The combined effect of simulated microgravity and radiation on chromosome aberrations in human peripheral blood lymphocytes

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Abstract

To understand the combined effect of microgravity (μG) and space radiation on cells is an important requirement for the anticipated space travel by humans. Cultured cells, such as human fibroblasts and lymphoblasts, were used in previous studies, and we observed changes in the expression of some cell-cycle-related genes and increased chromosome aberrations (CAs) in these cells under simulated μG . In this study, we investigated the combined effect of μG and space radiation on human peripheral blood lymphocytes. Whole blood was irradiated with X-ray or carbon-ion (C-ion) beam while being exposed to simulated μG using a three-dimensional clinostat. The frequency of CA was assessed using the three-color fluorescence *in situ* hybridization on chromosome spreads of colcemid-induced prematurely condensed chromosomes in lymphocytes during the first cell division post irradiation. Compared with the cells irradiated at 1G, the frequency of CA was increased in cells simultaneously exposed to simulated μG and radiation even though cells were irradiated by the same doses (0.5–1.0 Gy). This result is similar to those of our previous studies in which we used human fibroblast

TK6 cells and human fibroblast 1BR-hTERT cells. These ground-based experiments give insights into the biological effects of the space environment where radiation and μG coexist. ©2021 Jpn. Soc. Biol. Sci. Space; doi:10.2187/bss.35.15

Introduction

Space radiation is a major environmental factor whose biological effects must be neutralized, minimized, and/or reversed for the crew of all space missions. For this reason, space radiation biology has been an active area of research for many years. Space radiation can be classified into two categories: solar particle rays, which are suddenly generated by perpetually-occurring explosions on the solar surface, and high-energy galactic cosmic rays, which originate from supernova explosions and shower the entire universe. These rays range from low-linear energy transfer (LET) radiation, such as electrons and protons, to various types of heavy-ion beams (Furukawa *et al.*, 2020; Takahashi *et al.*, 2018). The latter types are high-LET radiation, generate more DNA double-strand breaks (DSBs) (Li, 2007), and are expected to cause more serious biological consequences. However, there are only a limited number of studies on cells exposed continuously to space radiation at low doses and low dose rates. To directly estimate biological effects of space radiation, chromosome damages in peripheral blood lymphocytes is the most widely used biomarker.

The fluorescence *in situ* hybridization (FISH) method is a standard technique for gene mapping (Lichter *et al.*, 1990; Pinkel *et al.*, 1986) in which two or three chromosomes are targeted for staining. Compared with a simple Giemsa staining (Edwards and Lloyd, 1991; Sabatier *et al.*, 1987), this method is more effective in identifying complex exchanges because different chromosomes can be identified by color coding (Anderson *et al.*, 2000; Durante *et al.*, 1999; Griffin *et al.*, 1995). The FISH method has a higher resolution for the locations and orders of genes (Ferguson-Smith, 2015) than classical methods for showing genetic linkage based on recombination data from pedigree analysis. The multicolor FISH method has been developed to stain all 23 pairs of human chromosomes (Loucas *et al.*, 2001). Each chromosome is chromatically identified using a combination of five fluorescently-tagged probes, and chromosome aberration (CA) can be identified through just one hybridization (Schröck *et al.*, 1996).

To assess the effects of space radiation on returning astronauts, CAs in their peripheral blood lymphocytes have been used as an indicator. In one study, peripheral blood lymphocytes of astronauts were prepared before and after 120–198 days of stay at the International Space Station (ISS), and CAs were compared in the two lymphocyte samples from each astronaut. It was reported that the frequency of CA significantly increased after their stay in space (Obe *et al.*, 1997). ISS missions have been conducted since 2000 (Feiveson *et al.*, 2021; George *et al.*, 2013), and the length of stay has been steadily increasing from about 3–6 months to over a year; in fact,

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the longest single mission in space is 437.7 days. Moreover, some astronauts participate in multiple ISS missions, such that the longest cumulative stay in space is 2.2 years. It has been reported that astronauts receive about 0.4 mSv/day of radiation on the ISS, which stays in low Earth orbit (Sato *et al.*, 2018). The ISS is located inside the Van Allen Belt; therefore, heavy-ion beams coming from deep space cannot reach it because they interact with protons, alpha rays, and electrons trapped in Earth's magnetic field. In deep space outside of Earth's magnetic field, it is predicted that astronauts will receive about 1 mSv/day during the solar minimum (Sato *et al.*, 2018), or about 1 Sv during a 2.5-year mission to Mars (Hassler *et al.*, 2014) due to heavy-ion beams with higher LET. The longer the duration of future human space missions such as living on the Moon and Mars, the greater the exposure to space radiation, and the higher the potential health risk to humans will be.

In addition to radiation, the space environment is practically free of gravitational force, or the world of microgravity (μ G), and living organisms in space are exposed to these two environmental factors at the same time. We have been studying whether simultaneous exposure of cells to simulated μ G and space radiation is different from exposing them to only one of the two. A three-dimensional (3D) clinostat was used to simulate the μ G condition by continuously rotating it on two axes. By synchronizing the specimen position on the clinostat with the timing of irradiation, we were able to avoid unnecessary stoppage of rotation during irradiation and achieve simultaneous exposure to simulated μ G and radiation (Ikeda *et al.*, 2016, 2017). Using this system, we irradiated human fibroblasts while they were exposed to simulated μ G. Cells were first kept under simulated μ G for 3 or 24 hours and then irradiated with X-ray or carbon-ion (C-ion) beam while still being under simulated μ G. Control cells were kept on a stationary clinostat for the same lengths of time and irradiated in the same manner. We

found that the expression of some cell-cycle inhibitor genes was decreased while that of certain cell-cycle-promoting genes was increased (Ikeda *et al.*, 2019). In another study of ours, human fibroblasts irradiated under simulated μ G conditions showed a higher frequency of both simple and complex types of CA compared with cells irradiated under the 1G condition (Hada *et al.*, 2019). The results from combined effects of simulated μ G and C-ion beam indicates that cells damaged by the radiation are not properly arrested at the cell-cycle checkpoint and may proliferate without repairing the damage. Furthermore, our recent study showed that the frequency of CA was increased in human lymphoblastic cells, which are non-adherent cells, when exposed to simulated μ G and radiation (Yamanouchi *et al.*, 2020). Our studies have suggested that different cell types are affected differently by the simultaneous exposure to simulated μ G and radiation, thus making it important to analyze the combined effects on various cell types in the human body. In this study, we investigated the combined effects from the viewpoint of CAs in using human peripheral blood lymphocytes.

Materials and Methods

Human blood

This study was conducted in accordance with accepted ethical and humane practices, and approved by the Institutional Review Board (IRB) of Gunma University (IRB no. HS2019-232) and Prairie View A&M University (IRB no. 2019-079). All experiments were performed using whole peripheral blood drawn from a healthy, non-smoking volunteer just before the experiment.

Cell cultures simultaneously exposed to simulated μ G and radiation

A sterile polypropylene bag was formed and filled completely with 5 mL of the freshly drawn whole blood (Fig. 1) and fitted into a diamond-shaped cavity of the

Figure 1. The process of making a sterile blood bag. A sterile polypropylene sheet was folded in half (a), and the folded side was thermo-compressed (b). The two longer sides of the sterile bag were thermo-compressed, and three edges were thermo-compressed to form a diamond shape (c). Blood was carefully injected into the bag with a syringe so as not to puncture it (d). Some air bubbles will form (e). The remaining shorter side of the sterile bag is thermo-compressed (f), and the last edge of the diamond-shaped bag is then thermo-compressed while excluding air bubbles from the blood filled compartment (g).

sample folder on the clinostat. To make the bag, a polypropylene sheet (Hybrid MekkinBag HM-1304, HOGY, Osaka, Japan) was folded in half (Fig. 1a), and the folded, short side was thermo-compressed (Fig. 1b). The two long sides were then thermos-compressed, followed by thermo-sealing three of the diamond-shaped bag edges (Fig. 1c). The blood was carefully injected into the bag with a syringe (Fig. 1d), during which some air bubbles may form (Fig. 1e). The one remaining side of the sterile bag was then thermo-compressed (Fig. 1f) after all bubbles and air space were chased out from the bag (Fig. 1g).

The bag containing blood was mounted on the 3D clinostat sample stage, in order to have efficient heat transfer from the stage (37°C) to the blood in the bag. This also ensured stable mounting of the bag onto the clinostat sample stage. The clinostat was turned on so that the cells were acclimatized to simulated μ G for 6 hours before irradiation. The 3D clinostat rotates on two axes, 11 rpm (66°/second) in the X-axis direction and 13 rpm (78°/second) in the Y-axis direction. By constantly changing the sample orientation relative to the direction of Earth's gravity, the time-averaged gravity vector acting on cells can be brought to close to zero. Samples were exposed for 0.2 seconds of each 60-second cycle when the stage becomes perpendicular to the axis of radiation beam. For X-ray irradiation, we used the X-ray generator (200 kV, 14.6 mA, 0.3 mm aluminum filter, MultiRad225: Faxitron Bioptics, LLC, Tucson, AZ, USA) with a high speed shutter (Accelerator Engineering Co., Chiba, Japan) which was synchronized to the clinostat rotation so that the shutter opened only when the sample bag was correctly oriented relative to the beam axis. For synchronized C-ion beam irradiation, a synchrotron

(Gunma University Heavy Ion Medical Center, Gunma, Japan) and the respiratory gating system were used. The dose-averaged LET was 50 keV/ μ m at the center of the 6-cm spread-out Bragg distribution with an energy of 290 MeV/n. Simultaneous exposure to simulated μ G and radiation was performed without stopping clinostat rotation. For 1G control, cells were mounted on an appropriately oriented stationary clinostat and irradiated in the same manner as described earlier (Ikeda *et al.*, 2016, 2017; Ohno *et al.*, 2011; Takahashi *et al.*, 2017). The dose rate for both X-ray and C-ion beam was approximately 0.03 Gy/minute.

Chromosome collection from the whole blood sample

Figure 2 shows the overall flow of the experiment. The method of chromosome collection was described in our previous reports (Hada *et al.*, 2019; Yamanouchi *et al.*, 2020). Samples were irradiated with 0.5 or 1.0 Gy of X-ray, or 0.1, 0.25 or 0.5 Gy of C-ion beam 6 hours after gravitational treatment started and kept under 1G or simulated μ G condition for 24 hours to allow DNA damage to repair. The whole blood was then transferred into T25 flask with PB-max karyotyping medium and cultured at 37°C for 52 hours. Chromosomes in G2 and mitotic stages in the first cell division were collected using the chemically induced premature chromosome condensation (PCC) method described previously (Durante *et al.*, 1999; George *et al.*, 2015). 50 nM Calyculin A (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) was added to the culture 30 minutes prior to harvesting, and cells were treated with a hypotonic solution (0.075 M KCl) for 15 minutes at 37°C and fixed with methanol/acetic acid (3:1).

Figure 2. The overall flow of experiment.

FISH

The FISH method was described in our previous reports (Hada *et al.*, 2019; Yamanouchi *et al.*, 2020). Fixed cells were dropped on a slide and hybridized *in situ* with the three-color fluorescence whole-chromosome human DNA probe (Aquarius, Cytocell, Oxford Gene Technology, Oxfordshire, UK) to stain chromosomes 1 to red, 2 to green, and 4 to yellow, according to the protocol recommended by the manufacturer. After staining these target chromosomes, all others were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). When breaks occurred in any two chromosomes (resulting in dicentric or translocations due to the exchange), this was defined as a simple type of CA (George *et al.*, 2009). Incomplete translocation and incomplete dicentric were also classified as a simple type of CA, assuming that the mutual fragments were below the detection level. A complex type of CA was defined as the occurrence of three or more breaks and exchanges in two or more chromosomes; each type of CA was counted as one exchange. When more than two chromosomes were damaged in a single cell, they were separately evaluated. We aimed to analyze at least 1,000 cells or to detect 50 aberrant cells in each type of radiation and each dose.

Statistical analysis

Statistical analysis was performed as previously described (Hada *et al.*, 2019; Yamanouchi *et al.*, 2020), and the ratio between the scored and the total cells analyzed was defined as the frequency of CA. It has been reported that radiation damage randomly occurs among chromosomes and that CA is proportional to the DNA content of the chromosome analyzed, with some variability in the data (Luomahaara *et al.*, 1999). The

following formula was used to convert the frequency of CA of chromosomes 1, 2, and 4 to whole-genome equivalents (Lucas *et al.*, 1992):

$Fp = 2.05[fp(1 - fp) + fp1fp2 + fp1fp3 + fp2fp3]FG$, where fp is the fraction of the whole genome comprised of painted chromosomes; $fp1$, $fp2$, and $fp3$ are the fractions of the genome for chromosomes 1, 2, and 4, respectively; and FG is the whole-genome aberration frequency. Fp is the combined frequency of exchanges in all painted chromosomes.

Standard errors for aberration frequencies were calculated assuming Poisson statistics. The error bars in the figure represent the standard errors of the mean values. The Fisher's exact test was used to assess whether the difference in frequency of chromosome exchanges is significant between different treatment groups. Logistic regression analysis was performed to examine the effect of radiation dose and gravity on total exchanges, using total exchange as dependent variable and radiation dose and gravity as independent variables.

Results

CAs in human peripheral blood lymphocytes simultaneously exposed to simulated μG and radiation

Table 1 shows the whole-genome equivalent frequency of CAs per 100 cells in human peripheral blood lymphocytes irradiated by X-ray or C-ion beam under the 1G or simulated μG conditions. The p -values were calculated by the Fisher's exact test between the 1G and the simulated μG conditions at each dose. Values less than 0.05 are shown in bold. Six cases were found to be $p < 0.05$: the frequencies of simple and total CAs in cells

Table 1. Whole-genome equivalent for the frequency of CAs per 100 human peripheral blood lymphocytes irradiated by X-ray or C-ion beam under 1G or simulated μG condition. P -values were obtained from the Fisher's exact test between 1G and simulated μG conditions at each dose. $P < 0.05$ values are shown in bold.

Radiation	Gravity	Total spread scored	No. of aberrant spreads	Simple exchanges	%	Complex exchanges	%	Total exchanges
Control (0 Gy)	1G	3018	18	0.77±0.26	100.00	0	0.00	0.77±0.26
	μG	1008	6	1.02±0.51	100.00	0	0.00	1.02±0.51
		(p -value)		0.748		1		0.748
X-ray (0.5 Gy)	1G	1037	37	7.93±1.40	91.46	0.74±0.43	8.54	8.67±1.47
	μG	782	50	12.82±2.05	82.98	2.63±0.93	17.02	15.45±2.25
		(p -value)		0.0497		0.065		0.0085
X-ray (1.0 Gy)	1G	685	50	15.38±2.40	93.16	1.13±0.65	6.84	16.51±2.49
	μG	419	50	32.51±4.47	96.35	1.23±0.87	3.65	33.74±4.55
		(p -value)		0.000211		1		0.00021
C-ion beam (0.1 Gy)	1G	1018	18	2.78±0.84	78.75	0.76±0.44	21.53	3.53±0.94
	μG	1025	25	5.01±1.12	86.83	0.75±0.43	13.00	5.77±1.20
		(p -value)		0.147		1		0.184
C-ion beam (0.25 Gy)	1G	1050	50	9.79±1.55	76.90	2.94±0.85	23.10	12.73±1.77
	μG	894	50	13.51±1.97	81.04	3.16±0.95	18.96	16.67±2.19
		(p -value)		0.152		1		0.168
C-ion beam (0.5 Gy)	1G	403	50	24.23±3.93	65.50	12.75±2.85	34.47	36.99±4.86
	μG	264	48	45.75±6.67	77.05	13.63±3.64	22.95	59.38±7.60
		(p -value)		0.0019		0.859		0.0051

*The results are presented as mean±standard error of the mean.

Table 2. Logistic-regression analysis of the effect of radiation dose and gravity on total exchanges.

Radiation	Factor	Coefficient	SE* of coefficient	p-value	OR**	95%CI*** for OR	
						Lower	Lower
X-ray	Dose	2.732	0.203	3.65×10^{-41}	15.370	10.318	22.895
	Gravity	0.760	0.150	4.00×10^{-7}	2.138	1.594	2.868
	Constant	-5.247	0.178	1.85×10^{-191}			
C-ion beam	Dose	6.284	0.355	3.13×10^{-70}	535.886	267.397	1073.961
	Gravity	0.466	0.124	1.66×10^{-4}	1.594	1.250	2.031
	Constant	-5.490	0.146	$<1.00 \times 10^{-191}$			

*SE, standard error; **OR, odds ratio; ***CI, confidence interval.

Figure 3. Frequencies of total chromosome exchanges induced by X-ray (●○) or C-ion beam (▲△) while cells were under either 1G (●▲) or simulated μ G condition (○△). Error bars indicate standard error of the mean. The “n” value for each data point is shown in Table 1. The dotted and solid lines show the results of linear approximation.

irradiated with 0.5 or 1.0 Gy X-ray, or 0.5 Gy C-ion beam. Looking at the total exchange, simulated μ G per se does not appear to cause a significant change in CA in peripheral blood lymphocytes, but both X-ray and C-ion beam at the dose higher than 0.5 Gy induce CAs under the simulated μ G condition. Note that low doses (0.1 and 0.25 Gy) of C-ion beam irradiation did not show increased CAs under simulated μ G.

Table 2 shows the results of the logistic-regression analysis of the effect of radiation dose or gravity on the frequency of total CAs. For the odds ratio for X-ray, the dose was 15.370 and the gravity was 2.138. The odds ratios for C-ion beam were 535.886 and 1.594 for the dose and the gravity, respectively. These results indicate that CAs were increased 2.1 times by X-ray and 1.6 times by C-ion beam in the cells irradiated under simulated μ G compared with 1G, regardless of irradiation doses. These results also show that C-ion beam causes many more CAs than X-ray.

Figure 3 shows the relationship between irradiation doses and total exchanges. The frequency of CAs increased in a dose-dependent manner for both X-ray (blue) and C-ion beam (red). The frequency of CAs was

Table 3. Ratio of frequencies of CAs in cells irradiated with C-ion beam to cells irradiated with X-rays at the same 0.5 Gy dose.

Type of CAs	Gravity	Radiation		Ratio
		X-ray	C-ion beam	
Total	1G	8.67	36.99	4.27
	μ G	15.45	59.38	3.84
Single	1G	7.93	24.23	3.06
	μ G	12.82	47.75	3.72
Complex	1G	0.74	12.75	17.22
	μ G	2.63	13.63	5.18

higher in cells irradiated under simulated μ G compared with those irradiated at 1G for both types of irradiating beams. The graph also shows that C-beam is more hazardous to lymphocytes than X-ray. Table 3 shows the ratio of frequencies of CAs in the cell irradiated with C-ion beam to the cell irradiated with X-rays at the same 0.5 Gy dose. The ratios were calculated by dividing the frequency of CAs in the cells irradiated by C-ion beam with that in the cells irradiated with X-ray. The frequency of CAs increased 4.3 times at 1G and 3.8 times at simulated μ G in the cells irradiated with C-ion beam than in the cells irradiated with X-ray.

Figure 4 shows a more detailed analysis on the occurrence of simple (blue bar) and complex (red bar) types of CAs under various conditions. No complex type was detected in unirradiated control cells. Compared with C-ion beam irradiated cells, the frequency of CAs was lower in X-ray irradiated cells. In Table 3, at the same dose of 0.5 Gy, the frequency of the simple type of CAs increased 3.1 times at 1G and 3.7 times at simulated μ G. The frequency of complex type increased 17 times at 1G and 5.2 times at simulated μ G. These ratio data show that C-ion beam induces more CAs than X-ray.

Discussion

Advantage of using human peripheral blood lymphocytes

Many studies have found increased levels of CAs in astronauts' blood lymphocytes after space missions. For example, it was reported that increased frequencies of CAs were detected in blood lymphocytes of eight astronauts who returned from separate long space missions (George *et al.*, 2001). Another study reported

Figure 4. Frequencies of simple and complex types of CAs induced by X-ray or C-ion beam while cells are under either 1G or simulated μ G condition. The blue column shows the simple type of CAs, and the red column shows the complex type. Error bars indicate standard error of the mean. "n" is shown in Table 1.

that lymphocytes of astronauts who were in a 115 day-space mission exhibited a significantly higher number of CAs compared with abnormalities scored in preflight samples from the same individuals (Yang *et al.*, 1997). Additionally, when the frequency of CAs was measured in the peripheral blood lymphocytes of 33 astronauts who participated in space missions over a period of 11 years, a statistically significant increase was found in astronauts from long-term missions (Durante *et al.*, 2003). However, there were no changes in the CA frequencies of two astronauts who experienced only a nine-day space-shuttle mission (Wu *et al.*, 2001), and no significant differences were observed after short-term taxi flights (Durante *et al.*, 2003). In another study, when CA frequencies were determined in 9 individuals who spent varying lengths of time in space, no correlation was found between CA and the number of flights or the duration of the astronaut's stay in space (Greco *et al.*, 2003). Despite a short exposure to simulated μ G for only 54 hours, this *in vitro* study shows an increase in the frequency of CAs in human peripheral blood lymphocytes under simulated μ G compared with 1G (Fig. 3). Under a human research program by NASA, preflight blood samples from astronauts were exposed *ex vivo* to varying doses of gamma rays, and the dose-response for CA rate and the background (i.e. base line) aberration have been determined for each astronaut. It was found that the CA rate observed in the crewmembers can be fairly well predicted by the radiosensitivity determined by the preflight *ex vivo* experiments after adjusting for the actual dose received during the mission and the base line CA levels (Feiveson *et al.* 2021). Since peripheral blood lymphocytes are used as biodosimetry for risk assessment of astronauts, there is an advantage to use these cells in ground based experiments in order to

obtain and predict biological effects of the space environment. Such data will complement the limited availability of data from astronauts.

The difference between X-ray and C-ion beam irradiation

The frequency of the complex type of CAs increased in cells irradiated by C-ion beam compared with those irradiated by X-ray with the same dose (Fig. 4). It has been reported that blood lymphocytes of astronauts after prolonged space flights show a slightly higher frequency of complex CAs perhaps due to exposure to high-LET radiation (George *et al.*, 2001). DSB is the most dangerous type of DNA damage (Shibata and Jeggo, 2014), and LET is defined as the amount of energy given to a tissue by ionizing radiation per unit length (Danzker *et al.*, 1959). X-rays are a form of low-LET radiation and mostly produce single-strand breaks (SSBs), with only a smaller number of DSBs. On the other hand, high-LET heavy-ion beams in space produce dense DSBs due to their high-energy density (Löbrich *et al.*, 1996) and have a high biological lethality. The relative biological effects of C-ion beam compared with X-ray is 2-3 times higher for human lymphoblastic TK6 cells (Yamanouchi *et al.*, 2020). It has also been suggested that heavy-ion irradiation induces complex DNA damages, called a clustered DNA damage, which is defined as the damage that includes DSBs and SSBs within one or two helical turns of DNA (Hada *et al.*, 2008; Pinto *et al.*, 2005; Rydberg *et al.*, 1998). These clustered damages have been reported to be either irreparable or likely to result in misrepair due to delayed repair (Pinto *et al.*, 2005; Shibata and Jeggo, 2014). Although the mechanism for the increased CA formation by high-LET radiation is not fully understood, clustered formation of DNA damage may play a role in inducing a higher frequency of the complex type of CAs

by C-ion beam irradiation.

The total exchanges in human peripheral blood lymphocytes compared with human lymphoblastic TK6 cells

We have earlier reported combined effects of radiation and simulated μG on TK6 cells which are stable human lymphoblastic cell line (Yamanouchi *et al.*, 2020). Suppl. 1 shows the fold increase in total number of CAs in human blood lymphocytes compared to TK6 cells. By comparing the total number of CAs in 100 human blood lymphocytes with that in 100 TK6 cells, we found that the CA frequency was lower in human lymphocytes than in TK6 cells at the baseline (i.e. without irradiation at 1G). However, when cells were irradiated, more CAs were observed in lymphocytes obtained from the peripheral blood of healthy humans than in TK6 cells with both X-ray and C-ion beam irradiation. At all doses and in both cell types, more CAs were observed at simulated μG than at 1G. Although the number of CAs in the control lymphocyte samples was less than that of TK6 cells, human blood lymphocytes are considered to be radiosensitive because they produce more CAs upon irradiation. Cell survival data are also needed to evaluate more accurately the radiation/gravity effects on different cell types.

The total exchanges in human peripheral blood lymphocytes compared with human fibroblasts 1BR-hTERT cells

We have also reported earlier on the effects of simultaneous exposure of human fibroblasts 1BR-hTERT cells to simulated μG and radiation (Hada *et al.*, 2019). Suppl. 1 shows the data from this earlier study. The comparison of data from the lymphocytes and 1BR-hTERT cells indicate that under the same irradiation doses, more CA was observed in human peripheral blood lymphocytes than in 1BR-hTERT cells with both X-ray and C-ion beam irradiation. At both doses and in both cell types, more CAs were observed at simulated μG than at 1G, and the rate of increase in CAs was not higher at simulated μG .

Suppl. 1. Total number of CAs in peripheral blood lymphocytes, TK6 cells and 1BR-hTERT cells irradiated by X-ray or C-ion beam under 1G or simulated μG condition.

Radiation	Gravity	Total number of CAs		
		Human blood lymphocytes	TK6 cells*	1BR-hTERT cells**
Control (0 Gy)	1G	0.77±0.26	2.36±0.75	0.73±0.30
	μG	1.02±0.51	3.14±0.87	1.37±0.41
X-ray (0.5 Gy)	1G	8.67±1.47	5.03±1.10	3.39±0.91
	μG	15.45±2.25	8.59±1.43	5.58±1.00
C-ion beam (0.25 Gy)	1G	12.73±1.77	6.71±1.27	
	μG	16.67±2.19	10.31±1.77	
C-ion beam (0.5 Gy)	1G	36.99±4.86	25.63±3.82	15.42±2.44
	μG	59.38±7.60	36.02±5.09	18.83±2.87

*Yamanouchi *et al.*, 2020, **Hada *et al.*, 2019.

The combined effect of simulated μG and radiation

Five out of 10 comparisons of combined effects yielded statistically significant differences ($p < 0.05$) between 1G and simulated μG (Table 1). Logistic-regression analysis was performed to investigate the effect of simulated μG on CA frequency in detail, and it was found that simulated μG made a significant contribution (Table 2). Compared with the 1G condition, 2.1 times more CAs were produced in cells irradiated with X-ray and 1.6 times more were produced in cells irradiated with C-ion beam under simulated μG , regardless of the dose. Although the odds ratios for dose are as large as 15.370 for X-ray and 535.886 for C-ion beam, the dose contribution is expected to be very small because the space-radiation dose is small.

Conclusions, limitations and future plans

Studies on astronauts' blood lymphocytes have repeatedly suggested that long-term space missions increase CAs, presumably caused by both space radiation and μG . Although they provide precious information, these studies are severely limited by a small case number. Also, quality and quantity of the radiation that astronauts are exposed during ISS missions are different from the expected radiation in the future deep space missions. For these reasons, more ground-based experimental model systems need to be developed. The system we used for the current study has been described in detail (Ikeda *et al.*, 2016, 2017) and may be one such model system because cells can be exposed simultaneously to simulated μG and radiation, albeit for a shorter period of time, and the results obtained using this system are remarkably similar to many of the findings obtained using astronauts' peripheral blood lymphocytes. Our results demonstrate the usefulness of this system for predicting certain effects of the space environment on human cells in space. Moreover, this study demonstrates that the system is amenable for testing not only adherent cells but also floating cells, and perhaps *ex vivo* tissue pieces. Using the current system, we are planning to expand the current study by collecting more samples from healthy donors and to determine whether disparities exist among individuals, ages and sex.

However, this study has a few limitations. First, the experiment was conducted at the cellular level, and applying such *in vitro* results to organismic physiology is challenging. Second, the experiments performed on the ground do not fully recapitulate biological events that takes place in the actual space environment. The Artemis Project proposed by NASA aims for human beings to inhabit on the Moon and Mars. The gravitational forces to which astronauts will be exposed in these projects are 1/6G on the Moon and 3/8G on Mars as well as μG in space while traveling. Thus, it is important to study also the effects of partial gravity. To ensure safe and low-risk living in space, it is critical to elucidate molecular mechanisms for biological effects of the space environment. Such information is necessary for developing ways to mitigate negative effects of the space environment on life. In addition to simulated μG , we have

recently developed devices for ground experiments that simulate various partial gravity (Takahashi *et al.*, 2020). In the future work, we plan to compare our chromosomal aberration findings with simulated μG to partial gravity conditions using these devices. It is also important to investigate if and how the biological system, at both the cellular and organismic levels, is affected by rapid changes in gravitational environments such as departure from Earth or returning to Earth. Studies on the effects of changing gravity from 1G to simulated μG on gene expressions and protein modifications are in progress using our devices.

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Conflicts of Interest

The authors declare no competing interests.

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