Effect of Space Flight on the Frequency of Micronuclei and Expression of Stress-responsive Proteins in Cultured Mammalian Cells

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Space shuttle experiment/Space radiation/Frequency of micronuclei/p53/Cultured mammalian cells

Results of past space experiments suggest that the biological effect of space radiation could be enhanced under microgravity in some cases, especially in insects. To examine if such a synergistic effect of radiation and microgravity also exists in human cells, frequencies of chromosome instability and cellular levels of several stress-responsive proteins were analyzed in cultured human and rodent cells after space flight. Human (MCF7 and AT2KY), mouse (m5S) and hamster (SHE) cell lines were loaded on the Space Shuttle Discovery (STS-95 mission) and grown during a 9-day mission. After landing, the micronuclei resulting from abnormal nuclear division and accumulation of stress-responsive proteins such as p53 and mitogen-activated protein kinases (MAPKs), which are involved in radiation-induced signal transduction cascades, were analyzed. The frequencies of micronuclei in all the four mammalian cell strains tested were not significantly different between flight and ground control samples. Also, the cellular amounts of p53, p21 (WAF1/SDI1/CIP1) and activated (phosphorylated) forms of three distinct MAPKs in MCF7 and m5S cells of flight samples were similar to those of ground control samples. These results indicated that any effect of space radiation, microgravity, or combination of both were not detectable, at least under the present experimental conditions.

INTRODUCTION

With the coming International Space Station era and the possible future manned Mars Mission, an increasing number of humans will make exploration in space and sojourns there will be longer and longer. Under such circumstances, exposure to space radiation is unavoidable. Thus, there is increasing concern of radiation risk during long-term space flight. In fact, the frequencies of chromosome aberrations detected in peripheral lymphocytes from astronauts who stayed in the Russian Mir Station for 3 to 6 months were much higher than those of astronauts who returned from space flights of a few weeks1,2). Furthermore, in past space experiments, several investigators suggested that microgravity could enhance various effects of radiation in living organisms (see a review by Horneck3)). This synergistic effect of radiation and microgravity was particularly evident in insects3–5). If such an effect indeed exists in humans, the risk from space radiation during long-term space flight

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would be much greater than the present estimate. Therefore, it is imperative to clarify whether human cells show synergistic effects of radiation and microgravity.

The primary objective of the present study is to analyze the effects of radiation and microgravity on the structure and functions of genomes in cultured mammalian cells during space flight, and if possible, to test whether the synergistic effects of radiation and microgravity can be detected or not. For this, we sent 4 kinds of mammalian cells including a radiation-sensitive human ataxia telangiectasia (AT) cell strain into space and examined the frequencies of micronuclei induced during a 9-day flight in the Space Shuttle Discovery (STS-95 mission). Micronuclei are efficiently induced by radiation, especially by high LET radiation, which is relatively abundant in the space environment. Since the scoring of micronuclei is much easier than the analysis of chromosome aberrations which requires a high level of skill, the micronucleus assay has become a popular method for detecting genotoxic agents. Recently, it was used in biodosimetry on residents near the Semipalatinsk nuclear test sites. Also analyzed were changes in the cellular amounts of several stress-responsive proteins such as p53 and MAPKs (mitogen activated protein kinases) during the space flight. These proteins are involved in a variety of signal transduction pathways, thereby modulating radiation responses.

**MATERIALS AND METHODS**

**Cell strains**

Two human, one mouse and one hamster cell strains were used. Human MCF7 is a breast cancer cell line (ATCC HTB 22) and AT2KY cells are diploid skin fibroblasts derived from a patient with radiation-sensitive ataxia telangiectasia (JCRB 0316). Mouse m5S is an immortalized cell line established from the embryonic skin of an ICR mouse. Syrian hamster embryo (SHE) cells were prepared in our laboratory from 13- and 14-day embryos as described previously. All cells were cultured in the Dulbecco’s minimum essential medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and antibiotics.

**Space experiments**

A cylindrical cartridge, 23 mm in diameter and 130 mm in length (Celco Inc., Germantown, MD, USA), was used for cell culture in space. The cartridge contains about 300 cellulose acetate hollow capillary fibers and has a 7-ml extracapillary space (Fig. 1).

Three days before the scheduled launch, late log phase cells were inoculated into the cartridges, so that they could spread and grow on the surface of the fibers. The hollow space provides an environment that mimics microgravity conditions. The cells were harvested after a 9-day flight in space.
capillary fiber cartridges were originally developed for the culture of cells growing in suspension. The results of several pre-flight tests done in our laboratory at Kyoto University showed that the cell growth of monolayer cultures in the cartridges was much slower than in ordinary cultures using plastic dishes or flasks, and further that m5S and SHE cells could not grow well due to a poor attachment of these cells to the surface of capillary fibers. Therefore, to improve cell attachment, we used modified cartridges containing capillary fibers coated with fibronectin for cultures of these two cell lines.

Numbers of cells inoculated per cartridge are listed in Table 1. Cartridges were then installed in the environmental hardware, Cell Culture Module (CCM; Walter Reed Army Institute of Research, Washington DC, USA). In the CCM units, both ends of each cartridge were connected to a reservoir containing 200 ml of culture medium which circulates in the cartridge through a gas-exchange oxygenator. The flow rate of the medium was 6 ml/min, and the system was kept at 37°C in a 5% CO₂-air at 1013 hPa. The CCM flight unit was installed in a mid-deck locker of the Space Shuttle Discovery 16 h before its scheduled launch, while an identical CCM unit was kept at the NASA Kennedy Space Center (KSC) for ground control. The Discovery launched on October 29, 1998, and the elapsed time for this STS-95 mission was 8 days 21 h and 15 min.

About 4 h after landing, cells were recovered by injecting trypsin-EDTA solution into cartridges, enumerated, and subjected to treatments for micronucleus assay and Western-blot analysis.

**Micronucleus assay**

Standard methods described previously were used with slight modifications. Briefly, about 2 × 10⁵ cells of each strain were seeded in 4 chambers of Lab-Tek tissue culture chamber/slide (Miles Lab., Naperville, IL, USA) and incubated for 24 h at 37°C in a tissue culture room at the KSC. Then, cytochalasin B (Sigma, St Louis, MO, USA) was added at a final concentration of 5 µg/ml and further incubated for 24 h. After incubation, cells were washed with phosphate-buffered saline (PBS), fixed with Carnoy’s solution and air dried. The samples were sent to Japan by air cargo.

In our laboratory (Kyoto Univ.), cell nuclei were stained with PI (propidium iodide) and binuclear cells harboring micronuclei were detected under a fluorescent microscope.

**Analysis of stress-responsive proteins**

The cellular levels of several stress-responsive gene products such as p53, its effector protein p21 (WAF1/SDI1/CIP1), and activated (phosphorylated) forms of 3 distinct MAP kinases (MAPK) in human MCF7 and mouse m5S cells were measured by Western blotting as described previously. Cells (about 10⁶ cells) recovered from each cartridge were suspended in 200 µl of lysis buffer [PBS containing 1% NP-40, 1 mM Na₃VO₄, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride and 1% protease inhibitor cocktail (Sigma)]. The cell mixtures were rotated in a microtube rotator for 30 min at 4°C, and DNA was sheared by sonication. The lysates were centrifuged at 13,000 rpm (9,000 × g) for 5 min at 4°C, and the supernatants were collected as the protein fraction. The concentration of protein was measured with Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). After equalizing the protein concentration among samples by adding lysis buffer, sample loading buffer (New England BioLabs Inc., Beverly, MA, USA) was added and boiled for 5 min. After cooling, all samples were frozen under liquid nitrogen and sent to Japan by air.

All samples were subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (Hybond-ELC, Amersham Pharmacia Biotech., Piscataway, NJ, USA). The proteins on the membrane were probed with anti-p53 (Ab-1, Oncogene Research Products, San Diego, CA, USA), anti-p21 (Ab-4, Oncogene Research Products) monoclonal antibodies, or polyclonal antibody specific to each activated (phospho-specific) MAPKs; PhosphoPlus ERK1/2 (p44/p22) MAP Kinase (Thr202/Tyr204), PhosphoPlus SAPK/JNK MAP Kinase (Thr183/Tyr185) and PhosphoPlus p38 MAP Kinase (Thr180/Tyr182) antibodies, all from New England BioLabs Inc. For comparison, anti-MAPK polyclonal antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) which recognizes each MAPK irrespective of its phosphorylation status was also used. Bound antigens were visualized with the ELC Plus Western Blotting Detection System (Amersham Pharmacia Biotech.).

**Measurement of radiation dose**

The accumulated radiation dose during the 9-day mission was determined by two types of passive dosimeters; thermoluminescent dosimeter (TLD) to primarily measure low LET radiation like high-energy protons and γ-rays, and CR39 plastic track detectors to measure high LET heavy charged particles. These dosimeters were attached to the lid of the CCM.
RESULTS

Number of cells recovered from cartridges

After the 9-day flight, cells were recovered from cartridges of flight and ground control groups. The numbers of cells collected are shown in Table 1. There was no great difference in cell numbers between flight and control samples in each cell strain. In the human tumor cell line MCF7, the numbers of cells recovered were 7 to 8 times those inoculated, while in m5S and SHE cells, the numbers recovered were very similar to those inoculated. In contrast, markedly fewer AT2KY cells were recovered than were inoculated. Similar characteristics of recovery efficiencies for these 4 strains were repeatedly observed with several pre-flight tests done in our laboratory. Also, the results of pre-flight and the flight experiments indicated that the method used to harvest the cells, by injecting trypsin-EDTA solution into cartridges, was not sufficient to recover enough of the AT2KY cells, skin fibroblasts, which firmly attach to the substratum of the cartridge.

Table 1. Number of cells inoculated into and recovered from each cartridge used for space flight or ground control

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>No. of cells inoculated</th>
<th>No. of cells recovered</th>
<th>No. of cells inoculated</th>
<th>No. of cells recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>5 × 10^5</td>
<td>4.0 × 10^6</td>
<td>5 × 10^5</td>
<td>3.4 × 10^6</td>
</tr>
<tr>
<td>AT2KY</td>
<td>5.6 × 10^6</td>
<td>1.9 × 10^6</td>
<td>5.6 × 10^6</td>
<td>1.6 × 10^6</td>
</tr>
<tr>
<td>m5S</td>
<td>5 × 10^6</td>
<td>5.8 × 10^6</td>
<td>5 × 10^6</td>
<td>6.0 × 10^6</td>
</tr>
<tr>
<td>SHE-1^a</td>
<td>1 × 10^7</td>
<td>3.4 × 10^6</td>
<td>1 × 10^7</td>
<td>4.8 × 10^6</td>
</tr>
<tr>
<td>SHE-2^a</td>
<td>1 × 10^7</td>
<td>7.5 × 10^6</td>
<td>1 × 10^7</td>
<td>6.9 × 10^6</td>
</tr>
<tr>
<td>SHE-3^a</td>
<td>5 × 10^6</td>
<td>4.8 × 10^6</td>
<td>5 × 10^6</td>
<td>5.8 × 10^6</td>
</tr>
<tr>
<td>SHE-4^a</td>
<td>5 × 10^6</td>
<td>6.0 × 10^6</td>
<td>5 × 10^6</td>
<td>6.6 × 10^6</td>
</tr>
</tbody>
</table>

^aFour cartridges were used for SHE cells in both flight and ground control experiments.

Micronucleus formation

Micronuclei present in binuclear cells after the blocking of cytokinesis by cytochalasin B were detected in 4 cell strains; human tumor MCF7, diploid human AT2KY, immortalized mouse m5S and hamster embryonic SHE cells. For individual strains, 3 to 9 thousand binuclear cells were analyzed, and the frequency of micronuclei was determined as the number of binuclear cells harboring micronuclei divided by the total number of binuclear cells examined (Table 2). In 3 strains, MCF7, AT2KY and SHE, the micronucleus frequencies of the flight samples were almost the same as those of ground controls. The frequency for the flight group in m5S cells was slightly higher than that of control samples. However, the difference was not significant. As a whole, these results indicate that, at least under the present experimental conditions, no excess micronuclei were induced in cultured mammalian cells by exposure to the space environment.

Table 2. Frequencies of micronuclei in four different mammalian cell strains after space flight

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Flight group</th>
<th>Ground control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>4.68% (388/8291)</td>
<td>5.06% (447/8841)</td>
</tr>
<tr>
<td>AT2KY</td>
<td>5.36% (161/3003)</td>
<td>5.48% (179/3269)</td>
</tr>
<tr>
<td>m5S</td>
<td>5.04% (419/8320)</td>
<td>4.04% (321/7942)</td>
</tr>
<tr>
<td>SHE</td>
<td>2.13% (110/5160)</td>
<td>2.07% (106/5129)</td>
</tr>
</tbody>
</table>

^aNumerals in parentheses show the number of binuclear cells with micronuclei/total number of binuclear cells examined.

Induction of stress-responsive proteins

To know whether the space environment is stressful to cultured mammalian cells, we measured expression of typical stress-responsive proteins, p53, p21 and MAPKs, in MCF7 and m5S cells during the space flight. Although relatively large numbers of SHE cells were recovered from cartridges, they were reserved for the analysis of cell transformation. Figure 2 shows cellular levels of tumor suppressor proteins p53 and p21, and activated (phosphorylated) forms of MAPKs, as revealed by Western blotting. MCF7 cells showed a high level of p53 proteins, and also the accumulation of p21 whose expression is induced by p53. However, there were no significant differences in the amounts of these proteins between flight and ground control samples. In m5S cells, only small amounts of p53 proteins were present in both flight and control groups, and accordingly, there was no detectable level of p21.

As for MAPKs which mediate stress-inducible signal transduction cascades, all 3 forms of activated (phosphorylated) MAPKs were present in both MCF7 and m5S cells, though their amounts differed markedly depending on the cell strain and the types of MAPKs. In MCF7 cells, the amounts of activated SUPK/JNK and p38 MAPK in the flight group were similar to those of the control group, but activated ERK1/2 in flight samples was slightly less abundant than in control samples. In m5S cells, levels of all 3
activated MAPKs appear to be similar between flight and control samples, judging from the total amount of MAPKs which is a measure of the amount of proteins loaded on each lane.

Altogether, these results also indicate that the present space flight conditions did not activate significant amounts of 5 classes of stress-responsive proteins in cultured human and mouse cells.

**Radiation dose during the mission**

The measured dose of low LET radiation was 12.8 mGy as determined by TLD, and that of high LET (higher than 10 keV/μm) radiation was 6 mSv (0.787 mGy) as estimated by CR39. This gave a total dose-equivalent of 18.8 mSv.

**DISCUSSION**

The results of the present study showed that no significant formation of micronuclei was induced by a 9-day space flight (STS-95 mission) in 4 mammalian cell lines, including 2 human strains. Radiation is an effective agent to induce micronuclei. The measured dose of space radiation during the mission was 18.8 mSv, comprising of 12.8 mGy of low LET radiation and 0.787 mGy (6 mSv) of high LET radiation. The observed dose rate, 2 mSv/day, was 5 to 10 times those of ordinary Space Shuttle missions flying at about 300 km above the earth. This is due to the unusually high altitude (555 km) of the STS-95 mission, since the dose rate of space radiation rapidly increases with increasing altitude. Nevertheless, the total dose (18.8 mSv) seems to be too small to cause significantly higher frequencies of radiation-induced micronuclei in flight samples compared with ground controls, as the lowest dose at which micronuclei are detected is thought to be about 10 cGy (100 mGy) for X- or γ-rays. Therefore, it may be reasonable that we were unable to detect excess levels of micronuclei in flight samples, even for extremely radiation-sensitive human AT cells. These results further suggest that microgravity did not enhance the effect of radiation on micronucleus induction, at least under the present experimental conditions.

With the STS-95 mission, we have analyzed mutations induced by space flight in human tumor HCT116 cells which are hypermutable due to a defect in the DNA mismatch repair system, and in repair-proficient MCF7 cells. However, frequencies of microsatellite mutations were not significantly different between flight and ground control samples in both HCT116 and MCF7 cells. Similarly, the frequency of ouabain-resistant mutations in HCT116 cells of the flight group was almost the same as that of controls. These findings are consistent with the results of the micronucleus assay. Therefore, it was concluded that possible synergistic effects of space radiation and microgravity on the genomic integrity of cultured mammalian cells were not detectable under the present conditions.

In the present study, we also analyzed the cellular content of p53, its effector protein p21, and 3 distinct groups of MAPKs which control different signaling cascades, in human MCF7 and mouse m5S cells. However, there was virtually no excess accumulation of these 5 kinds of stress-responsive proteins induced by space flight.
Radiation is known to increase cellular levels of p53 and p21\textsuperscript{12, 13}. In fact, a significant accumulation of these proteins in human tumor cells was reported after a small dose of X-rays, as low as 10 cGy\textsuperscript{23}. Similarly, all the 3 classes of MAPKs studied here are known to respond to various chemical and physical stresses including radiation\textsuperscript{13, 14}. A very low dose (2 cGy) of X-rays activated p38 MAPK in m5S cells and the activation reached a maximum level at 2 h after irradiation, followed by a decrease with time\textsuperscript{18}. In contrast, ERK1/2\textsuperscript{24} and JNK/SAPK\textsuperscript{25} are reported to be activated by large doses of radiation in several cell lines, although no significant activation of these MAPKs was detected in m5S cells after X-irradiation at 3 Gy\textsuperscript{18}. Therefore, a significant effect of the space flight on either the accumulation of p53 and p21, nor the activation of MAPKs, can be explained by assuming that the present dose of space radiation was too small to cause any detectable level of changes in these stress-responsive proteins. Alternatively, the low dose-rate nature of space radiation may require a much larger total dose to induce radiological responses of these proteins than those used in the aforementioned laboratory experiments, because both the extent and time course of activation of stress-responsive proteins greatly depend on the dose-rate of radiation, as has been shown with p53 and p21\textsuperscript{25}.

Microgravity, another characteristic factor inherent in the space environment, modulates the expression of early responsive genes such as c-fos and c-jun in cultured mammalian cells\textsuperscript{26, 27}, and alters the amounts of IL-1, IL-2 and IL-2 receptor in cultured lymphocytes\textsuperscript{27}. Interestingly, Ohnishi et al reported the accumulation of p53 protein in rat skin and muscle after a 14-day space flight (STS-58 mission)\textsuperscript{28, 29}. We assume that microgravity may be a primary cause of p53 accumulation in rat tissues, because the dose of radiation received by rats was much less than the dose of STS-95 mission, due to the lower altitude of the STS-58 mission (average 250 km). Their result contradicts ours, suggesting that microgravity may be a considerable stress to animals, but not to cultured cells.

In conclusion, the results of the present space experiments indicated that neither space radiation nor microgravity caused any detectable effect on the structures and functions of genomes in cultured mammalian cells, at least when the formation of micronuclei and activation of stress-responsive proteins were used as radiobiological endpoints.

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