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Effects of Hyperbaric Pressure on Cellular Morphology, Proliferation and Protein Expression of Jurkat Cell

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Abstract

The application of high pressure on cellular morphology, proliferation and protein expression of Jurkat cells (human T lymphocyte cell line) has been extensively investigated. In the present study, we manufactured a novel pressure chamber that modulates 5% CO_2, temperature and pressure (up to 3 ATA). Jurkat cells was incubated 2 ATA pressure and analyzed cellular morphology and growth using an electron microscopy and MTT assay. The cells showed the morphological changes in the cell surface, which appeared to cause a severe damage in cell membrane. The growth rate of the cells under 2 ATA pressure decreased as cultured time got increased. Furthermore, a long term exposure of high pressure on Jurkat cells may act as one of the important cellular stresses that leads to inducing cell death. Cellular proteomes were separated by 2-dimensional electrophoresis with pH 3-10 ranges of IPG Dry strips. And many proteins showed significant up- and -down expressions with hyperbaric pressure. Out of all, 10 spots were identified significantly using matrix-assisted laser desorption/ionization-time of fight (MALDI-TOF) mass spectrometry. We and found that 9 protein expressions were decreased and one protein, heat shock protein HSP 60, was increased in Jurkat cells under 2 ATA. Identified proteins were related to lipid metabolism and signal transduction.

Keywords: hyperbaric pressure, Jurkat cells, cell growth, 2-dimensional electrophoresis, HSP60

Hydrostatic pressure studies have been carried out in two major fields that are concerned with of low pressures such as the pressure in blood vessels or a hyperbaric pressure and high pressures such as the deep-sea pressure. A high pressure is defined as being more than 38 megapascal (MPa), which is equivalent to the sea depth of 3800 m[1]. Many high pressure studies have shown the effects of pressure on biological macromolecules, which cause structural changes or alterations to the cellular process. These changes are characterized by a wide variety of reactions including: ligand-protein interactions, redox reactions, protein-protein interactions, interactions between proteins and nucleic acids, etc[2].

The sensitivity for high pressure of supramolecular assemblies, such as microtubule and flagellin to high pressure, suggests that the cells are quite sensitive to pressure. The suggestion that the cells are sensitive to pressure is supported by the observations on virus and bacteria[3]. Pressure induces the formation of inactive rotaviruses[4], which causes the gastroenteritis responsible for millions of fatal diarrhea cases in children from developing countries. The new conformation of the pressurized particle did not result in a loss of immunogenicity, but pressure altered the receptor-binding protein, VP4 by triggering similar changes to those products when the virus interacts with the target cells[4]. Two kbar pressures have also been used to inactivate the bacteria leptospira, which is the causative agent of the important zoonotic and human disease. Electron microscopy revealed the dislocation of the outer membrane, the partial loss of the helical shape and an extrusion of the axial filament from the cytoplasmic cylinder of the pressurized leptospiras[5].

In addition, high pressure regulates the gene expression resulting in changes of macromolecules, proteins, lipids and nucleic acids[1]. When a deep-sea bacterium Photobacterium profundum strain SS9 is subjected to a different high pressure, 0.1, 28 and 50

MPa, the level of monounsaturated fatty acid and oleic acid increase with increasing pressure causing an increase in the fluidity of plasma membrane[6].

On the other hand, many studies concerning the effects of low pressure on cells and humans have been reported. Watase *et al.* reported that low pressure, 105 mmHg or 120/90 mmHg pressure increased the proliferative rate in smooth muscle cell without any cytotoxicity[7]. It was also reported that a pressure of 40 to 120 mmHg promoted the cell proliferation and DNA synthesis in rat intestinal epithelial cells but a pressure of 160 mmHg inhibited cell proliferation and DNA synthesis[8]. Similar results were early obtained from human aortic endothelial cells immortalized with the simian virus 40 (SE-1) at an ambient pressure. The amount of [^{3}H]-thymidine incorporated in SE-1 reached the maximum at 150 mmHg, while the level of incorporation was decreased at a 200 mmHg pressure[9]. Pressure (from 70 to 90 mmHg) activates the mitogen-activated protein kinase, which induces the activation of the tyrosine kinases, and enhances the proliferation of mesangial cells, probably through cyclin D1 expression[10]. In another study, mesangial cell proliferation as well as apoptosis was evaluated under a simulated normal glomerular pressure (30-35 mmHg), as well as simulated glomerular hypertension (55-60 mmHg). However, simulated glomerular hypertension (55-60 mmHg) promoted mesangial cell apoptosis and cathepsin-B and clusterin[11].

These two contrasting results in low pressure studies would originate from the difference in the experiment facilities with a 5% CO_2 supplying system. Therefore, we made a novel hyperbaric pressure chamber, in which the pressure is controlled from 1 to 3 ATA with an automatic supplying system of 5% CO_2 and temperature could also be controlled up to 70°C.

It would be valuable to examine the effects of hyperbaric pressure in the cellular processes, because most hyperbaric oxygen therapy treatments are performed at 2 to 3 ATA. Many studies concerning hyperbaric oxygen therapy have mainly dealt with their clinical or toxic effects without determining the actual effects of the hyperbaric pressure (2 to 3 ATA). In present study, we determined the effects of pressure on cellular morphology and growth under 2 ATA. In addition, we performed proteomic analysis of found results that the expression of 10 proteins in Jurkat cells were changed in 2 ATA condition compared to normal pressure.

Construction of a Hyperbaric Pressure Chamber and the Determination of the Chamber Conditions

The pressure chamber was manufactured by the Dae-Il chemical company according to the design of the pressure chamber established by Watase *et al.*[7] (Fig. 1). The pressure chamber consisted of the following four major parts: digital control pads (Fig. 1A ①, ②, and ③), a chamber box (Fig. 1D ④), a CO_2 control system (Fig. 1D ⑥ and ⑨), a temperature control water tank (Fig. 1D ⑤), and a second pressure tank (Fig. 1D ⑪). The digital control pads regulated the temperature, CO_2 concentration and the pressure. The second pressure tank was connected to an air compressor installed on the outside, and the pressure tank was also connected to a chamber box with a pressure control solenoid valve (Fig. 1D). The pressure in the second pressure tank was maintained up to 4 ATA and the pressure in incubation chamber was then controlled from 1 to 3 ATA. The equilibra-

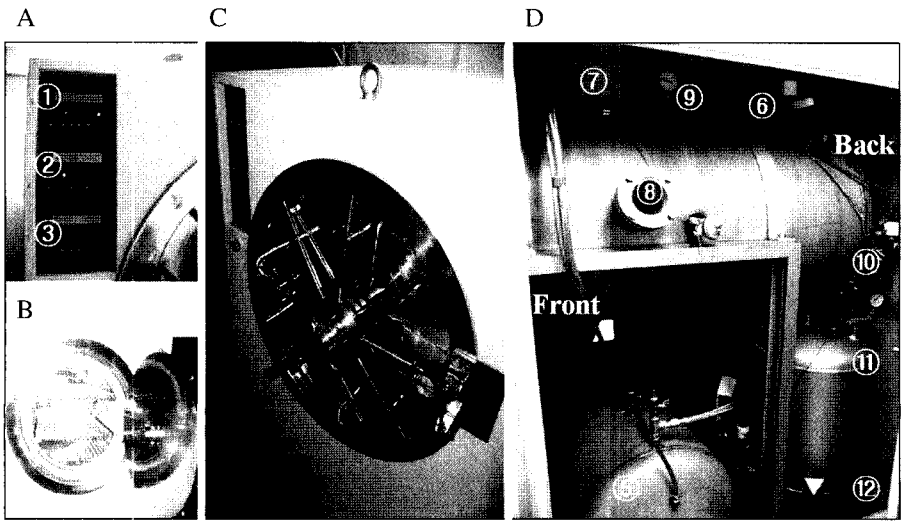

Fig. 1. Hyperbaric pressure chamber: 1: Temperature control pad, 2: CO_2 control pad, 3: Pressure control pad, 4: Incubation chamber, 5: Temperature control water tank, 6: CO_2 control solenoid valve, 7: CO_2 sensor, 8: Incubation chamber window, 9: Pressure gauge, 10: Pressure control solenoid valve, 11: Second pressure tank, 12: Air compressor.

tion of the CO_2 concentration up to 5% takes 5 minutes (Fig. 2). Table 1 shows the conditions of pH and DO in the media under 2 ATA. The pH in the media was measured under a normal, 1 ATA and 2 ATA hyperbaric pressure. These changes in the pressures led a shift of pH from 7.40 to 7.35 ($p<0.05$), and these results were not significantly differ from the previous reports[12]. The DO concentration in the media was also determined under a normal, 1 ATA and 2 ATA hyperbaric pressure. The concentrations of DO in the media under 1 ATA and 2 ATA were 2.53 and 2.40, respectively ($p<0.05$).

Table 1. Concentration of pH and dissolved O_2 in the media under a 2 ATA hyperbaric pressure

	1 ATA	2 ATA	pValue
pH	7.40±0.02	7.35±0.03	0.023
DO_2	2.53±0.14	2.4±0.19	0.028

The pH was measured in RPMI 1640 media containing penicillin /streptomycin and 10% FBS under 1 ATA and 2 ATA conditions. The results are from six experiments performed in triplicate. Statistical comparisons were performed using a Student's unpaired t test.

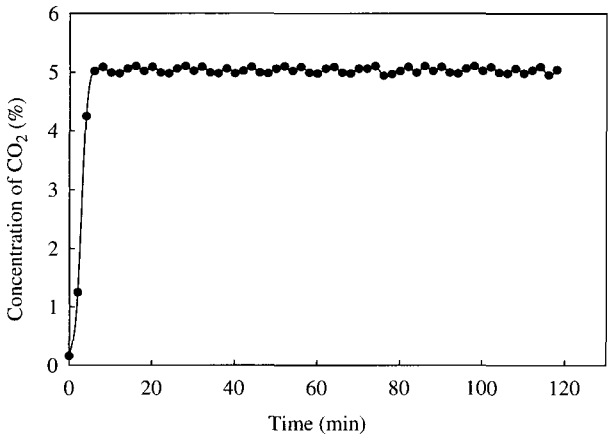

Fig. 2. Concentration of CO_2 in a hyperbaric chamber. The concentration of CO_2 was measured using a Portable Carbon Dioxide (CO_2) Plus Toxic Gas/Oxygen Analyzer (Data Ltd, UK) for 2 hours.

Cell Morphology under Hyperbaric Pressure

The morphology of the cells cultured under 2 ATA pressure chamber was determined by electron microscopy. The cells were harvested after 1 day of the third passage and were photographed by scanning electron microscopy. After cultivating the cells under a 2 ATA for a long time, the cells showed the morphological changes in the cell surface, which appeared to cause a severe damage in cell membrane (Fig. 3A and B)

Determination of Cell Proliferation under Pressure

The cells were incubated under the normal and 2 ATA pressure, and cell growth was determined by a MTS assay. In the normal pressure condition, the maximum growth of cells was shown at 4 days. However, the growth rate of the cells under the 2 ATA pressure decreased with increasing culture times. After three passages, the growth of cells was inhibited completely by the 2 ATA pressure and the cells stopped growing, and so the cells died after 1 day (Fig. 4). These results indicate that long term exposure of high pressure on Jurkat cells may act as one of important cellular stresses to induce cell death.

Proteomic Analysis of Jurkat Cells under 2 Ata

Jurkat cells were grown at 1ATA or 2ATA to examine the differences of protein expressions. The 2DE-pattern of Jurkat cells exposed to pressure using 3-10 range of a pI strip is shown in Figure 5. Total cellular proteins from three serial passages (P1-P3) and control (under normal pressure) were analyzed and separated by 2-DE (Fig. 5). Total of 10 proteins

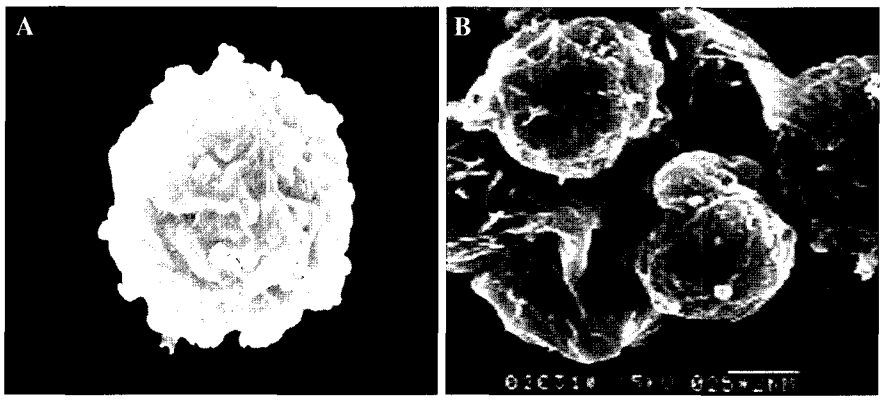

Fig. 3. The electron microscopic photograph of Jurkat cells grown under 1 ATA and 2 ATA pressure conditions. (A) Normal pressure (1 ATA). (B) Hyperbaric Pressure (2 ATA); the cells were cultured for 3 passage and harvested on the first day of the third passage (× 2500).

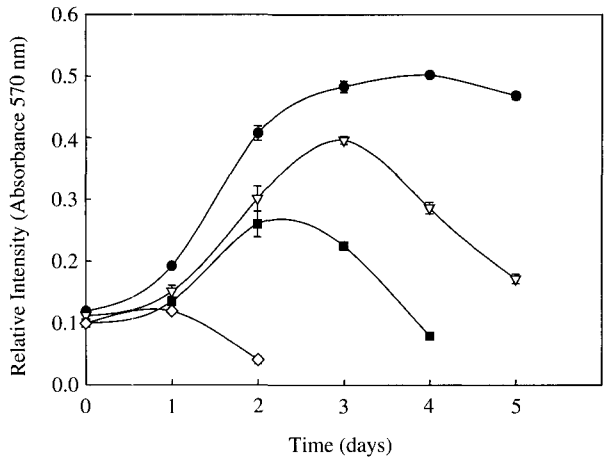

Fig. 4. Growth curve of the Jurkat cells in 1 ATA normal (closed circle) and 2 ATA pressure conditions: open triangle for 1 passage, closed square for 2 passage and open square for 3 passage. Cell growth under normal pressure showed no variation according to passage. Cell proliferation was analyzed by a MTS assay.

exhibited different expressions between the two pressure conditions. This was accomplished by image analysis and the protein were identified by MALDI-TOF (Fig. 5 and Table 2). As shown in Table 2, 9 proteins including; adenosine deaminase, tropomyosin 4, tubulin-specific chaperone A, protein kinase C, thioredoxin peroxidase 2, Rho GDP-association inhibitor 2, hydroxyacyl-coenzyme dehydrogenase, esterase and 26 S proteosome were decreased. Only heat shock protein, 60 (HSP60) was increased.

Hyperbaric oxygen therapy has been used to treat various diseases including carbon monoxide and cyanide poisoning, acute traumatic ischemia, decompression sickness, osteomyelitis and skin grafts or flaps. However, many other complications including; barotraumas, oxygen toxicity, reversible visual changes and claustrophobia have been encountered[13]. This hyperbaric oxygen therapy involves the intermittent inhalation of 100% oxygen under a pressure ≥ 1 ATA[14]. In addition, the 2 ATA pressure is encountered when working at the sea depth of 10 m. With these concerns, studies of the effects of hyperbaric pressure around 2 ATA on biochemical systems would be beneficial in view of medical and environmental aspects.

In this study, a novel hyperbaric pressure chamber was constructed and the effects of hyperbaric pressure were determined in cellular growth and morphology. In practice, many types of pressure chambers have been established and used for study. In 1950, the first pressure chamber was made by Zobell and Oppenheimer to evaluate the effects of high pressure on the growth of ocean bacteria[15]. During the last five decades, many pressure chambers for high or low pressure studies have been made. However, the pressure chamber designed by Watase *et al.* had advanced functions, which regulated the CO_2 automatically using solenoid valves and cultured cells[7]. Our hyperbaric pressure chamber was made according to his design with other functions, which provided suitable conditions, including CO_2 concentration, temperature and moisture, for examining the effects of pressure on cells.

Jurkat cell growth at third passage under a hyperbaric 2 ATA pressure was completely inhibited at day 2. In general, a pressure ≥ 160 mmHg inhibited cell proliferation and DNA synthesis[8,9]. The 2 ATA pressure condition caused significant morphological changes in the cell surface, which appeared to have severe effects on the intracellular biochemical interactions. The time to reach the maximum growth of cells at each passage under a hyperbaric 2 ATA pressure was reduced gradually from 3 days to 1 day. In the mesangial cells, the stimulated glomerular hypertension generated DNA fragmentation[11]. In this study, a hyperbaric pressure of 2 ATA may exert genotoxic effects for the long time exposure that results in cell death.

Table 2. Protein identities showing differential expression under 2 ATA verses 1 ATA in Jurkat cells

Spot no.	Protein name	PI	Mw (Da)	Coverage (%)	Accession number	Change
1	Adenosine deaminase	5.6	40803	43	P02813	Decrease
2	Tropomyosin 4	4.6	26000	37	P07226	Decrease
3	Tubulin-specific chaperone A	5.4	14000	63	O75347	Decrease
4	Protein kinase C	6.4	13800	76	P49773	Decrease
5	HSP, mitochondrial	8.0	10800	66	P22392	Increase
6	Thioredoxin peroxidase 2	8.27	22100	56	Q06830	Decrease
7	Rho GDP-sissociation inhibitor 2	6.4	22400	54	P52566	Decrease
8	Hydroxyacyl-coenzyme dehydrogenase	6.2	24800	59	Q99714	Decrease
9	Esterase D	6.7	35500	39	P10768	Decrease
10	26S proteasome	7	41400	55	P47210	Decrease

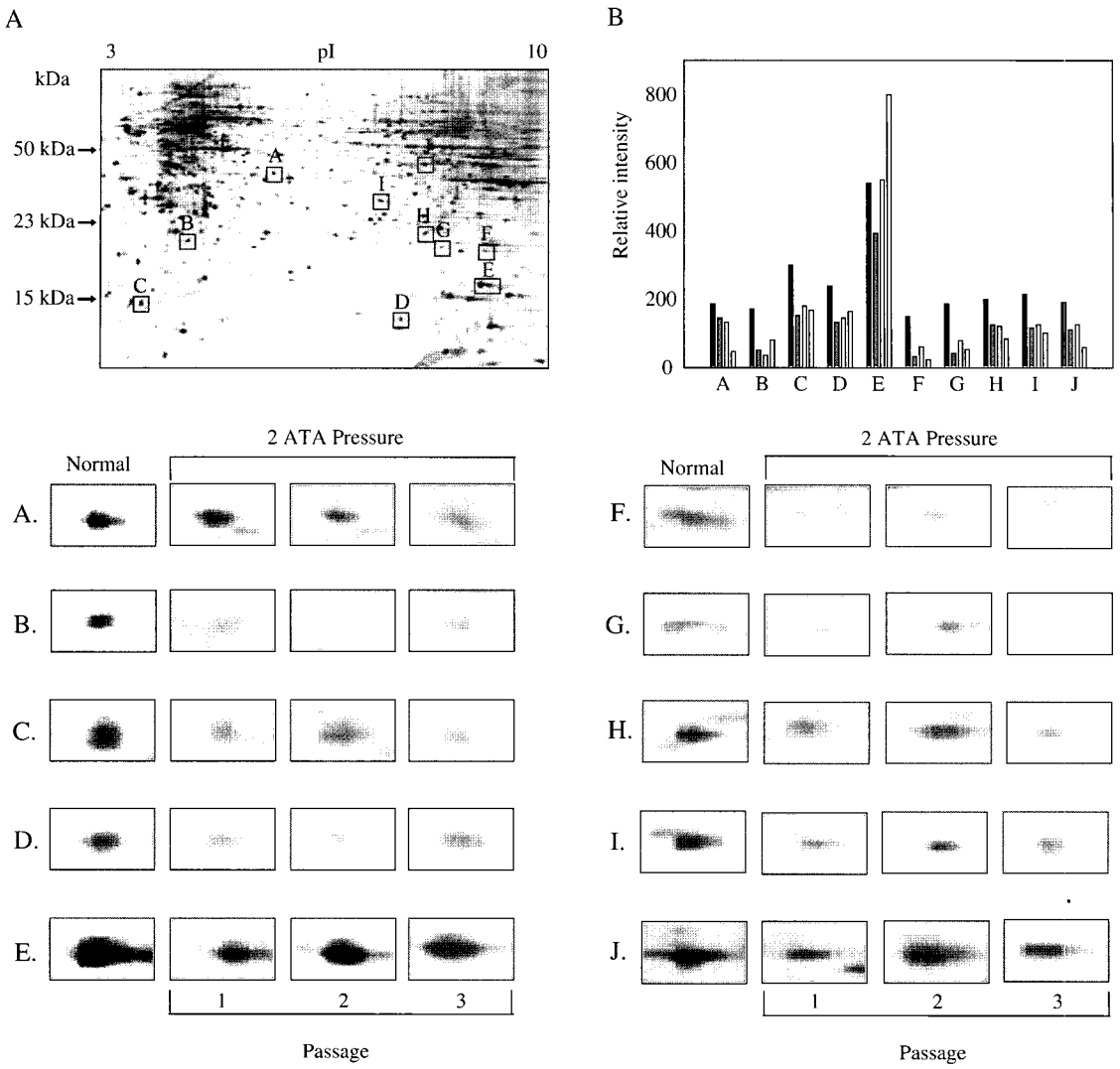

Fig. 5. Proteomic analysis of Jurkat cells treated with 2 ATA. Cells growing in normal pressure were subcultivated and divided into two groups; one for control and the other for pressure treatment. Then the cells under each pressure condition were cultivated for 3 passages and at each passage, cellular proteins were prepared and 2-dimensional electrophoresis was performed followed by silver staining as described in Material and Methods. (A) 2-DE gel pattern obtained using a pI strip (3-10). Gels were visualized by silver staining. Protein spot images were analyzed using Image Master 2-DE Elite Software program (Amersham Pharmacia Biotech, Amersham, UK). (B) Spot volumes were calculated by normalization versus total spot volumes. The quantity represented by each spot is expressed as a relative intensity.

On the other hands, we investigated the difference of protein expression in Jurkat cells grown at between normal and 2 ATA pressure conditions. Many previous studies showed that pressure range 60 to 160mmHg has an effect on cellular proliferation and DNA synthesis as mentioned before. However, the regulation of proteins whether specifically up-regulated or down-regulated under high pressure condition were not identified. In the present study, we identified 9 proteins that shows down-regulation and one protein (HSP 60) that shows up-regulation. Since HSP 60, the chaperone of the Hsp 60 family, is one of the most important components of the protein folding system in the mitochondrial matrix[16], hyperbaric pressure in our system acts as one of cellular stresses resulting in up-regulation of HSP 60. Thioredoxin peroxidases are antioxidants that function as peroxidase only when coupled with a sulfhydryl reducing system and protects from apoptosis induced by serum deprivation, ceramide or etoposide[17]. Under high pressure condition, the cell growth was inhibited completely after three passages, and the cells began

to die with DNA damage, which causes apoptosis. In addition, several proteins involved in biological metabolism, such as esterase, protein kinase C and hydroxyacyl-coenzyme dehydrogenase[18,19,20].

In summary, we manufactured a novel pressure chamber to investigate effects of hyperbaric pressure on cellular morphology, proliferation and protein expression of Jurkat cells. We found that the cells showed the morphological changes in the cell surface, which appeared to cause a severe damage in cell membrane and the growth rate of the cells under a 2 ATA pressure decreased with increasing culture times. Furthermore, proteome change was evaluated inJurkat cells with long term exposure of high pressure. Cellular proteomes were separated by 2-dimensional electrophoresis with pH 3-10 ranges of IPG Dry strips and many proteins showed significant up and down expressions with hyperbaric pressure. Of these, significant 10 spots were identified using matrix-assisted laser desorption/ionization-time of fight (MALDI-TOF) mass spectrometry. We found that 9 protein expressions decreased and one protein, which heat shock protein HSP 60, increased in Jurkat cells under 2 ATA. Identified proteins were related to lipid metabolism and signal transduction.

Methods

Chemicals

Urea, thiourea, CHAPS, DTT, acrylamide, N, N′-methylene-bisacrylamide (Bis), iodoacetamide, acetonitrile, sodium thiosulfate and TFA were purchased from Sigma (St. Louis, MO, USA). Protease inhibitor cocktail tablets were obtained from Roche (Mannheim, Germany). All other reagents were from various commercial sources and of the highest grade available.

Cell Line and Culture

Jurkat cells were obtained from American Type Culture Collection (TIB-152) and were maintained in RPMI 1640 containing 10% fetal bovine serum albumin (Gibco BRL, Grand Island, USA), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 in air atmosphere. Cells cultured for 3 passages and growth curve was determined by MTS assay.

Hyperbaric Pressure Chamber

The hyperbaric pressure chamber was designed according to the pressure chamber made by Watase *et al.* (7) and was manufactured by the Daeil Chemical Co. (Seoul, Korea) (Fig. 1). The hyperbaric pressure chamber was equipped with several systems where the pressure was controlled from 1 to 3 ATA, and CO_2 and temperature were regulated automatically. These systems are visualized and operated digitally in front of the hyperbaric chamber (Fig. 1).

Determination of pH and Dissolved Oxygen (DO) in Medium under 2 ATA Condition

In order to check for any changes in the pH of the culture medium, a serum conditioned medium, which had been incubated in the hyperbaric chamber under 2 ATA pressure with 5% CO_2, was collected and its pH was measured using an expandable ion Analyzer EA920 (Orion Research, MA). The DO concentration in the medium was measured using a Sension Dissolved Oxygen Electrode (HACH Co., Loveland, Colorado).

Determination of CO_2 in Hyperbaric Chamber

The CO_2 concentration in the hyperbaric chamber was analyzed by a Portable Carbon Dioxide (CO_2) Plus Toxic Gas/Oxygen Analyzer (Data Ltd, UK) for 2 hours. It takes 5 minutes for the CO_2 to increase to 5% in the chamber, and the CO_2 concentration in the hyperbaric chamber was maintained automatically ($5.0 \pm 0.01\%$).

Cell Growth Assay

The level of cell growth was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTS) assay (Promega, Madison, WI). After incubating the cells in 96 well plates under pressure, MTS (20 μl) was added to each well, and the plates were read at 490 nm wavelength two hours later using an ELISA reader (Molecular Devices Co. Sunnyvale, CA, USA). The cell growth patterns were analyzed up to 3 passages under a hyperbaric pressure.

Electron Microscopy

The pressurized cells were harvested after 3 passage of culture and subjected scanning electron microscopic analysis. Cells were suspended in 0.1 M cacodylate buffer (pH 7.3) and fixed in 2.5% glutaraldehyde solution at room temperature for 4 hrs. Cells were then rinsed twice with PBS and post-fixed in 1% osmium tetroxide. The sample was dehydrated I graded ethanol series (70-100%), after they dried with hexamethyldisilazane. Dehydrated specimens were mounted on holders, coated with gold in an ion-coater system (IB-5, Eiko) and observed and photographed in a scanning electron microscope (Hitach S-4700).

Sample Preparation

The cells were harvested and washed three times with a buffer containing 10 mM Tris (pH7.4) and 150 mM sucrose. The cells were subjected to sonication with lysis buffer (PBS, pH 7.2, 1% Triton X-100, protease inhibitor cocktail, 1 mM EDTA). The lipids and salts were removed from the sample using a molecular cutoff column (3 kDa; Amicon, Millipore, Bradford, MA, USA). After sample preparation, the protein concentration was measured using the Bradford assay.

IEF and SDS-PAGE

In the first dimension of the 2-DE, the proteins were separated only according to their p*I*. The protein sample (45 μg) was mixed with a rehydration buffer containing 8 M urea, 4% CHAPS, 40 mM Tris, 0.5% IPG buffer, 65 mM DTT and a trace of bromophenol blue (BPB) to a total volume a 450 μL. The IEF was carried out with commercially available immobilized pH gradients (pH 3-10 nonlinear, 24 cm), using the IPGphor (Amersham Biosciences, Amersham, UK) apparatus. The gel was rehydrated in the presence of the sample for 12 h and focused for 81500 Vhr. After IEF, the IPG gel strips were equilibrated twice for 15 min, under gentle shaking at room temperature, first in the solutions (equilibration buffer: 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 1% w/v SDS) containing 1% DTT, and in an equilibration buffer containing 2.5% iodoacetamide. In the second dimension SDS-PAGE, the proteins were resolved solely on the basis of their molecular masses in 12.5%gradient polyacrylamidegels (35 × 45 cm) using an Owl separation system runner (Owl Separation System, Portamouth, NH, USA). The IPG strips were embedded in 0.5% w/v melted agarose prior to running on the SDS -PAGE slabs. The agarose contained 0.001% w/v BPB as a tracking dye. The running conditions were 2.5 W/gel for 15 min and 140 W/total for 6 h until the BPB reached the end of the gel.

Visualization and Image Analysis

After separation in the SDS-PAGE gels, the proteins were visualized using a silver staining kit (Amersham Biosciences), according to the manufacturer's instructions. The silver-stained gels were scanned using an 800 × 1600 dpi instrument (UTA 2100XL, UMAX, Taipei, Taiwan) and the image files were transformed into TIF format with linear gray scale values. The computer analysis of the 2-D image was carried out using ImageMaster 2D Elite software V4.01 (Amersham Biosciences) according to the manufacturer's protocol. The spot finding parameters were gel-specific and were obtained using the 'spot detection wizard' in an ImageMaster program. Spot finding was edited manually so that all artifacts detected (*e.g.*, dust and streaks) were removed, the spot boundaries were defined and the spot 'chains' were split. The reference gel image was nominated and the 'test' gel images were matched to the reference gel (creating a match set). The matches were edited manually in order to ensure the correct spot matches and to ensure a more consistent determination of the spot volume. The normalization factors were determined based on the total spot volume and the matched spot volumes were normalized across the whole match. The normalized spot volumes of the images in a match set were compared (if the data was identical, all ratios = 1, for different data, ratios were $>$ or $<$ 1). Difference thresholds (defined as over 300%) were applied based on the normalized volume of the spot. Histogram of the difference between the samples (increase or decrease in a dose-dependent manner) in each p*I*, the range was shown next to the relative intensity.

Protein Identification by MALDI-TOF MS Analysis

The gel spots were excised with a scalpel, crushed, and destained by washing them with 25 mM NH_4HCO_3, 50% acetonitrile (ACN). The gels were dehydrated by adding ACN, rehydrated in ice by adding 10-20 μL of 25 mM NH_4HCO_3 with 10 μg/mL of sequencing grade trypsin (Promega, Madison, WI, USA), and incubated at 37°C for 12-15 h. The peptides were extracted by adding 25 μL of a solution containing 50% ACN, 0.1% TFA. The extraction was repeated three times and completed by adding 20 μL of ACN. The extracted solutions were pooled and evaporated to dryness in a SpeedVac centrifuge. The samples were reconstituted in 10 μL of 0.1% TFA and treated with ZipTips containing C18 resin (Millipore) according to the manufacturer's instructions. The washed peptides were eluted with a saturated matrix solution (CHCA in 60% ACN, 0.1% TFA). The monoisotopic masses (M11) of the tryptic fragments were measured in a Perspective Biosystem MALDI-TOF Voyager DE-STR mass spectrometer (Framingham, MA, USA). The trypsin peaks were used to internally calibrate the spectra obtained. The proteins were identified by a search of a *Homo sapiens* subset of the NCBInr (NCBInr.10.21.2003) database using the MS-Fit program http://prospector.ucsf.edu/ucsfhtml4.0/msfit. htm). The known keratin masses and trypsin autodigest products were excluded from the searches. The parameters were set as either missed cleavage or acrylamide modification. The protein identities were assigned if at least five

peptide masses matched within a maximum error of 50 ppm, and the candidate agreed with the estimated p*I* and Mr from the 2-DE gel.

Acknowledgements

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