bioreactor (BIO) as a rotary cell culture system: a slow turning lateral vessel BIO (Synthecon, Houston, TX) was used to model some aspects of microgravity. BIO is a nonperfused, horizontally rotating vessel with a fixed volume capacity of 55ml with a large radius and a short width. The vessel is connected to a variable speed motor mounted on a fixed base and used at 16 rpm. It has separate sample ports for adding media or reagents, as well as for sampling.

Cells were cultured at a concentration of 1×10^5/ml with Cytodex beads (1g/L). Since B16

melanoma cells are anchor dependent and grow normally in a flask, cytodex beads were added to the medium as an anchor platform to evaluate growth behavior in BIO. After culturing for 24 and 48 hr, the cells grown on cytodex beads were washed twice with phosphate-buffer saline (PBS), treated with 0.25% trypsin and resuspended in PBS. This suspension was teased through stainless steel screens and cells were separated from cytodex beads. Cells were counted on a hemocytometer and cell viability determined by trypan blue (2g/L, GIBCO, Rockville, MD). Cell proliferation was measured in a ^{3}H-tymidine uptake assay. The cells were seeded in a 96 well plate at 5×10^{4} cells/well and 1μCi of ^{3}H-tymidine (6.7 Ci/ mmol specific activity, Amersham Pharmacia Biotech) was added to each well. After 48hr incubation, cells were washed twice with PBS and lysed with 0.1% Triton-X100 since melanoma are adherent cells. Cell lysates were then transferred to 10ml plastic disposable scintillation vials to which 5 ml of scintillation fluid (Liquiscint, National Diagnostic) were added. The samples were counted on a Beckman LS-9000 liquid scintillation counter. In addition, doubling time was calculated for both culture populations by the Patterson method (1979).

Animals. 6-8 week-old C57BL/6 female mice obtained from Harlan Sprague-Dawley (Indianapolis, IL). Animals were housed under normal housing conditions at constant temperature (25℃) and humidity (50-70%) with a 12-hour light /dark cycle. Food and water were provided ad libitum. The Animal Care and Facilities Use Committee at The University of Texas is compliant with IACNE and National Institute of Health animal care standards. Animal protocol was approved by the Animal Welfare Committee of UTHSC at Houston.

In vivo tumor growth and properties. Melanoma cells were cultured in either regular tissue culture or BIO as described above. After 48 hr incubation, cells were collected, washed with PBS twice and resuspended in complete DMEM at 1×10^{6} cells/ml. The mice were inoculated in the right flank with 1×10^{5} melanoma cells in 0.1mL, either grown in flask or BIO. Tumor growth was measured every other day by calipers in three directions and diameter readings were averaged for determining the tumor volume as an indicator of growth. On day 14 after inoculation, tumors were excised from all animals and levels of melanin and apoptosis were measured.

Spectrophotometric determination of total melanin. Total melanin content of tumor cells was determined according to Kowalczuk et al (2001). In brief, 5×10^{6} Tumor cell was pelleted, washed, and treated with 1M NaOH; supernatants were stored at 4℃ for 48 hours. Supernatants were filtered through 0.45mm filter and absorbance measured at 400 nm. A standard curve was plotted using synthetic melanin (Sigma) as a standard with concentrations between 0 and 100μg -1mg in 1M NaOH. Protein was determined by the Bradford method (1976).

Assessment of apoptosis. Single cell suspensions were prepared from tumors as well as cells cultured in FL and BIO. Cells were assayed for apoptosis by flow cytometry after Annexin-V binding reaction (Roche, Inc., Germany).

Statistical analysis. Data are expressed as the mean ± SE of at least three independent experiments. Statistical analysis was performed by the unpaired Student's T-test. A p value of < 0.05 was considered statistically significant.

Results

1. Effect of microgravity on proliferation of melanoma cells

Melanoma cells were cultured in FL or BIO and cell growth was evaluated according to number of viable cells and ^{3}[H]-thymidine up take. The cell numbers at 24 hr and 48 hr of culture are given in Figure 1a. The viable cell numbers decreased in the BIO culture for 24 and 48 hr as compared with

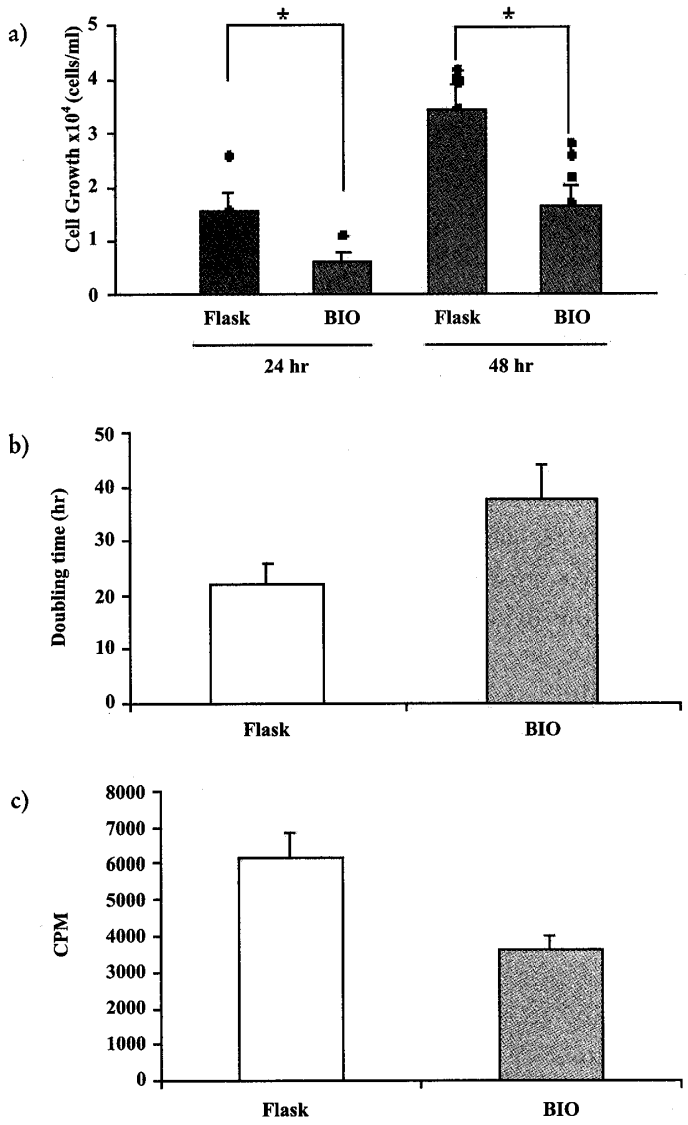

Figure 1. Effect of microgravity on proliferation of melanoma cells. a) cell growth, b) the doubling time calculated at 48hr, and c) the cell growth as measured by tritiated thymidine uptake.

those in FL ($p<0.05$).

The doubling time was calculated for both culture populations at 48hr. The results were inversely correlated with the cell growth. The doubling time for culture population in flask was 22 hr. and it was 38 hr for BIO cultures (Figure 1b).

Figure 1c shows the cell growth as measured by tritiated thymidine uptake by melanoma cells.

2. Melanin synthesis in simulated microgravity.

Figure 2 shows time-dependent production of melanin in simulated microgravity. Melanin is regarded as a differentiation marker in melanoma cells. B16-F10 melanoma cells were cultured for 24 hr and 48 hr in BIO or FL. Melanin levels increased about two-fold in BIO cultured cells as compared to FL cultured cells. This increase in differentiation marker supports and provides one possible explanation for the increased invasive property of *in vitro* BIO cultured cells.

3. Effect of simulated microgravity on apoptosis.

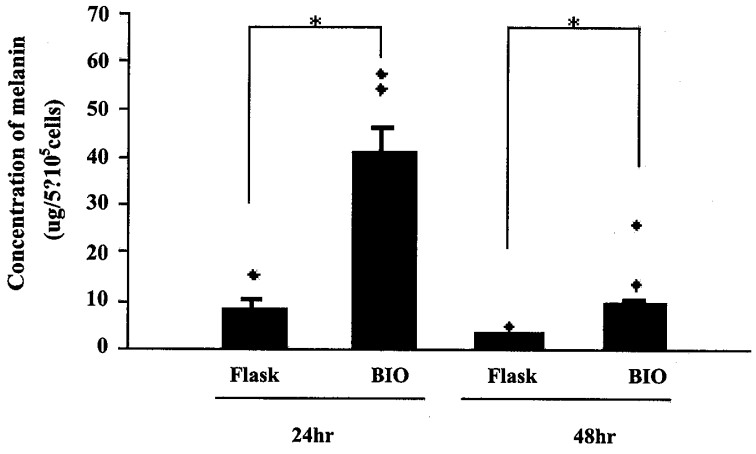

Figure 2. Melanin synthesis in simulated microgravity. B16-F10 melanoma cells were cultured for 24 hr and 48 hr in BIO or FL.

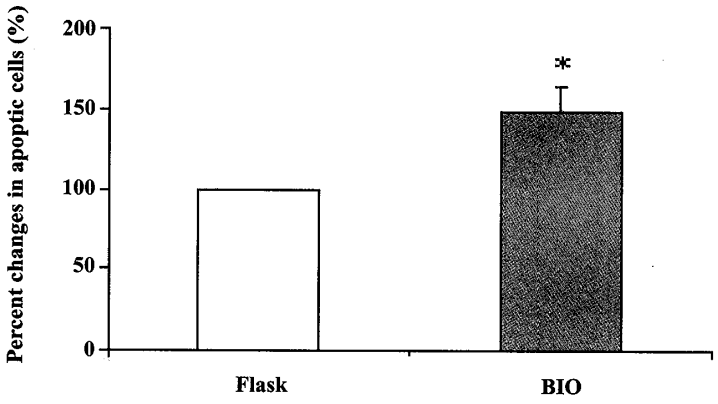

Figure 3. Effect of simulated microgravity on apoptosis.

Since the level of apoptosis varied with growth in both BIO and FL cultures, the percentages of apoptotic cells were assayed by flow cytometry after the Annexin-V binding reaction. The rate of apoptotic cells was assayed for both culture populations at 48hr. Flow cytometry analysis showed that there was an increase in the apoptotic cells in the BIO when compared with that in the FL (Figure 3).

4. In vivo tumor growth analysis:

After 48 hours of culture in FL and BIO, cells were inoculated subcutaneously in C57BL/6 mice. There were no significant differences in body weights between the groups (Figure 4). Tumor growth increased significantly with BIO cell inoculum as compared to FL cells ($p<0.01$) (Figure 5). Eight days after tumor cell inoculation there was a significant increase in growth and tumor size in BIO cell inoculum as compared to control FL culture inoculum. Flow cytometry analysis showed that there was an increase in the percentage of apoptotic cells in the BIO when compared with that in the FL ($p<.05$) (Figure 6).

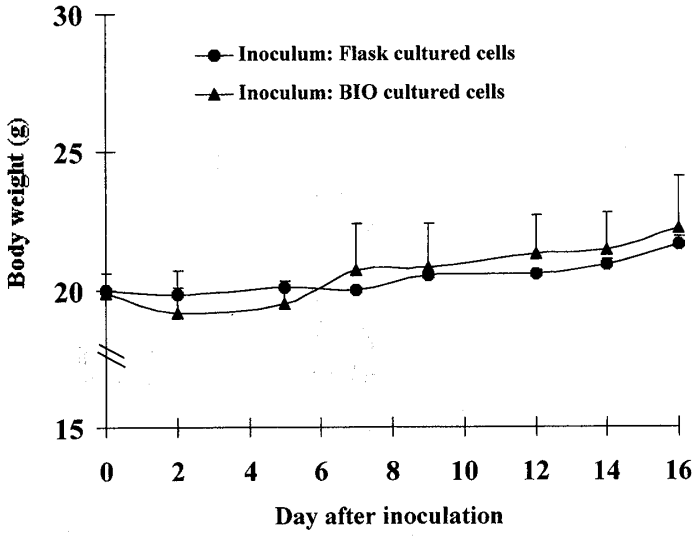

Figure 4. Changes of body weight after inoculation with B16-F-10 melanoma cells cultured either in FL or BIO for 48 hours.

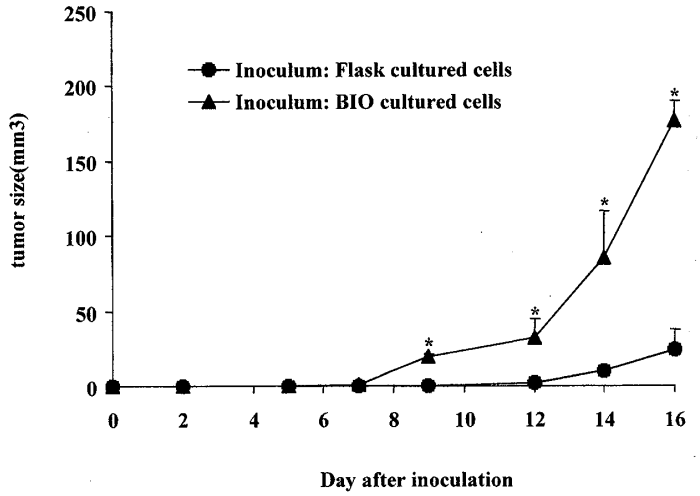

Figure 5. Tumor size after inoculation with B16- F10 melanoma cells cultured either in FL or BIO for 48 hours.

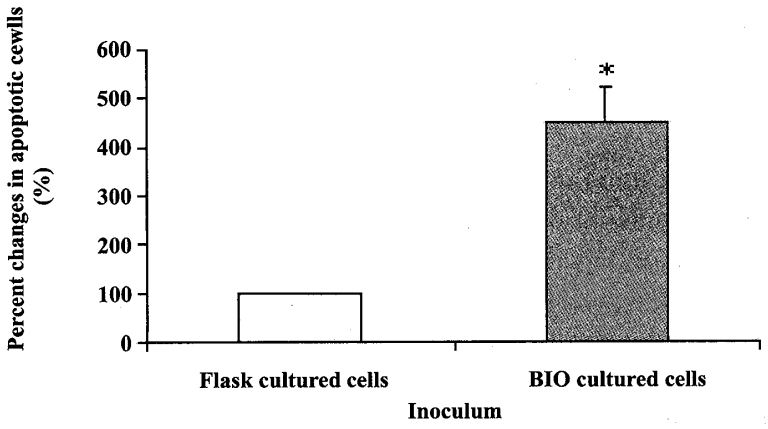

Figure 6. The percentage of apoptotic cells in the tumor 16 days after inoculation with B16-F10 melanoma cells.

Discussion

It is difficult to predict cancer risks from the space environment and unavoidable space radiation during flight. Humans in space are exposed to both microgravity and space radiation. Interaction of these two is not yet clearly understood but it is very important in risk estimations and of practical relevance to space flight missions. While microgravity countermeasures can be devised at least for some of the effects of microgravity, radiation exposure and its effects are unavoidable at present, at least logistically. It is vitally important to assess risks in ground-based microgravity analogs and to study a representative tumor and its response in the microgravity environment in order to develop measures for human protection. The assessment of radiation risk in space is dependent on the estimations made on Earth; however, it is not our aim here to study radiation as the initial insult causing carcinogenesis. The short-term or long-term exposure effects of radiation can be mutagenic and induce radiogenic cancers. Cosmic radiation consists of protons, alpha particles, heavier ions and a wide energy range. Neutrons form an important part of the secondary radiation spectrum as a result of interaction with the atmosphere and spacecraft material (Keifer 1999). Many investigators have raised critical concerns related to carcinogenic and mutagenic effects in the space environment (Cucinotta and Wilson 1995, Stanford and Jones 1999, Setlow 1999, and Hamm et al. 1999). Setlow in his report on the U.S. NRC's view on the radiation hazards in space emphasizes the uncertainty over the amount of exposure and its ill effects on astronaut health. Hamm et al. (1999) studied occupational radiation exposure and cancer risks among commercial airline pilots and astronauts. Comparing the health and physical fitness and cancer risks of these two groups, Hamm et al noted increased cancer incidence in pilots and raised serious concerns for astronauts as the mission crew will be exposed to space radiation hazards at increased levels during the planned long-term missions for space and planetary explorations. One goal that is important and pertinent to this proposal is to carry out experimental research in ground-based models. Without concentrating on the radiation effects, and with knowledge of the effects of the spaceflight environment, we have focused on studying a representative tumor to critically analyze its response to microgravity analogs. Based on the preliminary data and results obtained from this research, critical questions are raised on the application and extrapolation for spaceflight research to confront the yet unknown issues of cancer risk and concern for space travel. Advances made thus far in the field of medicine from NASA research are noteworthy and humans have benefited immensely for life on the Earth. We feel that our research will extend opportunities in the space biomedical discipline to better care for astronaut health and lead to treatment to conquer the difficult cancers such as malignant melanoma.

Incidence of cancer among commercial airline and military aircraft pilots and crew is high for certain types of cancers (Hammer et al. 2002, Boice et al. 2000, and Rafnsson et al. 2000). The occupational work environment is somewhat similar to astronauts in that these individuals fly at high altitude, experience altered circadian rhythm, exposure to cosmic radiation, electromagnetic fields and radiofrequency radiation, etc. Particularly notable is the observation of the differences between short-duration flights of a few hours and long-duration flights across several time zones. Increased cancer incidence is defined as Standardized Incidence Risk (SIR) among cohorts for all types of cancers as compared to normal individuals. The most notable incidence is development of malignant melanoma in these commercial and military aircraft crews. The longer the exposure the greater is the incidence of cancer. In a longitudinal study of astronaut health Hamm et al. (1998) analyzed the risk of cancer mortality among astronauts and normal individuals. Their study showed a higher age-specific risk of cancer morbidity and mortality, and this is comparable to the studies on

Air Canada pilots by Band et al. (1996). Astronauts, over the airline pilots, have the added risk factor of hypogravity which has adverse effects on many aspects of physiology. With longer duration spaceflights such as stays on the ISS, Mars and lunar missions or deep space exploration, the risk probability is greater and well founded yet unknown. Astronauts are healthy individuals, however, risk factors in these individuals are unknown or not cataloged as regards their prior exposure to various elements such as time spent sunbathing and radiation exposure, etc. As a matter of fact, the majority of the astronaut class is made up of former occupational pilots. In order to study the correlation between spaceflight and incidence of tumor growth, we chose a mouse melanoma as a representative cancer in ground-based microgravity analogs. Preliminary results are shown in Section C.

Many investigators have shown that radiation induces tumorigenisis in space environments. However our hypothesis in the present study was the microgravity itself affects tumor cells. In order to confirm this we used the bioreactor system which is developed by the National Aeronautics and Space Administration (NASA) (Schwarz RP et al., 1992), which provides a unique environment of low shear force, high mass transfer, microgravity, and the ability for three-dimensional cell growth in a conventional laboratory tissue culture incubator. We have shown here that the B16-F10 melanoma cell displayed altered cell growth and tumorigenicity in simulated microgravity. Cell growth of B16F10 was significantly decreased at 24 and 48 hr of culturing in BIO in microgravity analog. We also found out that the tumorigenicity for B16-F10 melanoma cells according to melanin production was increased under conditions of simulated microgravity compared with the control tissue in the culture flask. Significant melanin production was found in cells measured 24hr after the start of horizontal rotation, and it became more at 48hr. We observed both cell growth and melanin production, but cell confluence was over 90% for the control tissue in the culture flask.

Grimm et al. (2002) suggested that simulated microgravity alters differentiation and increases apoptosis in human follicular thyroid carcinoma cells. Since apoptosis is shown in clinorotated cells but not in incubator controls and ground samples, they concluded that it appears to be related to the microgravity. Our results with melanoma B16-F10 are consistent with their results from thyroid carcinoma cells, showing a high percentage difference in apoptotic cells when comparing the BIO incubated culture with the flask incubated culture (Figure 3). This high potential for cellular suicide in the *in vitro* microgravity analog might point to one of the risks of long-duration spaceflight. Melanoma cells were cultured in flask and BIO for 48 hrs, and were inoculated subcutaneously in C57Bl/6 mice. Tumors were excised from all animals on day 14 and apoptosis was measured. Percentage changes in apoptotic cells in tumors from animals inoculated with the BIO cultured cells were significantly higher than in those from control animals. Once cells are brought to the microgravity environment, those changes seem to be stable and may be able to cause problems in the human body. To our knowledge, this is the first experimental evidence of cell properties being altered *in vitro* by microgravity not by radiation. Furthermore the changes observed affected invasiveness *in vivo*.

The results of our study have demonstrated that simulated microgravity may have altered the tumor cell characteristics and enhanced invasiveness.

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