

Hypergravity Induces Phosphorylation of p53 at Serine 15, but not an Expression of p53-downstream Genes

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DNA microarray/Hypergravity/p53/Phosphorylation/Signal transduction

We investigated the p53 signaling pathway induced by hypergravity in the human glioblastoma cell line A172. Hypergravity ($20 \times g$) induced the accumulation of p53 and the phosphorylation of p53 at Ser-15. The phosphorylation of p53 with hypergravity was not inhibited by wortmannin, the PI3-kinase inhibitor. This indicated that the p53 signal pathway induced by hypergravity is different from other p53 signal pathways, such as that of the DNA damage signal. Hypergravity did not induce an expression of the genes Waf-1 or Bax, located downstream from p53. We also examined the expression of genes with hypergravity by using a DNA microarray containing oligo DNA from 30,000 human genes. Hypergravity ($20 \times g$, 6 h) did induce the expression of some genes concerned with the cell signaling pathway and cytoskeleton of the cell, but not any of the p53-downstream genes. DNA microarray revealed the induction of many genes to enable the cells to adapt to the hypergravity environment.

INTRODUCTION

Hypergravity causes considerable changes in cells. Cogoli *et al.*¹⁾ reported that it stimulated the proliferation of human lymphocytes. Hypergravity enhanced the expression of *c-myc*²⁾, *c-fos*, and *erg-1* and the activity of protein kinase C³⁾ in human and mouse cells. These genes and protein kinase C are concerned with cell growth and differentiation. We wondered whether hypergravity affects the accumulation of p53 and the expression of p53-dependent genes. The protein of p53 controls cell growth as well as cell death. The signal transduction of p53 is very sensitive, not only to various forms of radiation, such as X-rays or heavy-ions⁴⁾, but also to various stresses, such as hypoxia, heat shock, low pH, and osmotic shock⁵⁾. As a transcription factor, p53 causes a halt in the progression of the cell cycle by regulating certain gene products, including Waf-1, which has been identified as a potent inhibitor of several cyclin-Cdk complexes⁶⁾. It is also capable of inducing apoptosis by regulating gene products such as Bax, which plays an important role in this response⁷⁾. Nomura *et al.*⁸⁾ reported that microgravity and/or gravity-changing stress by free-fall accumulated p53 and induced apoptosis in human cells.

We had previously examined the effect of hypergravity on

the human glioblastoma cell line A172 and revealed that hypergravity suppressed the signal transduction of ionizing radiation through p53⁹⁾. Hypergravity at $20 \times g$ induced p53 accumulation with 3 h of incubation. After irradiation at 0.5 Gy and incubation under conditions of hypergravity ($20 \times g$), the peak of p53 accumulation shifted from 1.5 h to 3 h, and the induction of Waf-1 and Bax expression was suppressed entirely⁹⁾.

In this study, we examined the effect of hypergravity on p53 phosphorylation and the induction of gene expression in a human glioblastoma cell line, A172.

MATERIALS AND METHODS

Cell Culture

Human glioblastoma A172 cells (provided by JCRB, Tokyo, Japan), bearing the wild-type p53 gene, and GM638, the SV40-immortalized human fibroblast cell line¹⁰⁾, were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum.

Centrifugal Hypergravity

Cells in tissue culture flasks (25 cm², Iwaki Glass, Tokyo, Japan) were centrifuged in a swing-type centrifuge designed for cultured cells (Kawasaki Heavy Industries, Inc., Kobe, Japan) in an incubator at 37°C.

Irradiation

Cells in the growth phase were irradiated with a 200 kV X-ray source (Toshiba X-ray machine, Tokyo, Japan) at a dose rate of 0.308 Gy/min, at room temperature.

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Western Blot Analysis

The cells were washed once with PBS, and the total protein was extracted. Thirty micrograms of protein were subjected to Western blotting analysis. After electrophoresis on a polyacrylamide gel containing 0.1% sodium dodecyl sulfate, the proteins were electrophoretically transferred to a nitrocellulose membrane. They were then incubated for 1 h with specific monoclonal antibodies against human p53 (Anti-Human p53 Oncoprotein, Upstate Biotechnology, Inc., Lake Placid, NY; or Phospho-p53 antibody, Cell Signaling Technology, Inc., Beverly, MA); human p21 (Purified mouse antihuman Sdi1 monoclonal antibody, Pharmingen, San Diego, CA); and human Bax (N21, Santa Cruz Biotechnology Inc., CA). Other details of the Western blotting procedure have been described previously¹¹. Protein levels were analyzed by the use of an enhanced chemiluminescence system (ECL) (Amersham, Piscataway, NJ). The band densities were measured with a Macintosh computer with the IPLab Spectrum and IPLab Gel program (Scanalytics, Inc., Vienna, VA).

DNA microarray

We extracted total RNA by using RNeasy (Qiagen,

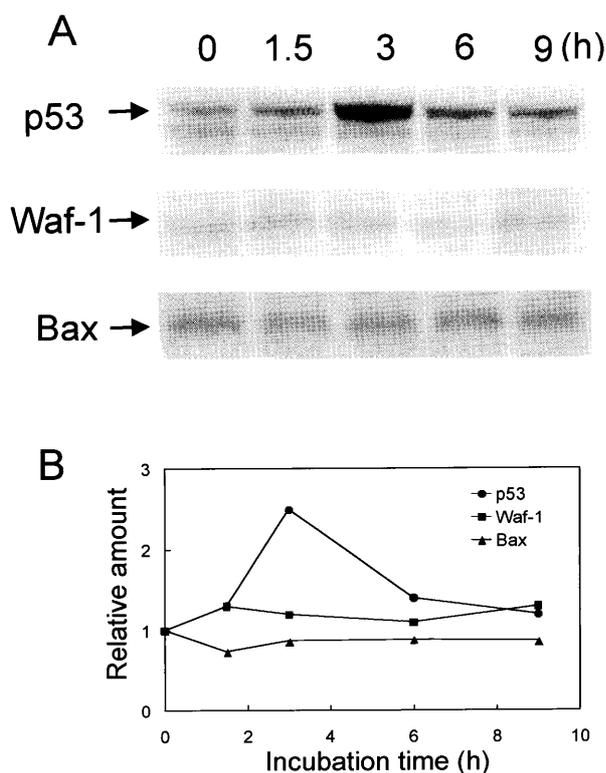


Fig. 1. Expression levels of p53, Waf-1, and Bax under conditions of hypergravity. A172 cells were centrifuged at $20 \times g$ and incubated at 37°C . (A) The expression levels of p53, Waf-1, and Bax were detected by Western blot analysis. (B) The relative amounts of each protein were shown in a graph.

Hilden, Germany). The labeled cDNA targets were made from 20 μg of total RNA. An oligo(dT) (1 μg , 12–18-mer) primer was added to the RNA, heated at 70°C for 10 min and cooled on ice. A reaction cocktail containing 3 μl of 0.1 M DTT, 6 μl of 5 \times First Strand Buffer (Gibco BRL, NY), 3 μl of dNTPs (5 mM each dATP, dCTP and dGTP, 3 mM dTTP and 2 mM amino-allyl-dUTP (Amicon, Beverly, MA)), 0.5 ml of RNase Inhibitor (30 units/ μl), and 2 μl of SuperscriptII (200 units/ μl , Gibco BRL, NY) was added, and the mixture was incubated at 42°C for 2 h to generate amino-allyl-labeled cDNA targets. The starting RNA was then alkali-degraded by adding 5 μl of stop solution (0.5 N NaOH, 50 mM EDTA), followed by incubation at 70°C for 20 min. Samples were neutralized by 1N HCl. The amino-allyl-labeled cDNA was purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The amino-allyl-labeled cDNA was then dissolved in 9 μl of 0.2 M sodium bicarbonate buffer (pH 9.0). Following the addition of 1 μl of Cy-Dye (Amersham Biosciences, Piscataway, NJ) dis-

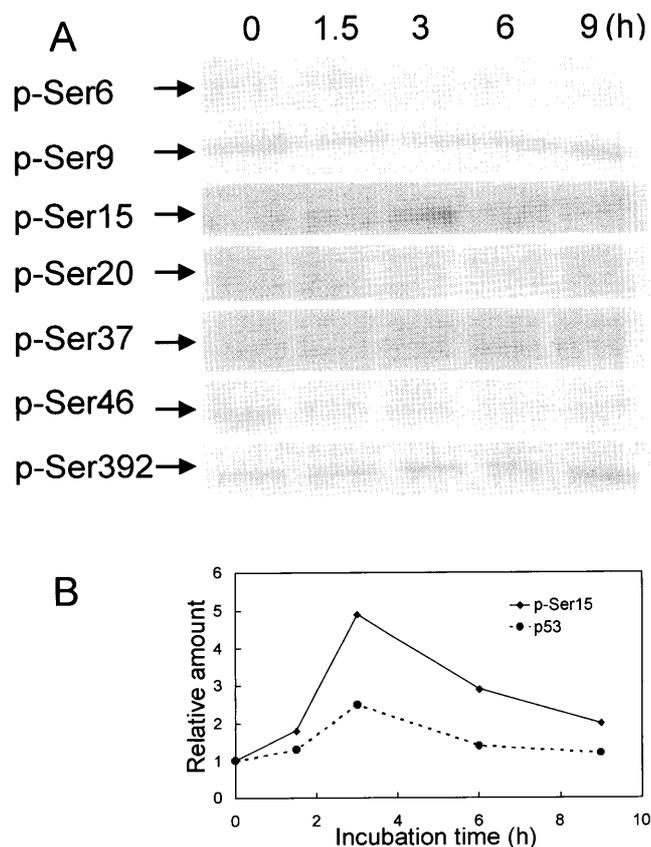


Fig. 2. Induction of p53 phosphorylation under conditions of hypergravity. A172 cells were centrifuged at $20 \times g$ and incubated at 37°C . (A) The amount of p53 phosphorylation at each site (Ser-6, Ser-9, Ser-15, Ser-20, Ser-37, Ser-46, and Ser-392) was detected by Western blot analysis. (B) The relative amount of phosphorylated Ser-15 was measured and compared with the relative amount of p53.

solved in 45 μ l DMSO, the reaction mixture was incubated at 40°C for 1 hr. Subsequently, 30 ml of RNase free water was added to the mixture and purified by P30 micro BIO spin column (BIO Rad, CA). The resulting sample of Cy-Dye-coupled and amino allyl-labeled cDNA (Cy-Dye labeled cDNA) was then concentrated by removing liquid with a Microcon-30 centrifugal concentration tube (Amicon, Beverlyon, MA).

Hybridization was performed by applying 50 μ l of a solution containing the purified Cy3- and Cy5-labeled cDNA targets, 5 \times SSC, 0.5% SDS, 4 \times Denhardt's solution, 10% formamide, 0.1 mg/ μ l salmon sperm DNA, and hybridization solution to an AceGene (Hitachi Software Engineering, Japan), covering it with a cover slip and incubating it at 42°C for 16 h in a humid chamber. The AceGene was washed for 5 min at 30°C in 2 \times SSC/0.1% SDS. A similar procedure was repeated with further washing solutions in the following order: 2 \times SSC, 1 \times SSC. The AceGene was then rinsed with 0.01 \times SSC at room temperature. After it was dried by air-spray, the fluorescence intensities were scanned with a ScanArray (PerkinElmer Life Sciences, Norwalk, CT) and analyzed by the use of QuantArray (PerkinElmer Life Sciences, Norwalk, CT).

The QuantArray data copied to the Excel sheet was treated on the following condition. The average value of the blank spots was regarded as the background (BG). The cut-off value was 1,000 < (Cy3 intensity - BG) + (Cy5 intensity - BG), and the global normalization method was applied. The ratios of Cy5 and Cy3 were then calculated. A ratio of more than 2 was considered significant.

RESULTS AND DISCUSSION

We examined whether hypergravity induces an expression of Waf-1 or Bax, located downstream from p53. A172 cells

were centrifuged at 37°C. Hypergravity at 20 \times g induced p53 accumulation after 3 h of centrifugation, but did not induce Waf-1 and Bax expression until 9 h (Fig. 1A). The density of the bands was measured by densitometer and showed in the relative amount of each protein (Fig. 1B). The relative amount of p53 reached 2.5 times after 3 h of centrifugation. But Waf-1 induced only 1.3 times after 9 h, and Bax did not induce until 9 h.

We then examined the amount of p53 phosphorylation at various phosphorylation sites of serine (Ser-6, Ser-9, Ser-15, Ser-20, Ser-37, Ser-46, and Ser-392). The only site of Ser-15 underwent phosphorylation from 3 h until 9 h under the condition of hypergravity (Fig. 2A). The relative amount of

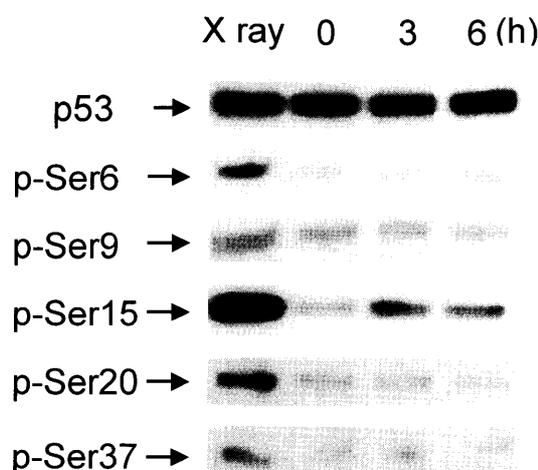


Fig. 3. Phosphorylation of p53 in GM638 cells by hypergravity or X-ray. SV-40 immortalized GM638 cells were centrifuged at 20 \times g and incubated at 37°C. For control, the cells were irradiated with 4 Gy X-rays and incubated for 2 h. The amount of p53 phosphorylation at each site (Ser-6, Ser-9, Ser-15, Ser-20, and Ser-37) was detected by Western blot analysis.

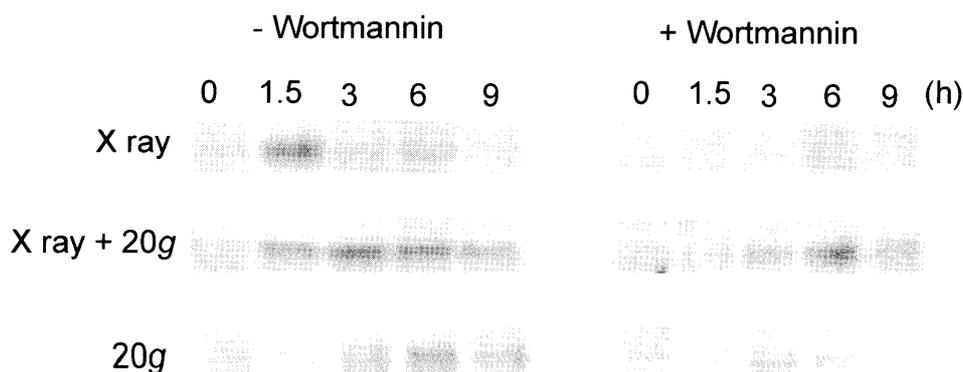


Fig. 4. Inhibition of phosphorylation of p53 at Ser-15 by wortmannin. X ray: A172 cells were exposed to 0.5 Gy of X-ray radiation and incubated with or without 50 μ M wortmannin. X ray + 20 g: A172 cells were exposed to 0.5 Gy of X-ray radiation and incubated under conditions of hypergravity (20 \times g) with or without 50 mM wortmannin. 20 g: A172 cells were incubated under conditions of hypergravity (20 \times g) with or without 50 mM wortmannin. The amount of p53 phosphorylation at Ser-15 was detected by Western blot analysis.

phosphorylated p53 at Ser-15 reached 4.9 times after 3 h of centrifugation (Fig. 2B). The ratio of the amount of phosphorylated p53 at Ser-15 compared with the total amount of p53 was about twice at 3 h. This result indicated that the increase of phosphorylation at Ser-15 was not only due to the accumulation of p53.

To confirm the induction of phosphorylation at Ser-15 by hypergravity, we used SV40-immortalized GM638 cells, which contain a large amount of p53. Similar results were observed in GM638 cells (Fig. 3). Although 4 Gy of X-ray induced phosphorylation of p53 at Ser-6, Ser-9, Ser-15, Ser-20, and Ser-37, increased phosphorylation of these serines was not observed with hypergravity, except of phosphorylation at Ser-15.

We focused on the type of kinase that might contribute to the phosphorylation of p53 at Ser-15 with hypergravity. In the case of irradiation with ionizing radiation, p53 is phosphorylated at Ser-15 by the ATM family. This phosphorylation is inhibited by wortmannin, a PI3-kinase inhibitor. Thus we next examined the inhibition of phosphorylation at Ser-15 by wortmannin under conditions of hypergravity.

Figure 4 shows that wortmannin at 50 μ M completely inhibited the phosphorylation at Ser-15 induced at 1.5 h by X-ray irradiation at 0.5 Gy (upper panel). On the other hand, Ser-15 phosphorylation with hypergravity was not inhibited by wortmannin (lower panel). When the cells were irradiated with X-rays and incubated under conditions of hypergravity, the peak in Ser-15 phosphorylation occurred from 1.5 h until 9 h after irradiation. It is possible that the Ser-15 phosphorylation peak at 1.5 h corresponds with the effects of the X-ray irradiation and that the phosphorylation after 3 h is due to the hypergravity. Wortmannin inhibited Ser-15 phosphorylation at 1.5 h, but not after 3 h (middle panel). This indicated that wortmannin inhibited only the phosphorylation induced by the X-rays, and not by hypergravity. From these data we concluded that the protein kinase, which phosphorylates p53 at Ser-15 under conditions of hypergravity, was not part of the ATM family.

Hypergravity induced the accumulation of p53 and the phosphorylation of p53 at Ser-15, but it did not induce the expression of gene products downstream from p53, such as Waf-1 or Bax. We then examined the expression of genes induced by hypergravity at 6 h by the use of DNA microarray, AceGene. This DNA microarray contained oligo DNA from 30,000 human genes, including the p53 target genes. The induction ratio of the genes under a condition of hypergravity (Cy3) compared with the control (Cy5) was shown as Cy3/Cy5 in Table 1. DNA microarray revealed that hypergravity failed to induce not only the expression of Waf-1 (1.09-fold) and Bax (1.26-fold), but also that of other p53 target genes. MDM2 induced to 1.84-fold and Bcl2 reduced to 0.84-fold by hypergravity, but they were not significant.

Table 2 shows the genes that were expressed at levels more than twice ratio of Cy3/Cy5. Hypergravity did induce

Table 1. Results of the expression of p53 target genes under hypergravity (20 \times g, 6 h) detected by DNA microarray.

No.	Gene name	Cy3/Cy5
1	Waf-1	1.09
2	Bax	1.26
3	MDM-2	1.84
4	Cyclin G	1.43
5	GADD45	0.99
6	Bcl2	0.84
7	p53	1.26

Table 2. List of the induced genes under hypergravity (20 \times g, 6 h) detected by DNA microarray.

No.	Gene name	Cy3/Cy5
1	fms-related tyrosine kinase	3.22
2	inhibitor of DNA binding 1	3.11
3	SWI/SNF related, subfamily a, member 2	3.08
4	sprouty 2	2.90
5	mannosidase, alpha, class 1A, member 2	2.55
6	solute carrier family 23	2.44
7	glutamate dehydrogenase 1	2.42
8	plectin 1	2.38
9	protein kinase, cAMP-dependent, catalytic, gamma	2.28
10	ubiquitin-activating enzyme E1	2.27
11	molybdenum cofactor synthesis 1	2.22
12	coronin, actin binding protein, 2A	2.21
13	integrin, alpha 7	2.17
14	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	2.08

the expression of some genes concerned with the cell signaling pathway and structure of cells. The fms-related tyrosine kinase 1 (Flt1), a receptor for vascular endothelial growth factor, is a transmembrane receptor in the tyrosine kinase family. Flt1 controls cell proliferation and plays a role in the tyrosine kinase signaling pathway¹². Sprouty 2 provides a control mechanism for the ras/MAPK signaling pathway¹³. These genes are concerned with signaling pathways.

The inhibitor of DNA binding 1 (Id1) is induced by bone morphogenetic protein 2 (BMP-2) and inhibits myogenesis¹³. Plectin is a cytoskeleton-associated protein that is abundantly expressed in a wide variety of tissue and cell types^{15,16}. The integrin family has at least 22 members: α 1 β 1, α 2 β 1, α 3 β 1, α 6 β 1, α 7 β 1, α v β 3, α 6 β 4, etc. The subunit of α 7 associated with β 1 is present in muscle tissue. It has been suggested that α 7 β 1 integrin directs the motility

of satellite cell-derived myoblasts along laminin-rich basement membranes during skeletal muscle regeneration¹⁷⁾. These genes are concerned with the morphology and structure of cells.

Kumei *et al.*²⁾ reported that hypergravity stimulates the proliferation of HeLa cells and enhanced *c-myc* gene expression after exposure to $35 \times g$ for 2 h. Nose *et al.*³⁾ reported that hypergravity transiently stimulated cultured mouse osteoblastic cells (MC3T3-E1) to induce genes such as *c-fos*, *egr-1* and protein kinase C exposed to more than $90 \times g$, $50 \times g$, and $900 \times g$, respectively. We could not detect an induction of the genes they reported. This may be because we used different cells and conditions.

We conclude that with hypergravity ($20 \times g$), the p53 signal pathway differs from other p53 signal pathways, such as that of the DNA damage signal, and does not induce an expression of genes downstream of p53, but it may induce the expression of other genes in the human glioblastoma cell line A172 as a means of adaptation to this environment.

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