Simulated Microgravity Effects on Nonsmall Cell Lung Cancer Cell Proliferation and Migration

Jae Ho Chung; Chi Bum Ahn; Kuk Hui Son; Eunjue Yi; Ho Sung Son; Han-Sung Kim; Sung Ho Lee

BACKGROUND: Despite improvements in medical technology, lung cancer metastasis remains a global health problem. The effects of microgravity on cell morphology, structure, functions, and their mechanisms have been widely studied; however, the biological effects of simulated microgravity on the interaction between cells and its eventual influence on the characteristics of cancer cells are yet to be discovered. We examined the effects of simulated microgravity on the metastatic ability of different lung cancer cells using a random positioning machine.

METHODS: Human lung cancer cell lines of adenocarcinoma (A549) and squamous cell carcinoma (H1703) were cultured in a 3D clinostat system which was continuously rotated at 5 rpm for 36 h. The experimental and control group were cultured under identical conditions with the exception of clinorotation.

RESULTS: Simulated microgravity had different effects on each lung cancer cell line. In A549 cells, the proliferation rate of the clinostat group (2.267 $\pm$ 0.010) after exposure to microgravity did not differ from that of the control group (2.271 $\pm$ 0.020). However, in H1703 cells, the proliferation rates of the clinostat group (0.534 $\pm$ 0.021) was lower than that of the control group (1.082 $\pm$ 0.021). The migratory ability of both A549 [remnant cell-free area: 33% (clinostat) vs. 78% (control)] and H1703 cells [40% (clinostat) vs. 68% (control)] were increased after exposure to microgravity. The results of the molecular changes in biomarkers after exposure to microgravity are preliminary.

DISCUSSION: Simulated microgravity had different effects on the proliferation and migration of different lung cancer cell lines.

KEYWORDS: simulated microgravity, lung cancer cells, proliferation, migration.

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It has been observed that prolonged spaceflights cause changes in human physiology, from bone loss and muscle atrophy to body fluid and electrolyte reduction. This phenomenon has led to extensive research on the effects of microgravity on cell morphology structure, functions, and their mechanisms, including studies of various cancer cells.[11,19,21] Although many investigations regarding the effects of real and simulated microgravity on cancer cells have been carried out, the biological effects of simulated microgravity on the interaction between cells and its eventual influence on the characteristics of cancer cells are yet to be discovered.

Lung cancer, which accounts for 13% of total cancer cases and 18% of total cancer deaths worldwide, remains a major health problem.[9] Despite improvements in diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, the progressive growth of metastases is the leading cause of most cancer deaths (90%). Therefore, preventing cancer metastasis is important for improving the overall survival of cancer patients.[22]

Metastasis is known as the spread of cancer cells from a primary site to form a new colony in a distant location and it involves multiprocess mechanisms. It is mainly divided into

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two steps consisting of acquisition of migration ability in the primary site and proliferation in the second site. The metastatic potential depends on these two factors.[2,14,22] These mechanisms have been widely examined in many cancer metastasis research in order to understand the concept of cancer cell metastasis that may lead to effective therapies.

Considering the limited opportunity and resource in space, a random positioning machine known as a "clinostat" has been developed to create a "simulated microgravity" environment.[13] This machine decreases or neutralizes Earth's surface gravity by constantly changing the direction of the effective g force with respect to the sample. To avoid centrifugal effects, the clinostat rotates slowly with a constant and sufficiently small angular velocity, and the cells are placed at the center of the axes of the clinostat.[5,23] Although clinostats cannot fully imitate the space environment, "simulated microgravity" via clinostats is useful for developing strategies for stem cell therapies, tissue bio-engineering, regenerative medicine, and cancer therapies.[17]

Using the clinostat machines, the biological effects of simulated microgravity on different types of cancer cells, including lung cancer cells, have been studied. For example, according to Infanger et al., simulated microgravity resulted in an increase in the apoptosis in human follicular thyroid carcinoma cells.[7] This result agrees with those of De Chang et al., who demonstrated that simulated microgravity weakens the metastatic potential of human lung adenocarcinoma cells by inhibiting cell migration and invasion, as well as by slowing cell proliferation.[3]

However, there are distinct subtypes and cell lines within human lung cancer cells. Even nonsmall cell lung cancer, which accounts for approximately 80–85% of lung cancers, is divided into many subtypes with distinct characteristics; adenocarcinoma accounts for approximately 40% of cases and squamous cell carcinoma accounts for approximately 25–30%. These subtypes involve pathologically and functionally distinct cell types and differ in typical location of appearance, metastatic sites, glucose metabolism, genetic expression, and genetic mutations.[6,8] Adenocarcinoma and squamous-cell carcinoma cells have different prognoses and effects under the same chemotherapy treatment, indicating that various methods of invasion and immunity are used by squamous-cell carcinomas and adenocarcinomas.[1,6,24] Given the many different subtypes and different cell lines in human lung cancer cells, including both adenocarcinoma and squamous cell carcinoma cells, additional studies on the biological effects of microgravity on lung cancer cells are needed. To further understand the mechanism of cancer cell metastasis, we examined the effects of simulated microgravity on the metastatic ability of different cell lines of human lung adenocarcinoma and squamous cell carcinoma using the clinostat system to produce a microgravity environment.

METHODS

Materials

For cell culture, human lung cancer cell lines of adenocarcinoma (A549) and squamous cell carcinoma (H1703) were grown in Dulbecco's modified Eagle medium (DMEM) (ATCC-formulated RPMI-1640 medium for H1703) supplemented with 10% fetal bovine serum, 100 U · mL^{-1} of penicillin, and 100 μg · mL^{-1} streptomycin. Cultures were maintained in a humidified incubator at 37°C under a 5% CO_2 atmosphere. Each cell line was passaged routinely every 2–3 d by treatment with 0.25% trypsin containing 0.02% EDTA.

Equipment

A clinostat system (3D clinostat ver. 2, Yonsei Academy of Science, Biophysics and Medical Engineering Institute, Seoul, Korea), known as an effective system for simulating microgravity on the ground, was used in this study. To create simulated microgravity during cell culture, 4×10^5 cells were plated in a membrane cell culture dish (SPLCoat™ Dish, SPL Life Sciences Co., Gyeonggi-do, Korea). Cells were adhered to the flask and then the flask was filled with culture medium. The flask was fixed carefully to the rotating panel of the clinostat system, which was placed in a humidified incubator at 37°C and 5% CO_2 atmosphere. The clinostat was continuously rotated at 5 rpm for 36 h. The control group was cultured in the same manner as the experimental group, but without clinorotation.

Procedures

Progressive growth of neoplastic cells plays a major role in the mechanism of cancer metastasis. Therefore, to examine the effect of simulated microgravity on lung cancer cell growth, proliferation assays using the cell counting kit-8 (Enzo Life Sciences, Inc., Farmingdale, NY) were performed on the clinorotated experimental group and control group. Separate tests were performed on A549 and H1703 cells to determine the different effects of microgravity on different types of lung cancer cells. The total exposure time in microgravity for the experimental group was 36 h, and the proliferation results of each different cell were collected at 12, 24, and 36 h of exposure to clinorotation.

Technically, A549 and H1703 cells were seeded at a density of 4×10^5 cells/well with 100 μL of complete medium in 96-well plates in triplicate for 24 h. The cells were incubated with a mixed solution of DMEM (2 mL) and CCK-8 solution (200 μL) for an additional 2–3 h at 37°C. Next, the medium was completely removed and the CCK-8 containing medium was replaced with DMSO to dissolve the water-insoluble formazan salt. Absorbance was measured using the Multiskan Ascent at a reference wavelength of 450 nm (Thermo Scientific, Waltham, MA). Cell survival was determined based on the OD value.

To evaluate lung cancer cell migration, an in vitro scratch wound healing assay was performed in a 24-well plate. A549 and H1703 lung cancer cells were seeded and grown to confluence in DMEM containing 10% serum. Next, a straight scratch was made on the cell monolayer using a sterile 200-μL pipette tip. The scratched cells were immediately and gently rinsed twice with DMEM. The cells were classified into a clinostat group and control group. Scratched cells in the clinostat group were incubated in a simulated microgravity environment in a humidified incubator at 37°C under a 5% CO_2 atmosphere for 36 h. Scratched cells in the control group were incubated in the

same environment as the experimental group except without clinorotation. The wounded area in each group was photographed at 12, 24, and 36 h after incubation using a computer-connected microscope. The cell migration effect in the wound healing assay was measured by calculating the percentage of the remaining wound area to the cell-free area of the initial scratch.

Matrix metalloproteinases (MMPs) are known as key enzymes in the degradation of the extracellular matrix, which progressively affects cell migration and invasion.[12] Tissue inhibitors of metalloproteinase-1 (TIMP-1), which is a member of TIMPs, is known to suppress metastasis and act as a potential tissue biomarker for lymphatic invasion and distant metastasis of lung adenocarcinoma.[4] In order to examine the effect of microgravity on biomarkers related to cancer cell migration and invasion, we performed western blotting and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays. We examined and reported the changes in relative expression of MMP-2, MMP-9, TIMP-1, and TIMP-2.

For western blot analysis, clinorotated experimental groups and control groups of A549 and H1703 cells were harvested and lysed on ice for 20 min in RIPA buffer (1% Triton X-100, 150 mmol · L^{-1} NaCl, 10 mmol · L^{-1} Tris–HCl, pH 7.4, 1 mmol · L^{-1} EDTA, 1 mmol · L^{-1} EGTA, pH 8.0, 0.2 mmol · L^{-1} Na_3VO_4, 0.2 mmol · L^{-1} phenylmethylsulfonyl fluoride, and 0.5% NP-40) containing protease inhibitor. The lysis solution was centrifuged at 12,000 rpm for 20 min at 4°C to remove cellular debris. The supernatants were collected and the protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA). Next, 60 μg of protein were separated by 12% SDS PAGE and transferred onto polyvinylidene fluoride membranes (Roche Diagnostics Korea Co., Seoul, South Korea). Membranes were treated overnight at 4°C with primary antibodies against MMP2, MMP9, TIMP1, and β-actin. Bands were detected using goat-antirabbit IRDye800 secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). Membranes were incubated for 2 h in a horseradish peroxidase-conjugated secondary antibody (1:10,000 diluted); target bands were detected with the enhanced chemiluminescence detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions. β-actin was used as the loading control. For the real-time quantitative RT-PCR assays, total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and the concentration was determined using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE). Reverse transcription was performed using the iScript™ cDNA synthesis kit (Bio-Rad) with 2 μg of total RNA in a 50-μL reaction volume. For RT-PCR amplification, a predenaturation step was performed at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min. After completion of the cycling steps, another extension was carried out at 72°C for 5 min and the mixture was stored at 4°C. Quantitative real-time PCR was performed with SsoFast EvaGreen supermix using the iQ5 Real-Time PCR Detection System (Bio-Rad). The β-actin, which was shown to be unaffected by exposure to the random positioning machine, was used as an internal control for normalization.[18,20] Each reaction was performed in duplicate in two independent experiments. The primer sequences used for MMP-2, MMP-9, TIMP-1, TIMP-2, and β-actin were based on an RT-PCR technique that was shown to be highly reproducible in previous studies (**Table I**).[15]

Statistical Analysis

All data were entered into an Excel spreadsheet (Microsoft, Seattle, WA). Data were analyzed using SPSS Statistics Version 19 (IBM, Armonk, NY). Univariate data analyses, including independent samples *t*-tests, were used for quantitative variables. Data are reported as the mean ± SD. A value of $P < 0.05$ was considered statistically significant.

RESULTS

For the proliferation assay, the influence of simulated microgravity on the proliferation rates of A549 cells and H1703 cells differed as shown in **Fig. 1A** and **1B**. In A549 cells, the mean proliferation rates of the clinostat group and control group after 12, 24, and 36 h of exposure to simulated microgravity and normal gravity were 2.085 ± 0.018 vs. 2.122 ± 0.035, 2.383 ± 0.040 vs. 2.444 ± 0.029, and 2.267 ± 0.010 vs. 2.271 ± 0.020, respectively. The mean proliferation rate of the clinostat group (2.267 ± 0.010) after 36 h of exposure to simulated microgravity did not differ from the control group [2.271 ± 0.020, $t(2) = -0.256$, $P = 0.822$]. In contrast, the proliferation rates of H1703 cells after 36 h of exposure to simulated microgravity differed in the clinostat group and control group. The mean proliferation rates of the clinostat and control group after 12, 24, and 36 h of exposure to simulated microgravity were 0.477 ± 0.023 vs. 0.541 ± 0.021, 0.462 ± 0.001 vs. 0.939 ± 0.015, and 0.534 ± 0.021 vs. 1.082 ± 0.021, respectively. The proliferation rates of the clinostat group (0.534 ± 0.021) were significantly lower than those of the control group [1.082 ± 0.021, $t(2) = 26.724$, $P = 0.001$].

To evaluate the effect of simulated microgravity on the migration ability of lung cancer cells, an in vitro scratch wound

Table I. Primers Used for RT-PCR and Quantitative PCR.

MRNA	SENSE PRIMER	ANTISENSE PRIMER	PCR PRODUCT (BP)
MMP-2	5′-GGCTGGTCAGTGGCTTGGGGTA-3′	5′-AGATCTTCTTCTTCAAGGACCGGTT-3′	225
MMP-9	5′-GCGGAGATTGGGAACCAGCTGTA-3′	5′-GACGCGCCTGTGTACACCCACA-3′	208
TIMP-2	5′-CTCGGCAGTGTGTGGGGTC-3′	5′-CGAGAAACTCCTGCTTGGGG-3′	364
TIMP-1	5′-ACCATGGCCCCCTTTGAGCCCCTG-3′	5′-TCAGGCTATCTGGGACCGCAGGGA-3′	627
β-actin	5′-TGCTATCCAGGCTGTGCTA-3′	5′-ATGGAGTTGAAGGTAGTTT-3′	443

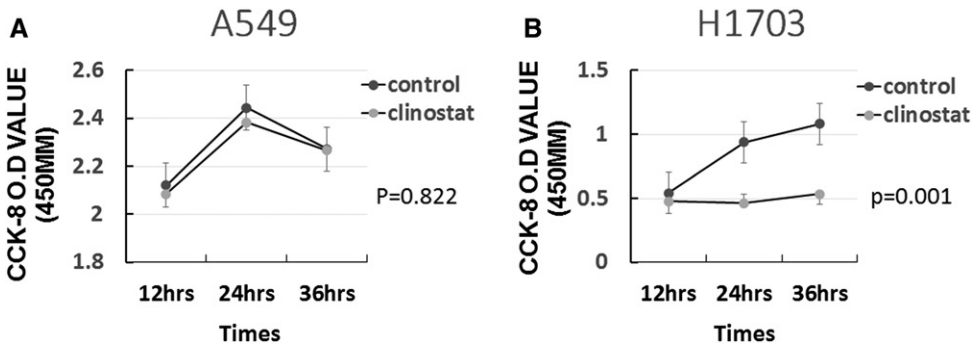

Fig. 1. Results of CCK-8 cell proliferation assay with A) A549 and B) H1703 cells. The x-axis represents the time of exposure in simulated microgravity and normal gravity. The y-axis represents the cell density measured by the methyl thiazolyl tetrazolium (MTT) labeling reagent absorbance at 450 nm.

healing assay was performed. Next, both A549 and H1703 cells were exposed to simulated microgravity for 36 h. As shown in **Fig. 2A** and **2B**, A549 cells exposed to simulated microgravity for 36 h migrated more quickly to the cell-free wound areas than those of the normal control group. The unhealed area of the clinostat group after 36 h of exposure to simulated microgravity was less than 33% of the wound area, while the unhealed area of the control group after 36 h in normal gravity was approximately 78% of the wound area [$t(3.714) = -2.374$, $P = 0.08$]. The results for H1703 cells were similar to those of A549 cells. At 36 h after scratching and exposure to simulated micro- and normal gravity, the mean percentage of the remnant cell-free area of the clinostat group (40%) was significantly lower than that of the control group [68%, $t(2) = -10.021$, $P = 0.01$]. Thus, the migration rate of the clinostat group was faster than that of the normal control group.

In the real-time PCR assay of A549 cells, the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 from cells in the clinostat (experimental group) were different after 36 h of exposure to simulated microgravity compared to those of the control group under normal gravity. As shown in **Fig. 3A**, the relative expression of MMP-2 [1.034 ± 0.374 vs. 24.722 ± 3.182, $t(2) = 10.454$, $P = 0.009$], MMP-9 [1.049 ± 0.448 vs. 30.061 ± 24.486, $t(2) = 1.675$, $P = 0.236$], TIMP-1 [1.008 ± 0.183 vs. 36.443 ± 7.926, $t(2) = 6.320$, $P = 0.024$], and TIMP-2 [1.003 ± 0.110 vs. 22.154 ± 3.619, $t(2) = 8.256$, $P = 0.014$] after 36 h of exposure to simulated microgravity were lower in the clinostat group compared to the control group. These results indicate that the expression of cell migration and invasion markers at the molecular mRNA level was relatively inhibited in A549 cells by simulated microgravity.

To analyze the effect of simulated microgravity on the expression levels of MMP-2, MMP-9, and TIMP-1 at the protein level, western blotting of A549 cells was also performed (**Fig. 4A**). MMP-9 and TIMP-1 could not be detected in the western blot assay of A549 cells. MMP-2 expressions in both the clinostat and the control groups were decreased after 36 h. When comparing the clinostat and the control groups, MMP-2 expression in the clinostat group (0.30) was slightly lower than or similar to that in the controls (0.67).

Both PCR and western blotting of A549 cells showed decreased expressions of migration and invasion biomarkers. In the real-time quantitative PCR assay of H1703 cells, the relative expression of MMP-2 [1.005 ± 0.134 vs. 1.979 ± 0.146, $t(2) = 6.938$, $P = 0.02$] and MMP-9 [1.000 ± 0.032 vs. 2.450 ± 0.075, $t(2) = 25.174$, $P = 0.002$] were lower in the clinostat group after 36 h of exposure to simulated microgravity compared to the control group. The relative expressions of TIMP-1 [1.176 ± 0.876 vs. 5.067 ± 3.930, $t(2) = 1.366$, $P = 0.305$] and TIMP-2 [1.001 ± 0.070 vs. 1.891 ± 0.904, $t(2) = 1.388$, $P = 0.3$] were also lower in the clinostat group compared to the control group, but the difference was not significant. However, the decrease in the expression of TIMP-1 in the control group was relatively greater. In conclusion, the expression of all four biomarkers in the clinostat group was lower than in the control group after 36 h of clinorotation (**Fig. 3B**). These results indicate that the expression of

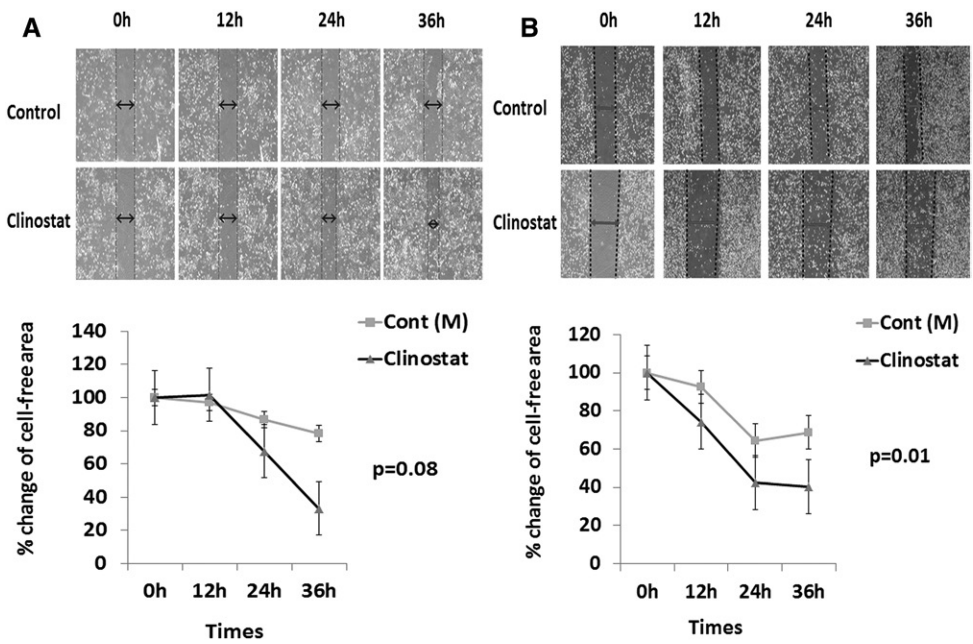

Fig. 2. Photographs of A) A549 cells and B) H1703 cells from the in vitro scratch wound healing assay (upper panels). Graphs of percent change in cell-free area were calculated from cell counts. Data are reported as mean ± SD (lower panels).

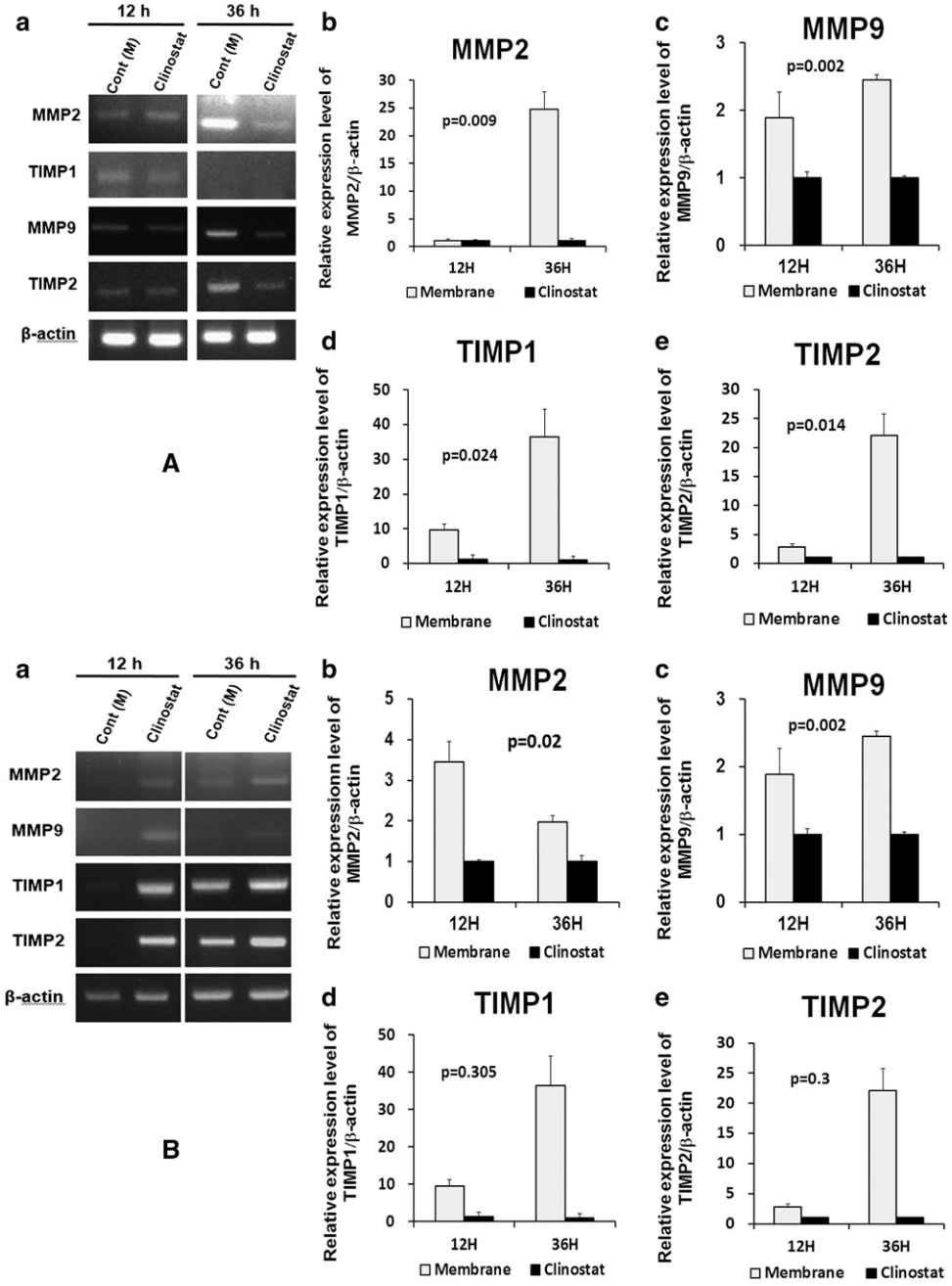

Fig. 3. Quantitative PCR results showing the relative mRNA expressions in A) A549 cells and B) H1703 cells. a) Results of quantitative PCR shown as photography of gels. The quantified relative gene expression levels of b) MMP-2, c) MMP-9, d) TIMP-1, and e) TIMP-2 after 36 h of exposure to simulated microgravity are shown. β-Actin mRNA expression from the same samples was measured as a loading control. Gene expression levels were normalized to the β-actin mRNA expression level. The x-axis represents exposure time in simulated microgravity and normal gravity. The y-axis represents the changes (-fold) in relative gene expression levels.

cell migration and invasion biomarkers at the molecular mRNA level is inhibited in H1703 cells after exposure to simulated microgravity.

In the western blot assay of H1703 cells, the expression levels of MMP-2, MMP-9, and TIMP-1 increased until 24 h of simulated microgravity exposure, but decreased after 36 h. When comparing the clinostat and control groups, the expression of MMP-2 (0.12 vs. 0.50), MMP-9 (0.04 vs. 0.43), and TIMP-1 (0.80 vs. 1.35) in the clinostat group was lower than that in the control group after 36 h of clinorotation (**Fig. 4B**). Finally, reduced expression of migration and invasion biomarkers was noted after the exposure of lung cancer cells to the simulated microgravity environment.

DISCUSSION

Several studies of the biological effects of clinostat-simulated microgravity on different types of cancer cells, including lung cancer cells, have been conducted. Simulated microgravity was found to alter the biological characteristics of cancer cells by decreasing their proliferation, invasion, migration, adhesion, and MMP-2 production abilities and increasing their apoptosis rate. These factors weakened the metastatic potential of various cancer cells.[3,10,16] Although the simulated microgravity created by the clinostat system may not have perfectly reproduced the zero gravity of real space, it was sufficient for increasing our understanding of cell physiology, including cell proliferation, invasion, adhesion, cell cycle, structure, and functions.[17,20,23] However, the simulated microgravity environment affects different cells in different manners and differentially affects specific stages of cell growth and processes; thus, additional studies are needed to fully clarify the potential effects on cells.

In this study, we focused on lung cancer cells, which are still one of the leading causes of cancer-related death worldwide. Previously, Chang et al.[3] have reported that simulated microgravity reduced the metastatic potential of human lung adenocarcinoma cells by altering the expression of MKI67 and MMP-2, thereby inhibiting cell proliferation, migration, and invasion. However, even nonsmall cell lung cancer consists of many other different types of cells, including squamous cell carcinoma. Furthermore, different clinical features of adenocarcinoma and squamous cell carcinoma and different effects and prognoses under the same chemotherapy indicate that there may be various distinct

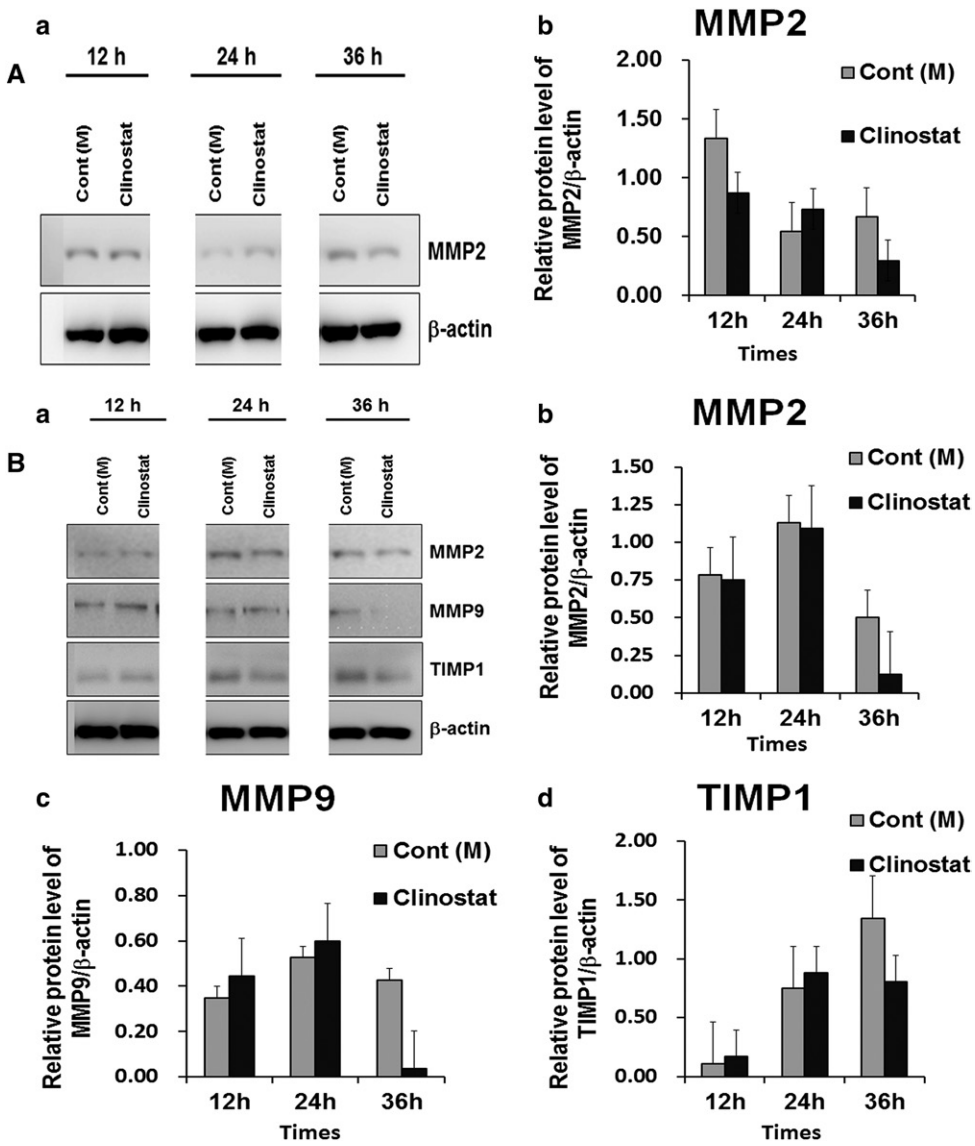

Fig. 4. Western blot analysis results showing the relative protein expressions in A) A549 cells and B) H1703 cells. a) The photographs show the gel image of the proteins analyzed by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes prior to immunoblotting. The relative protein expression levels of MMP-2, MMP-9, and TIMP-1 after 12, 24, and 36 h of exposure to simulated microgravity are shown. The graphs [b) MMP-2, c) MMP-9, d) TIMP-1] compare the scanning signal intensity of the proteins by ImageJ software (NIH, Bethesda, MD).

methods of invasion and immunity between adenocarcinoma and squamous-cell carcinoma.[1,8,24] Hence, studies focusing on adenocarcinoma would not be sufficient for improving the understanding of the effect of simulated microgravity on lung cancer cells. We therefore hypothesized that the simulated microgravity environment elicits different responses in two different cells and focused on both adenocarcinoma and squamous cell carcinoma. We evaluated the A549 and H1703 cell lines, which are commonly used in lung cancer cell studies.

In contrast to previous studies, the cells were evaluated at different time points to measure the effect of simulated microgravity exposure. While previous studies measured the changes in proliferation and migration of cells by comparing the cells that were previously exposed to simulated microgravity to the control group, we measured the effects of simulated microgravity on cells during exposure to simulated microgravity. We used this method to predict the actual changes occurring in cells while they are in space, and not after they have returned from space. Because the cells were in clinorotation, the artifacts that may have been caused by kinematic rotation such as centrifugal acceleration, tangential acceleration, shear forces, and convection due to fluid motion in the culture flask were increased.[13,23] In order to minimize these effects, we rotated the clinostat machine adequately slowly (5 rpm) at a constant angular velocity, and the culture dishes were placed at a moderate distance from the center of rotation based on values found in previous studies.[18,23] In addition, to obtain proliferation and migration results during clinorotation, we must also take into account that artifacts may have been caused by kinematic rotation and may have increased when using free-floating nonadherent cells. Additionally, although the effects of simulated microgravity on nonadherent cells may be greater because of free-fall in fast rotating systems, we slowly rotated the 3D clinostat random positioning machine and the gravity vector was nullified by the continuously and randomly changed direction and not by the free-fall during rapid rotation. Therefore, we used A549 and H1703 cells which adhere to the surface of the culture flasks rather than free-floating nonadherent cells.

Our results differed from those of previous studies. According to recent reports, the simulated microgravity environment showed a tendency to inhibit the cell proliferation, invasion, and migration abilities, and increase the apoptosis rate of cancer cells.[3,7,16] However, our results showed that the proliferation rates of A549 cells were not clearly influenced by the simulated microgravity environment, while the proliferation rates of H1703 cells exposed to simulated microgravity were reduced compared to the control group. The results of our study indicate that A549 cells are affected differently by simulated microgravity according to the time period of gravity exposure and collection of results. Thus, the proliferation ability of A549 cells may have decreased after exposure to simulated microgravity, but

may not be affected directly by the simulated microgravity during clinorotation. Unlike A549 cells, the proliferation ability of H1703 cells was clearly influenced by the simulated microgravity during clinorotation. As shown in Fig. 2B, the proliferation rate of H1703 cells was reduced by the simulated microgravity, which is similar to the results in other cancer cells. Further studies evaluating the changes in proliferation rates during and after exposure to the microgravity environment are needed to clarify the effects of microgravity on human lung cancer cells.

The results of cell migration under simulated microgravity also differed from the results of previous studies. The results of the scratch wound healing assay revealed that the wound area decreased faster in the clinostat group than in the normal control group. The results for A549 and H1703 cells were similar. While the change in the migration ability of A549 after exposure to simulated microgravity was nearly statistically significant, the results for H1703 were statistically significant. Thus, there was a notable increase in migration when A549 and H1703 cells were exposed to simulated microgravity compared to the control group.

Because cell migration was evaluated during clinorotation, there were some limitations to our study. First, confounding factors in the study results must be taken into account. Although we attempted to minimize such factors, artifacts caused by kinematic rotation such as centrifugal acceleration, tangential acceleration, shear forces, convection due to fluid motion in the culture flask, and lack of culture movement in the control groups may have affected the results. Second, Transwell migration and invasion assays could not be performed during clinorotation because continuous rotation may affect cell diffusion through the Transwell.

In addition, the results of PCR and western blotting of both A549 and H1703, which showed decreased relative expressions of MMP-2, MMP-9, and TIMP-1, also contradict the increased migration of the clinostat group observed in the wound healing assay. The PCR and western blot assay were expected to show an increase in MMPs together with increased cell migration. MMPs are a prominent family of proteinases associated with tumorigenesis and are known to play a crucial role in various physiological processes, including tissue remodeling and organ development, regulation of inflammatory processes, and diseases such as cancer. However, in addition to their known roles in extracellular matrix turnover and cancer cell migration by modulating the proteolysis of extracellular matrix components, recent studies of MMPs indicated that they also regulate signaling pathways controlling cell growth, inflammation, or angiogenesis, and that they may even function in a nonproteolytic manner. Moreover, the failure of MMP inhibitors as targets for anticancer therapy in clinical trials showed that depending on the circumstances, MMPs can either suppress or promote tumorigenesis, as well as act independently of their proteolytic activity.[12] TIMPs, which are endogenous inhibitors of MMPs, are thought to suppress cancer metastasis. However, clinical results suggest a controversial role for TIMPs in cancer progression. Recent studies showed that TIMPs carry out several MMP-independent functions that facilitate cancer cell progression. For example, TIMP-1 may inhibit apoptosis by stimulating the Akt, FAK/PIK-3, or MAPK signaling pathway and regulating cell proliferation. Chang et al. reported that the expression levels of TIMP-1 were significantly increased in patients with lymph node and distant metastasis. They also reported that the downregulation of TIMP-1 inhibits cell migration, invasion, and metastatic colonization in lung adenocarcinoma.[4]

The difference in our results between cellular and molecular biological studies indicate that the multiple functions of MMP and TIMPs in the tumor microenvironment may have been affected by the simulated microgravity, which eventually may have led to different results in the levels of cell expression and migratory and invasive abilities. Therefore, additional studies of the effects of simulated microgravity on cancer cells are needed.

This study focused on changes in two main cell types of non-small cell lung cancer and is the first study to measure these cell changes during clinorotation. Our future studies will further explore cellular changes in the microgravity environment and the mechanism of cancer cell metastasis.

In summary, simulated microgravity elicits different responses in different cell lines of lung cancer cells. The simulated microgravity environment did not significantly influence the cell proliferation of the A549 cell line of adenocarcinoma, but inhibited proliferation of the H1703 cell line of squamous cell carcinoma. Although the migratory ability of both the A549 and H1703 cell lines were increased after exposure to simulated microgravity compared to the control group under normal gravity, the molecular changes in biomarkers after exposure to simulated microgravity appear to be preliminary. Therefore, simulated microgravity may have different effects on different cell lines of lung cancer. Further studies of cell proliferation and migration in a microgravity environment involving other different subtypes of lung cancer cells may improve the understanding of the effect of simulated microgravity on lung cancer cells.

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Jae Ho Chung and Chi Bum Ahn contributed equally to this work.

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