CELL DIFFERENTIATION AND P38^{MAPK} CASCADE ARE INHIBITED IN HUMAN OSTEOBLASTS CULTURED IN A 3D-CLINOSTAT

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SUMMARY

A three-dimensional (3D) clinostat is a device for multi-direction G force generation. By controlled rotation of two axes, a 3D-clinostat cancels the cumulative gravity vector at the center of the device and produces an environment with an average of 10^{-3} G over time. We cultured a human osteoblast cell line in a 3D-clinostat and examined the growth properties and differentiation of the cells including morphology, histological detection of calcification and mitogen-activated protein kinase (MAPK) cascades. In a normal 1G condition, alkaline phosphatase (AlPase) activity was detected on day 7 of culture, bone nodules were formed on day 12, and calcium deposits were seen on day 20. In the 3D-clinostat, the cells looked larger and bulged. AlPase activity was detected on day 10 of culture. However, neither bone nodules nor calcification was found in the 3D-clinostat up to day 21. The expression levels of core-binding factor A1 (Cbfa1, a transcription factor for bone formation) and osteocalcin (a bone matrix protein) increased in the control culture but decreased in culture in the 3D-clinostat. Phosphorylation of $p38^{MAPK}$ (p38) was repressed in culture in the 3D-clinostat, while total p38 as well as total and phosphorylated forms of extracellular signal-regulated kinases (ERK1/2) and stress-activated protein kinase/jun N-terminal kinase (SAPK/JNK) were not changed in the 3D-clinostat. When a p38 inhibitor, SB 203580, was added to the culture medium in a normal 1G environment, AlPase activity, formation of bone nodules and calcium deposits were strongly inhibited. On the other hand, they were inhibited only partially by a MAPKK (MAPK kinase) inhibitor, U-0126. Based on these results, it is concluded that 1) osteoblasts differentiation is inhibited in culture in a 3D-clinostat, and 2) this inhibition is mainly due to the suppression of p38 phosphorylation.

Key words: osteoblasts; 3D-clinostat; simulating microgravity; MAPK;、p38; SAPK/JNK.

Running title: Osteoblast differentiation in 3D-clinostat

Abbreviations used: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; SAPK/JNK, stress-activated protein kinase/jun N-terminal kinase; MAPKK, mitogen-activated protein kinase kinase; AlPase, alkaline phosphatase; Cbfa1, core-binding factor A1.

INTRODUCTION

Transduction of physical force into a cellular response is an essential mechânism that enables living organisms to respond to the environment. It is generally accepted that bone tissue is sensitive to mechanical stress or loading and mechanical stresses affect the form of bone (Burger and Klein, 1998; Duncan and Turner, 1995). Subnormal mechanical stress as a result of bed rest or immobilization results in decreased bone mass and osteoporosis by disuse (Houde et al., 1995). Mechanical loading through exercise increases bone mass and retards bone loss (Bassey and Ramsdale ,1994; Chesnut, 1993; Prince et al., 1991; Simkin et al., 1987; Smith and Gilligan, 1990). Microgravity in space flight results in reduction in mineral content of bone (Rambaut and Goode, 1985; Russell and Simmons, 1985; Turner et al., 1985; Vico et al., 1987), bone matrix protein production (Patterson-Buckendahl et al., 1985) and bone formation (Morey and Baylink, 1978; Shaw et al., 1988; Wronski and Morey, 1983).

A microgravity condition can be produced by space flight or by a free fall. However, the duration of a microgravity condition produced by a free fall is usually too short to alter cell growth and differentiation. Because of limited access to space flight, many efforts have been made to establish alternative methods for simulating microgravity on Earth, and a clinostat is considered to be a device for simulating microgravity. Classic 1D-clinostats include that using horizontal rotation used by Marimuthu et al. (1970), that using vertical rotation used by Schatten et al. (2001), and that using horizontal or vertical rotation used by Sarkar et al. (2000) and Kobayashi et al. (2000). However, these 1D-clinostats may generate centrifugal force and are not able to remove the effect of gravity.

Mitogen-activated protein kinase (MAPK) pathways transmit environmental signals from the cell membrane to the nucleus through phosphorylation cascades, resulting in regulation of gene expression (Nebreda and Porras, 2000). In mammalian systems, three subgroups of MAPKs have been known: extracellular signal-regulated kinases (ERK1/2),

stress-activated protein kinase/jun N-terminal kinase (SAPK/JNK), and $p38^{MAPK}$ (p38). It has been shown that stress kinases, SAPK/JNK and p38, respond to various stimuli, including ultraviolet radiation (UV), oxidative stress, heat shock, and so on (Nebreda and Porras, 2000). However, cellular response to gravity environment has not been reported.

In the present study we used a 3D-clinostat, which is a device for multi-direction G force generation. By controlled simultaneous rotation of two axes, the 3D-clinostat cancels the cumulative gravity vector at the center of the device, producing an environment with an average of 10^{-3} G over time. We cultured osteoblasts in this 3D-clinostat and examined the growth properties, the differentiation of the cells including morphology, histological detection of calcification, and MAPK cascade.

MATERIALS AND METHODS

3D-clinostat.

The 3D-clinostat, produced by Mitsubishi Heavy Industries, Co., LTD. (Kobe City, Japan), is shown in Fig.1. By controlled simultaneous rotation of two axes, the 3D-clinostat cancels the cumulative gravity vector at the center around the device, producing an environment with an average of 10^{-3} G over time. This is accomplished by rotation of a chamber at the center of the device to disperse the gravity vector uniformly within a spherical volume at a constant angular velocity (Japanese Patent, Publication number: P2000-79900A, Date of filing: Sept. 22, 1998).

Cell culture.

A normal human osteoblast (NHOst) cell system was purchased from BioWhittaker Inc. (Walkersville, MD). The cells were seeded in 25-ml culture flasks (3107 with a vented plug seal cap, Becton Dickinson Labware, Franklin Lakes, NJ) and maintained in an osteoblast basal medium (OBM, CC-3208: BioWhittaker Inc., Walkersville, MD) supplemented with 10% fetal bovine serum (FBS: CC-4102), 200 nM ascorbic acid (CC-4398), and 200 nM gentamicin/amphotericin-B (CC-4381).

An osteoblast differentiation medium (ODM), which consisted of OBM supplemented with SingleQuots containing 200 nM hydrocortisone, 10 mM β-glycerophosphate and 10 μM 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$ D_3] (Calbiochem, La Jolla, CA), was used for NHOst cell differentiation. The cells were transferred to the ODM, and the cell concentration was adjusted to 1.2 x 10^4 cells/cm^3 with the aid of a hemocytometer. The cells were then seeded in culture flasks. After 24 h, the medium was removed and the flask was completely filled with fresh medium to eliminate air bubbles, and to diminish turbulence and shear forces (day 0 of culture). The cells were cultured up to 21 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was renewed every 3 days.

In this study, we used total number of 252 culture flasks. We used three flasks for each analysis.

Experimental groups.

To determine the effects of inhibitors of signal transduction pathways on NHOst cell differentiation, 10 μM SB 203580, a p38 inhibitor (Promega, Madison, WI), or 0.5 - 1 μM U-0126, a MAPKK (mitogen-activated protein kinase kinase) inhibitor (Promega, Madison, WI), was added to the ODM. The concentrations of the inhibitors used were based on the studies by Tokuda et al. (2002) and Bhat et al. (1998). The inhibitors were dissolved in dimethylsulfoxide (Me_2SO) to give concentrations of 1-50 mM, and 1 μl of this solution per 1 ml of the culture medium was added. The equivalent volume of Me_2SO was added to control cultures in each experiment. The medium was replaced with fresh medium (with or without inhibitors) every 3 days.

The cells were cultured in ODM in a normal 1 G environment (group C), in ODM in a 3D-clinostat (group CL), in ODM with 10 μM SB 203580 at 1 G (group C + SB), or in ODM with 0.5 and 1 μM U-0126 at 1 G (group C + U 0.5 μM and group C + U 1 μM).

For studying the effects of inhibitors, three culture flasks were used in each group on each culture day.

Analysis of cell permeability and DNA synthesis.

Twenty-one flasks of each group C and CL were used for a trypan blue-uptake study. On days 0, 1, 3, 5, 7, 14, and 21 of the culture (three flasks for each day), the cells were dispersed in Hank's balanced salt solution (HBSS: pH 7.5) containing 0.05% trypsin and 0.02% EDTA and suspended in 2% trypan blue in HBSS. The cells were counted under a microscope x 40 magnification using a hemocytometer.

Twelve flasks of each group C and group CL were used for monitoring DNA synthesis. Bromodeoxyuridine (BrdU) at the concentration of 0.01 % was added to ODM on day 0.5 (12 h) of culture, and the cells were incubated in this medium for 1 h (day 0), 1, 2, 3,

and 5 days. Cells in three flasks were fixed with absolute ethanol on each culture day. Cells labeled with BrdU were visualized using a cell proliferation kit (Amersham International Plc., Amersham, UK) and counted under a microscope with the aid of hemocytometer. In our preliminary experiments, almost 100 % of cells were labeled by BrdU on culture day 3 and thereafter (data not shown).

Data of trypan blue and BrdU positive cells were statistically analyzed for differences on a day-by-day basis. A probability value of <0.01 was considered to be statistically significant.

Counting bone nodules, alkaline phosphatase activity and calcium deposits.

Three flasks of each group C and group CL were used on every 0 to 21 day of the culture. The numbers of bone nodules were counted in 10 randomly selected 10 areas (0.5 cm x 0.75 cm, the area inside the photomask) for one flask, using an inverted phase contrast microscope (TE300 Eclipse, HB-10103AF, Nikon, Tokyo, Japan). These data were statistically analyzed for differences on a day-by-day basis. A probability value of <0.01 was considered to be statistically significant.

The same flasks were used for detecting alkaline phosphatase (AlPase) activity and calcium deposits on days 1, 7, 15, and 20 of the culture. The AlPase activity was detected histochemically by the naphthol AS-phosphate method (Wolff, 1975). In brief, cultures were washed in HBSS and distilled water and were then incubated in an incubation medium containing 3% naphthol AS-BI phosphate sodium salt, 5% *N, N'*-dimethylformamide, and 5% fast red violet LB salt in 0.1 M Tris buffer (containing 10% $MgCl_2$, pH 8.5) for 30 min at 37°C. Then the same cultures were fixed with 4% formaldehyde, and calcium deposits were stained by the von Kossa method (Bills et al., 1971). Namely, the cultured cells were stained with 3% silver nitrate for 1 h and then washed for 3 min in 5% sodium thiosulfate. The cells were examined under a light microscope.

Reverse transcription polymerase chain reaction (RT-PCR).

We examined core-binding factor A1 (Cbfa1) mRNA for day 0, 1, 3, 5, and 7 of culture, osteocalcine mRNA for day 1, 3, 6, 9, 12, 15, 18, and 21, and a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) on day 1, 3, 6, 9, 12, 15, 18, and 21. Three flasks were used for each day for each group C and group CL.

Total RNA from the cultured cells was extracted using Isogen® (Nippon Gene, Tokyo, Japan) based on the method of Chomczynski (Chomczynski and Sacchi, 1987). One μg of total RNA was used for reverse transcription with SuperScript II® (Gibco Laboratories, Rockville, MD). The primers used to amplify Cbfa1 cDNA fragments were: sense, 5'-TTT CTC GAG TGG TTA ATC TCT GCA GGT-3'; and antisense, 5'-TTT GAA TTC GCT CAC GTC GCT CAT CT-3'. The primers used to amplify osteocalcine cDNA fragments were: sense, 5'-AGC CCT CAC ACT CCT CGC CCT ATT G -3'; and antisense, 5'-GGA GAG GAG CAG AAC TGG GGT TGC C-3'. G3PDH cDNA was amplified using the primer supplied by TOYOBO Inc. (Osaka, Japan). The PCR was performed after denaturation for 3 min at 94 °C. The PCR conditions were 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 50 s at 72 °C followed by extension at 72°C for 10 min for Cbfa1; 30 cycles of 45 sec at 94°C and 45 sec at 55°C followed by extension for 72°C for 1 min for osteocalcin; and 24 cycles of 45 sec at 94°C and 45 sec at 60°C followed by extension for 2 min at 72°C for G3PDH.

Protein extraction and Western blot analysis.

Whole cell proteins of the cultured cells were extracted with Tricine SDS-PAGE sample buffer from culture day 1, 3, 6, 9, 12, 15, 18, and 21 (three flasks for each day for each group C and group CL). The proteins were subjected to Tricine SDS-PAGE and then electrophoretically transblotted onto a nitrocellulose membrane (Hybond-C super®, Amersham International Plc., Amersham, UK). A monoclonal anti-bovine osteocalcin antibody, clone OCG2 (Takara Shuzo Co., Ltd., Shiga, Japan), and monoclonal anti -β-actin antibody, clone AC-74, IgG2a (Sigma Chemical, St. Louis, MO), were used as

primary antibodies at a dilution of 1:1000. After the membranes had been blocked for 1 h at room temperature in PBS containing 0.1% Tween 20 and 5% non-fat dry milk, they were incubated overnight at 4°C with either primary antibody. Detection was carried out using an ECL Western blotting analysis system (RPN 2108: Amersham International Plc., Amersham, UK). The membranes were washed and incubated with LumiGLO®, and detected by exposure to ECL X-ray film. The cytosolic protein content was determined using a Bio-Rad Protein Assay kit (Bio-Rad, Mississauga, Ontario, Canada) using bovine serum albumin as a standard.

Analysis of phosphorylation status of MAPK.

The phosphorylation status of MAPK was analyzed from 0, 10, 30 min, 1, 3, 5 h and 7, 14, 21 days of culture by using three flasks for each time period for each group C and group CL. To obtain a protein sample, the cultured cells were washed with HBSS, lysed by adding an SDS sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25°C, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue), and sonicated. After heating to 95°C for 5 min, the protein samples were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Hybond-P, Amersham International Plc., Amersham, UK). A biotinylated protein maker was also loaded to determine molecular weight.

Western blots were performed for analysis of the activation of p38 (Thr180/Tyr182), ERK1 (p44) / 2 (p42) (Thr202/Tyr204), and JNK/SAPK (Thr183/Tyr185) using primary antibodies (rabbit polyclonal IgG) specific to the phosphorylated and the total forms of the three MAPKs (PhosphoPlus MAP Kinase Antibody Kit, New England Biolabs Inc., Beverly, MA). The membranes were blocked with blocking buffer (Tris buffered saline, pH 7.6, containing 0.1% Tween-20 and 5% non-fat dry milk) for 3 h at room temperature and incubated with the primary antibody with gentle agitation overnight at 4°C. After extensive washing, the membranes were incubated for 1 h at room temperature with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) and an anti-

biotin antibody conjugated to HRP (PhosphoPlus MAP Kinase Antibody Kit, New England Biolabs Inc., Beverly, MA) for detection of MAPKs and the biotinylated protein marker, respectively. The membranes were washed, incubated with LumiGLO® and exposed to ECL X-ray film (Amersham International Plc., Amersham, UK).

RESULTS

Uptake of trypan blue and BrdU.

There was no difference between the percentages of viable cells (trypan blue-negative cells) or between the percentages of BrdU-uptake cells in group C and group CL (Fig. 2). Almost all cells were exhibited positive reaction in BrdU-immunocytochemistry after day 3.

Morphology of cultured osteoblasts in the 3-D clinostat.

Differentiation of human osteoblasts in group C was compared with that in group CL. The osteoblasts cultured at 1 G (group C) were spindle-shaped on day 1 (Fig. 3 A). The cultured cells began to form colonies on day 3 and became confluent on day 5 (data not shown). The number and size of colonies increased, though each cell was oriented randomly in a colony on day 7. AlPase activity was detected on day 7 (Fig. 3 B). The accumulation of cells to form a three-dimensional nodule has been called a bone nodule (Bodine et al., 1996; Kaneki et al., 1999; Kim and Valentini, 1997). A few bone nodules appeared on day 12 (Fig. 3 C), and the number of bone nodules increased with elapse of culture time (Fig. 4). Calcification detected by von Kossa staining was first observed on day 20 (Fig. 3 D).

Figure 3 E – H show osteoblasts cultured in the 3D-clinostat (group CL). On day 1, the cells looked larger and bulged with flattened peripheral cytoplasm. The cells began to form colonies on day 5, and the number and sizes of colonies increased thereafter. AlPase activity appeared on day 10. Neither bone nodules nor calcium deposits were found throughout the culture period (Fig. 3, 4).

Expression of Cbfa1 and osteocalcin.

Expression of Cbfa1 was examined from day 1 to day 7 of the culture period by RT-PCR (Fig. 5). The expression level of Cbfa1 increased up to day 3 and decreased from

day 5 in group C. In group CL, however, it decreased on day 1 and could not be detected on days 3 to 7.

By Western blot analysis, expression of osteocalcin was first detected on day 9 and the expression level increased thereafter in group C (Fig. 6). On the other hand, osteocalcin was not detected in group CL throughout the culture period.

The expression profile of osteocalcin obtained by RT-PCR analysis of osteocalcin mRNA expression was the same as that obtained by Western blot analysis (data not shown).

Effects of MAPK inhibitors.

Figure 4 shows the numbers of bone nodules formed during the culture period. In group C, bone nodules were first formed on day 12, and the number increased thereafter. Bone nodules were not formed in group CL, and this was also the case in culture at 1 G in a medium containing a p38 inhibitor (group C + SB). The MAPKK inhibitor U-0126 partially inhibited the formation of bone nodules depending on its concentration (group C + U 0.5 μM and 1 μM).

Figure 7 shows the AlPase activities on day 7 of the culture. Strong activity was found in group C. However, the activity was hardly detectable in group CL and was strongly inhibited in group C + SB. U-0126 inhibited AlPase activity depending on its concentration.

Phosphorylation of MAPK cascades.

To determine the effects of culture in the 3D-clinostat on MAPK cascades, the phosphorylation statuses of various MAPKs, i.e., p38, ERK1/2 and SAPK/JNK, were examined (Fig. 8). Phosphorylated p38 in the control culture showed strong bands throughout the culture period. When the cells were cultured in the 3D-clinostat, the expression of the band was successively decreased at 10 min, 30 min, and 1 h after the start of culture, and no band was observed from 3 h to day 21. On the other hand, there were no changes in phosphorylated ERK1/2 and SAPK/JNK between group C and CL.

Total forms of p38, ERK1/2 and SAPK/JUN were not changed with the elapse of culture time in the both groups.

DISCUSSION

Microgravity affects important cellular processes in osteogenic cells such as proliferation, differentiation, and bone-related gene expression (Rambaut and Goode, 1985; Russell and Simmons, 1985; Turner et al., 1985; Vico et al., 1987; Patterson-Buckendahl et al., 1985; Morey and Baylink, 1978; Shaw et al., 1988; Wronski and Morey, 1983). Response of osteogenic cells to microgravity environment has been studied in space flight up to a wk: Van Loon et al. (1995) showed that 4 days of space flight inhibited matrix mineralization and stimulated osteoclastic resorption of mineralized matrix in organ cultures of bone rudiments from embryonic mice; Carmeliet et al. (1997) found that 4 days of space flight reduced expression of osteocalcin, AlPase, and collagen Iα1 mRNA in a human osteoblastic cell line MG-63; and Kumei at al. (1996) suggested that microgravity-induced production of prostaglandin E2 and interleukin-6 in rat bone marrow stroma cultures was related to alterations in bone resorption. These findings suggest that elimination of gravity results in inhibition of osteogenic functions.

The precise mechanism and long-term effects of a microgravity environment can only be elucidated in the long-term study in space, which is limited at the present. A clinostat has been considered as an alternative method for simulating microgravity environment on the Earth: 1D-clinostats have been developed to minic the microgravity environment by "nulling the gravitational vector" through continuous averaging (Sarkar et al., 2000; Kobayashi et al., 2000). However, it was difficult to "null the gravitational vector" by using these classic clinostats. A 3D-clinostat used in the present study has been considered as an instrument of better choice (Ichigi and Asashima, 2001). However, the cells cultured in the 3D-clinostat may be influenced by frequent changes of the direction of G force. The effects of this must be clarified in further studies in which effects of 3D-clinorotation and true microgravity environment during space flight will be compared.

There have been a few studies on the effects of clinorotaion. By using a 1D-clinostat that induced "vector-averaged gravity", Sarkar et al. (2000) found no change in the proliferation but increased apoptosis of cultured osteoblast-like ROS 17/2.8 cells for 2 day-clinorotation. In the present study, we found that neither cell proliferation nor cell death was affected in culture in the 3D-clinostat. The discrepancy between the results of the present study and that of Sarkar et al. (2000) may be due to the different physical environments: 3D-clinorotation in the present study and horizontal or vertical rotation in their study. Moreover, the observation period, 2 days, in the study by Sarkar et al. (2000) was too short to examine osteoblast differentiation. Kobayashi et al. (2000) found that vertical rotation for three days in a 1D-clinostat inhibited the responsiveness of human osteoblastic cells to tumor necrosis factor (TNF)-α by repressing nuclear factor (NF)-κB. The only previous study in which a 3D-clinostat was used was that by Ichigi et al. (2001). They incubated a *Xenopus* renal epithelial cell line (A6) and found a significant reduction in "dome formation" of cells, which is thought to be a sign of reduced active transport of the cells (Cohen-Luria et al., 1993). These previous and our results suggest that the culture in a "vector-averaged gravity environment" inhibits differentiation of cells.

The real microgravity environment is a relevant model to explore the bone cell response to minimal strains. Osteoblastic MC3T3-E1 cells became rounded and actin stress fibers were reduced in space flight for 4 days (Hughes-Fulford and Lewis, 1996). Guignandon et al. (1997), who cultured ROS17/2.8 osteoblast-like cells in space flight for 6 days, found that cell proliferation was not changed but the cell body was rounded. They suggested that cytoskeleton reorganization in the microgravity environment affected gene expression, which resulted in alterations in osteoblastic function (Hughes-Fulford and Lewis, 1996; Guignandon et al., 1997).

In the clinorotaion studies, Sarkar et al. (2000) found that cultured osteoblast-like ROS 17/2.8 cells were rounded, distribution of integrin β1 was changed, and actin cytoskeleton was greatly disorganized after 1D-clinorotaion for 24 h. We also found bulging of cultured osteoblasts after 24 h. These studies together with the studies in

space flight (Hughes-Fulford and Lewis, 1996; Guignandon et al., 1997) suggest that the both microgravity in space flight and an averaged gravity vector environment generated by clinorotation affect cell-to-matrix contact and cytoskeletal organization, although the gravity environments are greatly different between the two. The precise role of the cytoskeleton on cell differentiation in a microgravity or clinorotation remains to be elucidated.

The stress kinase (p38 and SAPK/JNK) pathways can be rapidly activated by a number of common stress agents including heat shock, osmotic shock, UV radiation, X- and γ-rays, oxidative stress, protein synthesis inhibitors, and cytokines (Nebreda and Porras, 2000). Activation of these pathways by stress leads to activation of multiple cellular responses through phosphorylation of signal transduction kinases. MAPK cascades, especially p38, have been shown to play an important role in differentiation of various cells, such as adipocytic differentiation of 3T3 Ll fibroblasts (Engelman et al., 1998), myogenic differentiation of C2C12 myoblasts (Cuenda and Cohen, 1999) and L8 myoblasts (Zetser et al., 1999), and neuronal differentiation (Iwasaki et al., 1999; Morooka and Nishida, 1998). Kumar et al. (2001) showed that p38 inhibitors inhibited bone resorption. The present study showed that the 3D-clinrotation inhibited osteoblasts differentiation mainly by repressing p38 phosphorylation. The further studies are needed to elucidate the effects of microgravity environments and multi-directional G force environment on MAPK cascade and cell differentiation.

Galileo in 1638 first discussed the importance of gravity in the determination of bone size and shape (Galileo, 1638). The space flight results in bone atrophy (Rambaut and Goode, 1985; Russell and Simmons, 1985; Turner et al., 1985; Vico et al., 1987) and decreased osteoblastic activity (Patterson-Buckendahl et al., 1985; Morey and Baylink, 1978; Shaw et al., 1988; Wronski and Morey, 1983). Based on the results of the present study, multi-directional G force environment in a 3D-clinostat inhibits osteoblasts differentiation by mainly repressing phosphrylation of p38. The 3D-clinostat described in

this paper is a powerful and versatile tool for the study of gravitational cell biology and for other fields of study such as physics and material sciences.

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Figure legends

FIG. 1.

(A) Photograph of a 3D-clinostat. A culture flask filled with a medium was placed in the center of the clinostat. (B) Schematic drawing of the 3D-clinostat.

FIG. 2.

Percentages of cells showing uptake of trypan blue (A) and Bromodeoxyuridine (BrdU: B). There were no differences between control culture (group C) and 3D-clinostat (group CL) in cell viability (trypan blue-negative) or BrdU-uptake. Values were shown in mean ± standard deviation (SD).

FIG. 3.

Phase-contrast microscopic examination of cultured osteoblasts (A, C, E, G) and conventional microscopic examination after staining with alkaline phosphatase (AlPase) and von Kossa's silver impregnation (B, D, F, H).

Group C: Cells were spindle-shaped on day 1 (A), bone nodules had formed on day 12 (C), and AlPase activity (red) was detected on day 7 (B) and day 20 (D). Calcium deposits (brown) were seen in bone nodules on day 20 (D, arrowheads).

Group CL: Cells looked larger and bulged on day 1 (E). Bone nodules were not found on day 12 (G). AlPase activity was detected on day 20 (H) but not on day 7 (F). Calcium deposits were not seen on day 20 (H).

FIG. 4.

Number of bone nodules in 0.5 x 0.75 cm^2. Bone nodules had formed on day 12 of culture and then increased in number in group C but were not formed in group CL or in cultures in a medium containing SB 203580 (C + SB). U-0126 inhibited the formation of

bone nodules depending on its concentration (C + U, 0.5 μM; C + U, 1 μM). Significant differences were found between two groups shown by asterisks: * $p<0.001$, ** $p<0.01$, and *** $p<0.01$, respectively. Values were shown in mean ± SD.

FIG. 5.

Expression of core-binding factor A1 (Cbfa1) was examined from day 1 to day 7 of the culture period by reverse transcription polymerase chain reaction (RT-PCR). The expression level of Cbfa1 increased up to day 3 and decreased from day 5 in group C. On the other hand, in group CL, it decreased on day 1 and could not be detected on days 3 to 7.

FIG. 6.

By Western blot analysis, osteocalcin was detected on day 9 and increased during culture in a normal 1 G environment. However it was not seen in culture in the 3D-clinostat. Expression level of a house-keeping protein, β-actin, did not change during culture period.

FIG. 7.

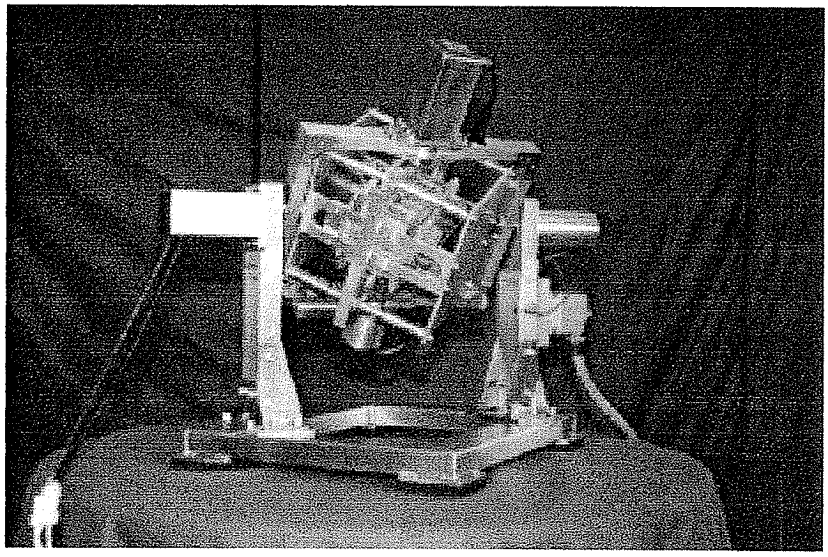
Effects of mitogen-activated protein kinase (MAPK) inhibitors on AlPase activity on culture day 7. Compared with that in group C, the activity was strongly inhibited in group C + SB but less strongly in group C + U 0.5 μM and group C + U 1 μM. The activity was hardly seen in culture in group CL.

FIG. 8.

Phosphorylation status of MAPK in control culture (C) and at 0, 10, 30 min, 1, 3, 5 h, and 7, 14, 21 days after transfer to the 3D-clinostat (CL). Phosphorylated p38 was repressed in culture in the 3D-clinostat after 10 min to 1 h of the culture and was not detected after 3h. Total forms of p38, as well as phosphorylated and total forms of

extracellular signal-regulated kinases (ERK)1/2 and stress-activated protein kinase/jun N-terminal kinase (JNK/SAPK), were not changed during culture in both C and CL.

A



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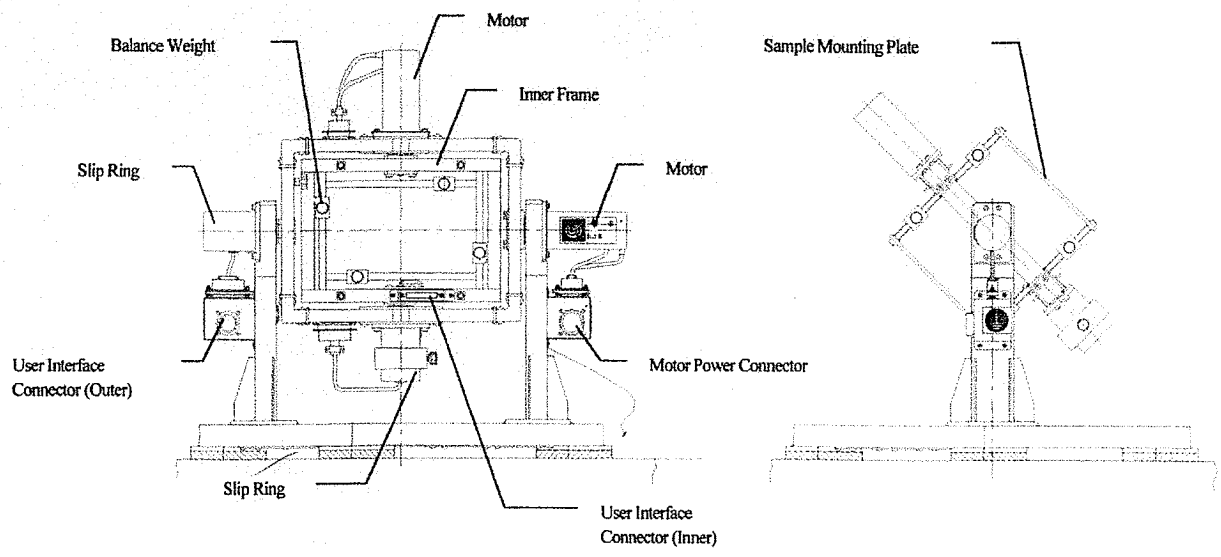

Fig.1

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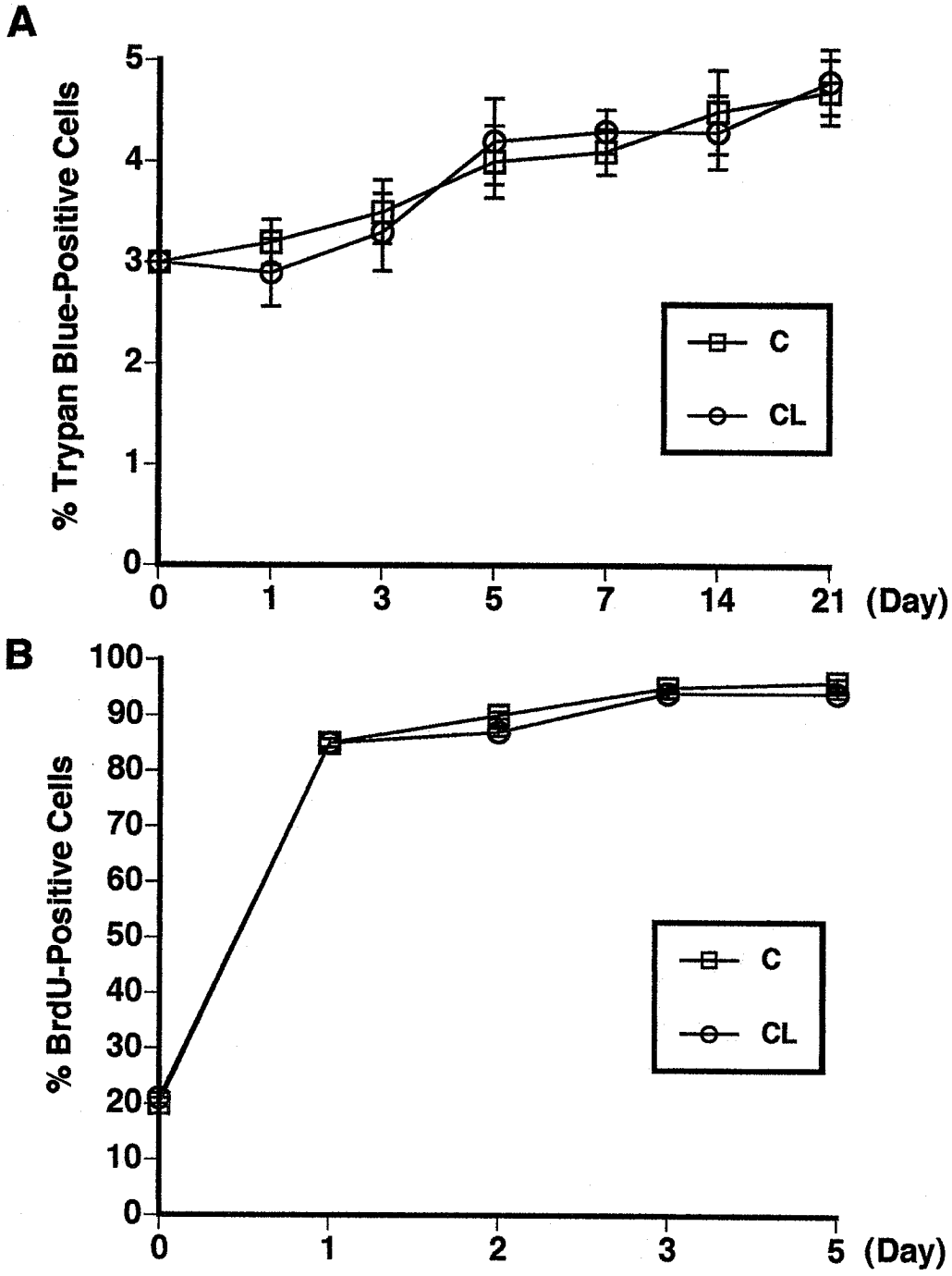

Fig.2

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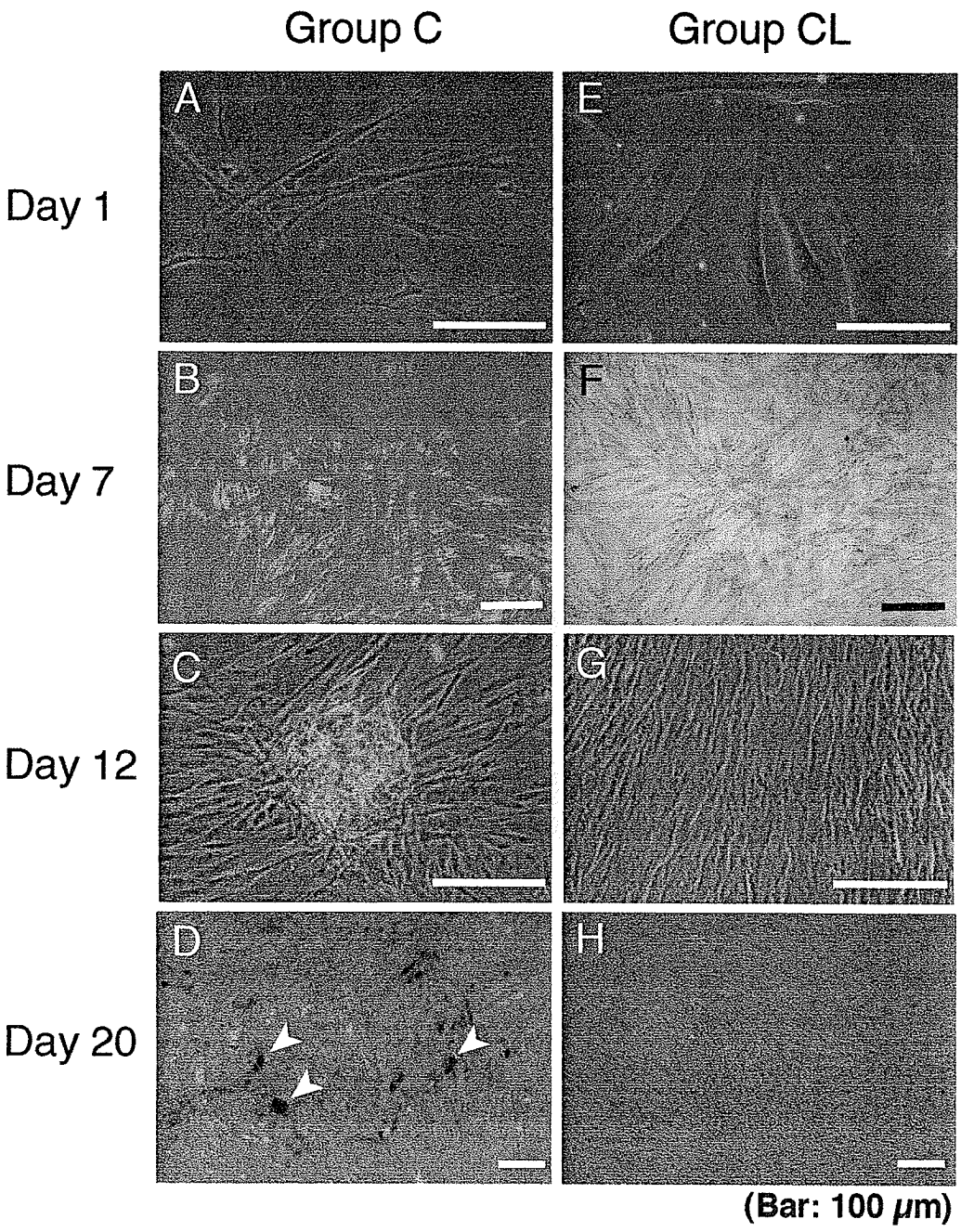

Fig.3

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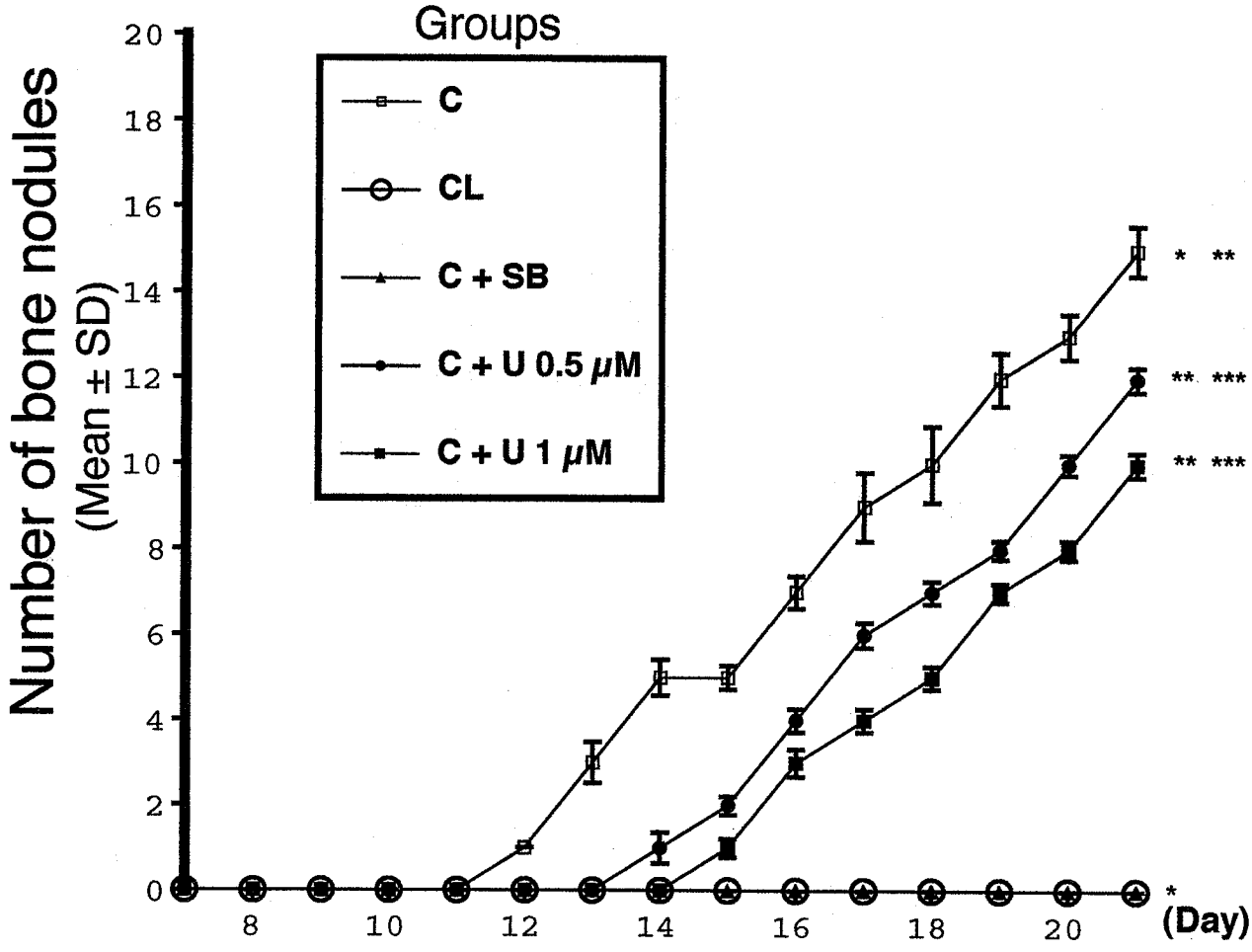

Fig. 4

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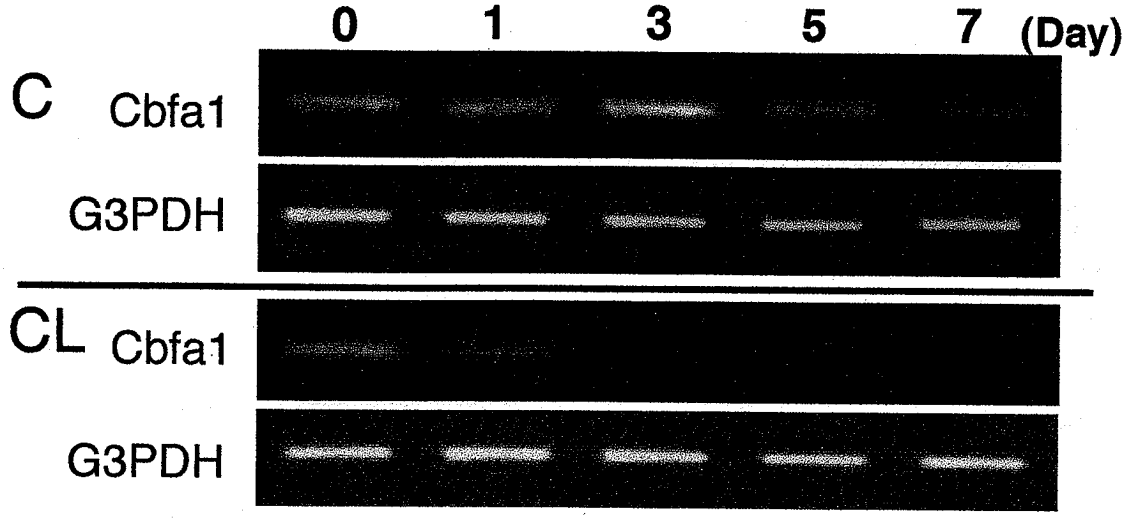

Fig. 5

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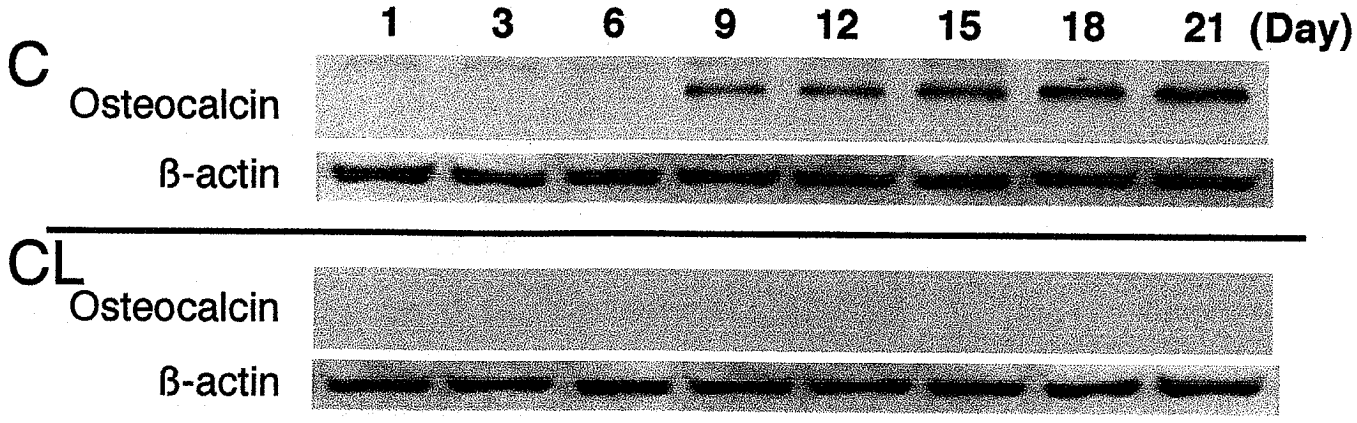

Fig.6

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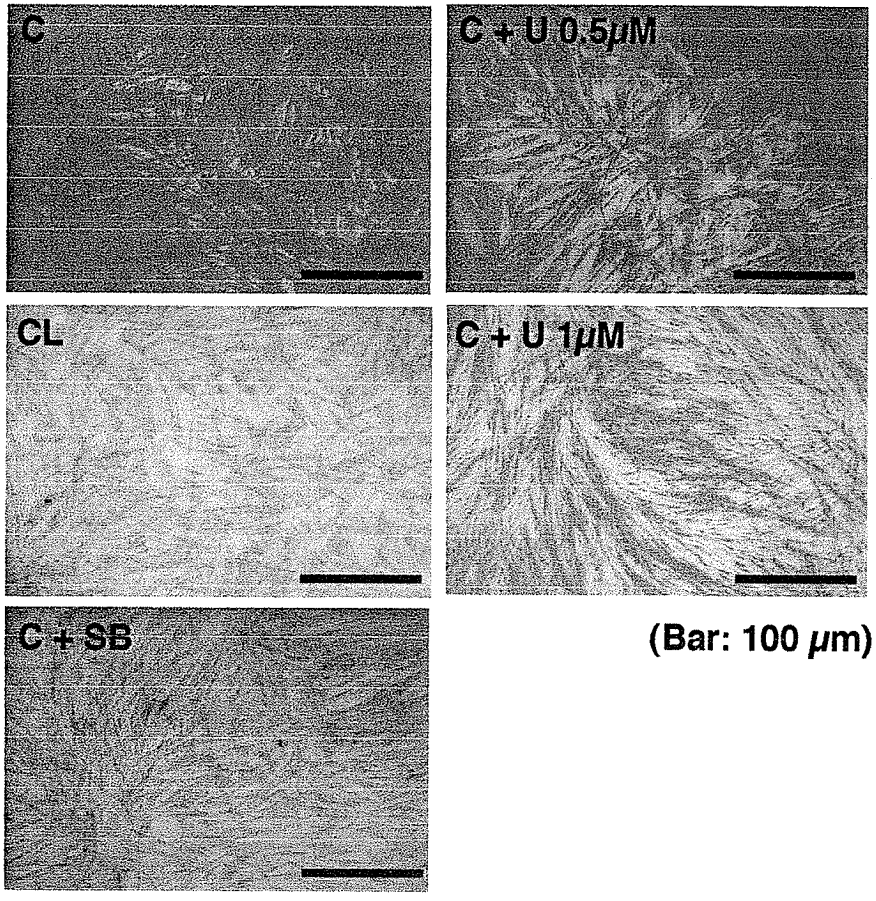

Fig.7

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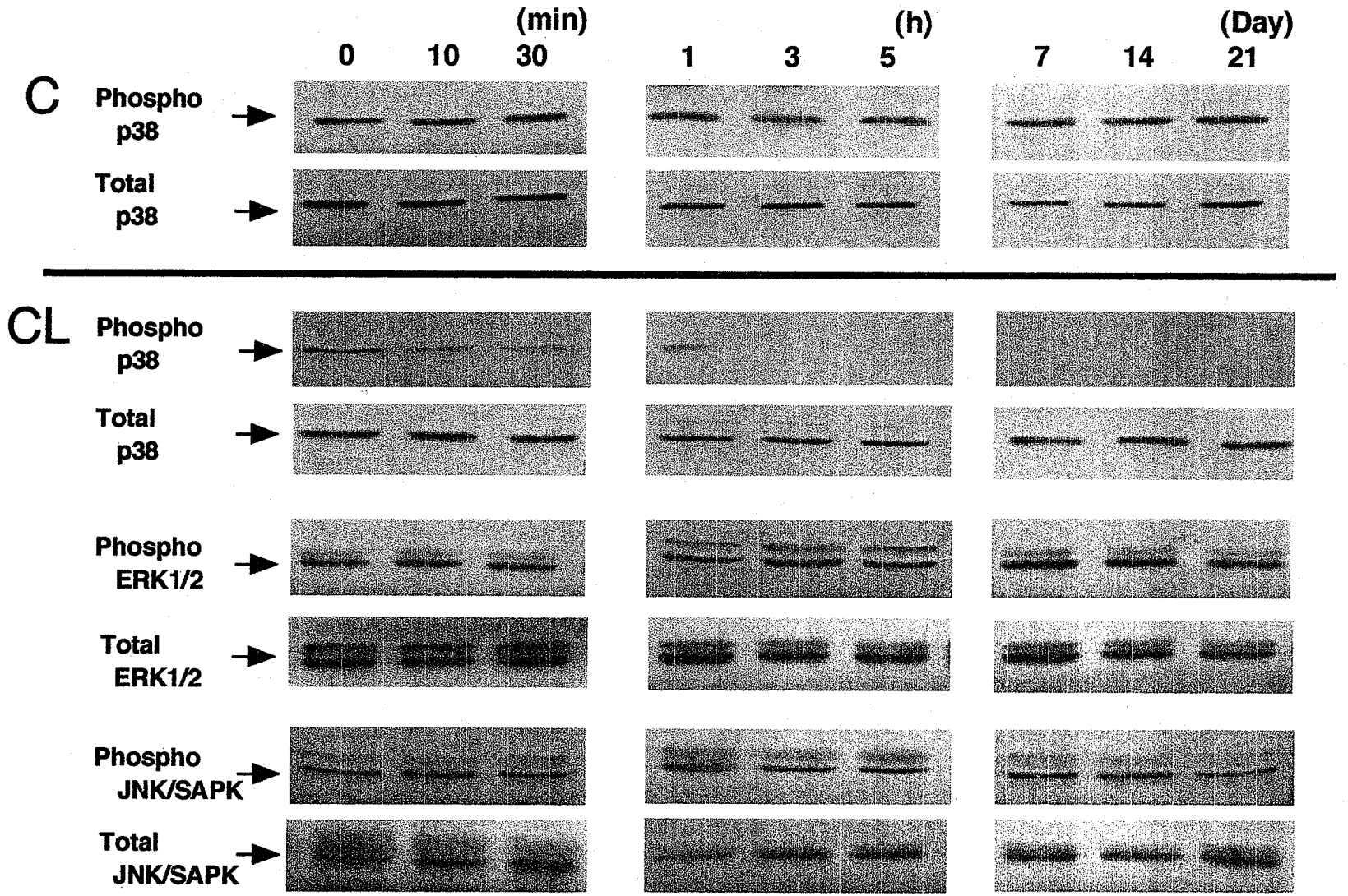

Fig. 8

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