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High Pressure Induces G2 Arrest in Murine Erythroleukemia Cells[1]

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The effect of high pressure on proliferation and cell cycle progression was examined using murine erythroleukemia (MEL) cells. The MEL cells were exposed to high pressures (0.1-130 MPa) and then cultured for 5 days at atmospheric pressure. The proliferation of MEL cells was unaffected up to 60 MPa, but was suppressed at 80-110 MPa. Above 120 MPa, the cells were fragmented. The cell cycle analysis of 80 MPa-treated MEL cells showed that the cells in S phase are most sensitive to high pressure and they arrest in G2 phase. Interestingly, G2-arrested cells reinitiated DNA synthesis, resulting in giant cells with high DNA contents. Furthermore, when such G2-arrested cells were exposed to caffeine, premature mitosis, characterized by chromosome pulverization, was observed. These results suggest that the suppression of proliferation in high-pressure-treated MEL cells is associated with G2 arrest following S phase delay. Thus, it seems valuable to apply high pressure to the investigation of the cell cycle.

Key words: cell cycle, DNA synthesis, G2 arrest, high pressure, premature mitosis.

Interesting results have been obtained from the application of high pressure in biological research. For example, anesthetic effects in tadpoles are reversed by a pressure of about 10 MPa (*1*). Agonist-induced platelet aggregation (*2*) and the biosynthesis of macromolecules such as nucleic acids (*3*) are inhibited under high pressure. The membrane structure of human erythrocytes is destroyed by a pressure of about 130 MPa (*4*). High-pressure-induced hemolysis is more sensitive to the interactions between membrane proteins. Recently, Crenshaw *et al.* have revealed that the cytoskeletal organization in HeLa cells is significantly perturbed by an application of high pressure (~40 MPa), as shown by the disruption of actin stress fibers and intermediate filaments such as vimentin and keratins (*5*). Similar changes, *i.e.*, cell rounding due to perturbation of the cytoskeleton, were reported to occur at pressures of 30-60 MPa in chicken heart fibroblasts (*6*) and kidney epithelial cells (*7*). These data suggest that high pressure is a useful tool to probe the biological systems.

Eukaryotic cells reproduce identical daughter cells around the cell-division cycle known as G1, S, G2, and M phases. The cell cycle is precisely controlled. For instance, before beginning the important events such as DNA synthesis and segregation of replicated chromosomes, the cells have mechanisms to check whether the previous process has been completed (*8, 9*). Like other cellular functions, the progression of the cell cycle is dependent on protein-protein interactions. For instance, cyclin-cyclin dependent kinase complexes play a crucial role in the cell cycle progression (*10*). The activity of such complexes is significantly modified by phosphorylation states of the kinases (*11*). In DNA synthesis, a large multienzyme complex, which is composed of many protein components such as DNA polymerase, DNA helicase, and DNA primase, is formed at a DNA replication fork (*12*). In mitosis, furthermore, microtubules which are formed by polymerization of tubulins play an essential role in the segregation of chromosomes (*13*). These protein associations or complexes are fundamentally formed *via* hydrophobic and/or ionic interactions. It is well known that such interactions are strongly affected by high pressure. Therefore, it is of interest to examine whether exposure of cells to high pressure would affect cell cycle progression. To examine the effects of high pressure not only on the cell cycle, but also on cell differentiation, we selected murine erythroleukemia cells (MEL cells). MEL cells are induced by Friend virus infection of erythroblast progenitor cells and have been used as a model of multi-stage transformation (*14*). Upon exposure to various chemicals, MEL cells can be committed to terminal differentiation to erythrocytes. In the present work, we demonstrate that the proliferation of MEL cells is suppressed by high pressure (80-110 MPa) and that the suppression is associated with G2 arrest following S phase delay.

MATERIALS AND METHODS

Chemicals—Aphidicolin, colcemid, caffeine, and mimosine were obtained from Wako Chemicals. 6-Dimethylaminopurine (6-DMAP), propidium iodide (PI), and ribonuclease A (RNase A) were obtained from Sigma. 5-Bro-

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Abbreviations: AO, acridine orange; BrdUrd, 5-bromodeoxyuridine; 6-DMAP, 6-dimethylaminopurine; FCS, fetal calf serum; MEL, murine erythroleukemia; PBS, 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4; PI, propidium iodide.

modeoxyuridine (BrdUrd)-labeling and detection kit I was obtained from Boehringer Mannheim. All other chemicals were of reagent grade.

Cell Culture and Pressure Treatment—MEL cells (cell line 745A) were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS), streptomycin (0.1 mg/ml), and penicillin G (100 U/ml) at 37°C in a CO_2 (5%) incubator. To synchronize cells in G0 phase, the cells were cultured with 0.5% FCS for 24 or 48 h. Reinitiation of the cell cycle was achieved by the addition of 10% FCS to the medium. To arrest the cells in G1 or G1/S phase, they were cultured in the medium containing mimosine (100 and 200 μM) or aphidicolin (0.3 μg/ml) for 14 h at 37°C and then washed in aphidicolin or mimosin-free medium. The synchrony of the cell cycle was checked using flow cytometry. These synchronized cells and exponentially growing cells ($0.5\text{-}1\times10^6$ cells/ml) suspended in the medium were subjected to various pressures (0.1-130 MPa) for 30 min at 37°C, as described previously in erythrocytes (*4*). After decompression, the cells ($0.5\text{-}1.0\times10^5$ cells/ml) were cultured for 5 days at 37°C. The cell number was calculated by using a hemocytometer. For colony-forming ability, high-pressure-treated cells were inoculated at a density of 100 cells/dish (35 mm) in the medium containing 0.8% methylcellulose (semi-solid culture), and cultured for 5 days. The number of colonies was counted by using a light microscopy (Olympus, model BHC).

Cell Cycle Analysis—MEL cells suspended in the medium were exposed to a pressure of 80 MPa for 30 min at 37°C and then cultured in the presence or absence of aphidicolin (0.3 μg/ml) for 12 h at atmospheric pressure. At appropriate intervals, aliquots ($0.5\text{-}1.0\times10^6$ cells) of cell suspension were centrifuged ($260\times g$, 5 min at 4°C). The cells were washed twice with 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4 (PBS), and fixed with 70% ethanol overnight at −20°C. Prior to flow cytometric analysis, samples were washed in PBS and treated with RNase A (10 μg/ml) in PBS for 30 min at 37°C. These cells were stained with PI (50 μg/ml) for 10 min at room temperature.

To examine the effect of high pressure on cell cycle progression, MEL cells were pulse-labeled with 10 μM BrdUrd for 30 min at 37°C in culture medium. These BrdUrd-labeled cells were subjected to a pressure of 80 MPa for 30 min at 37°C and cultured for 24 h at atmospheric pressure. At intervals, aliquots were harvested, washed with PBS, and fixed with 70% ethanol in 50 mM glycine, pH 2.0 for at least 12 h at 4°C. The incorporation of BrdUrd was examined by using a BrdUrd-labeling and detection kit I. For instance, fixed cells were washed with PBS and incubated with the kit's solution containing both anti-BrdUrd monoclonal antibody and restriction DNase for 1 h at 37°C. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG for 30 min at 37°C, washed with PBS, and stored at 4°C until flow cytometric analysis. RNase A treatment and PI counterstaining were conducted as stated above.

To estimate the rate of mitosis, MEL cells were exposed to a pressure of 80 MPa for 30 min at 37°C and then cultured for 0-24 h at 37°C and atmospheric pressure. Then, these cells were incubated in the presence or absence of colcemid (0.08 μg/ml) for 1 h at 37°C. They were washed with PBS, incubated for 5 min in 75 mM KCl at room temperature and fixed in methanol/acetic acid (3 : 1, v/v) at 4°C (*15*). The samples were mounted onto glass slides and stained with giemsa or Hoechst 33258 (1 μg/ml). Using a light microscopy, the number of mitotic cells was counted. To examine the effect of caffeine and 6-DMAP on G2 arrest, 80 MPa-treated MEL cells were cultured for 9 h at 37°C and atmospheric pressure and then incubated for a further 3 h in the presence or absence of caffeine (3 mM) or 6-DMAP (1 mM). The cells with condensed chromosomes were detected by flow cytometry. For instance, ethanol-fixed cells in 50 μl of PBS were placed in 250 μl of 0.1 N HCl, allowed to stand for 45 s at room temperature, and then mixed with 1 ml of acridine orange (AO, 30 μg/ml) in 0.1 M citric acid-0.1 M sodium phosphate, pH 2.6. The acid-AO-staining method makes use of the property that mitotic or mitotic-like cells are distinguished from interphase cells by the sensitivity of condensed chromosomes to acid denaturation (*16, 17*).

Flow Cytometry—Flow cytometric analysis was performed by using a FACScan (Becton-Dickinson). Excitation was accomplished by the use of an argon laser emitting at 488 nm with 15 mW power. Red fluorescence was detected using a 650-nm (for AO signal) or 585-nm (for PI signal) long-pass filter and green fluorescence was detected using a 530-nm band-pass filter.

RESULTS

Effects of High Pressure on Proliferation of MEL Cells—MEL cells were subjected to various pressures (0.1-130 MPa) for 30 min. After decompression, the cell viability was determined by trypan blue exclusion. Above 120 MPa, the cells were fragmented. In other cases (0.1-110 MPa), the dye was excluded from all the cells. These cells were cultured at atmospheric pressure to examine the effect of high pressure on proliferation (Fig. 1). Up to 60 MPa, the proliferation of MEL cells was almost unaffected. A delay of proliferation was observed at 80 MPa. At both 100 and 110 MPa, the cell density decreased, indicating that a certain population of the cells died during the culture. The suppression of proliferation was particularly apparent at

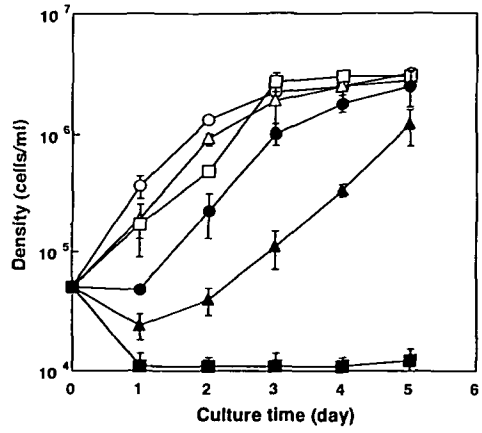


Fig. 1. **Effects of high pressure on proliferation of MEL cells.** MEL cells were subjected to various pressures (0.1-110 MPa) (○, 0.1; △, 40; □, 60; ●, 80; ▲, 100; ■, 110) for 30 min at 37°C. After decompression, the cells were cultured under atmospheric pressure. Values are the mean±SD of three independent experiments.

110 MPa.

The exposure time of MEL cells to high pressure may also affect the rate of proliferation at atmospheric pressure. So, MEL cells were incubated for 5, 30, or 60 min at 80 MPa, and then cultured at atmospheric pressure. The proliferation of MEL cells was unaffected by a short exposure (5 min) to high pressure, but was affected similarly at both cases of 30 and 60 min. For ease of comparison with our previous results concerning other cells (*4*), the same condition (*i.e.*, 30 min) was used in this work.

The growth curves of MEL cells subjected to high pressure suggest that the cells may be damaged by high pressure. To clarify this point, we examined the colony-forming ability of high-pressure (80 and 100 MPa)-treated MEL cells. The number of colonies decreased because of cell death and the formation of giant cells, but the size of colonies was almost the same as that of untreated cells (data not shown). Thus, the growth curves of cells exposed to 80-100 MPa are consistent with the idea that a part of the cell population is incapable of proliferating due to cell death and the formation of giant cells.

Cell Cycle Analysis of High-Pressure-Treated MEL Cells—The DNA content of high-pressure-treated MEL cells was analyzed by flow cytometry (Fig. 2). The MEL cells were subjected to a pressure of 80 MPa and then cultured at atmospheric pressure. The DNA histogram of the MEL cells measured immediately after decompression

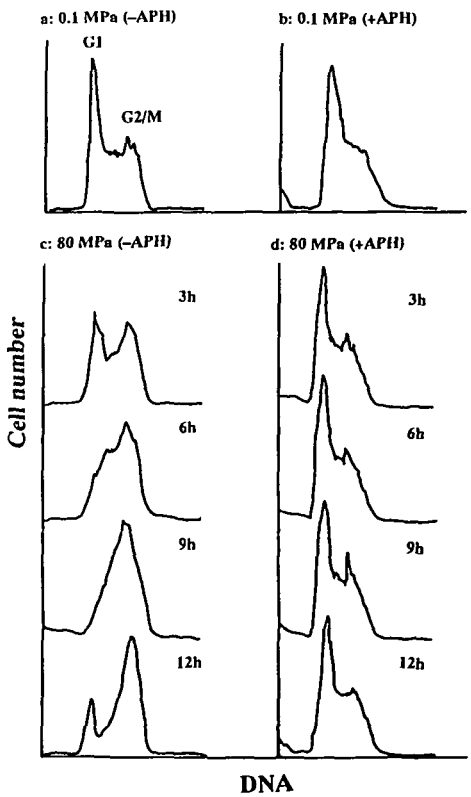


Fig. 2. **DNA histograms of 80 MPa-treated MEL cells.** High-pressure-untreated MEL cells were cultured for 12 h at 37°C in the absence (a) or presence (b) of aphidicolin (0.3 μg/ml). MEL cells, subjected to a pressure of 80 MPa for 30 min at 37°C, were cultured for 3-12 h in the absence (c) and presence (d) of aphidicolin at atmospheric pressure. At the indicated time, cells were harvested and used for flow cytometric analysis. APH, aphidicolin.

was almost the same as that of a high-pressure-untreated sample (Fig. 2a). During the culture, the number of cells in G1 decreased, whereas G2/M cell populations increased (Fig. 2c). After 12 h, the peak corresponding to G1 cells reappeared. To examine whether the decrease in G1 cell population is due to progression to the next stage or cell death, 0.1 MPa- and 80 MPa-treated cells were cultured in the presence of aphidicolin. Aphidicolin, a potent *in vitro* inhibitor of DNA polymerase α (*18*), is widely used to synchronize the cell cycle at the G1/S boundary (*19*). In high-pressure-untreated cells, G1/S boundary and S phase cells were accumulated due to inhibition of DNA synthesis. Similarly, upon aphidicolin treatment of 80 MPa-treated cells no decrease in the number of G1 cells was observed, indicating that the decrease of G1 is due to progression to the next stage (Fig. 2d). Thus, the cell cycle in 80 MPa-treated MEL cells seems to be arrested in the G2/M phase.

To analyze the sensitivity of each phase in the cell cycle to high pressure, MEL cells were synchronized by serum starvation or by the use of cell cycle inhibitors such as aphidicolin and mimosine (*19, 20*), and cultured for various times to allow cell cycle progression. These MEL cells were subjected to a pressure of 80 MPa. However, all of these synchronized cells were disrupted by high pressure (data not shown). This enhanced lethality may be related to unbalanced growth rather than cell cycle stage. Thus, it was difficult to analyze the difference in the response of each phase of the cell cycle to high pressure by using cell cycle synchrony methods. Therefore, we attempted to examine the cell cycle progression by using BrdUrd, a thymidine analog which is incorporated into cells in S phase. The distribution of exponentially growing cells pulse-labeled with BrdUrd is shown by using a dot plot in Fig. 3A (left panel). We set six windows (a-f) to estimate the cell cycle progression, where windows a (or d), b (or e), and c (or f) correspond to G1, S, and G2/M phases in unlabeled (or labeled) cells, respectively.

The dot plot pattern after 6 h shows that labeled (*i.e.*, S phase) cells divide and enter the G1 phase and that unlabeled cells progress to the G2/M phase from the G1 phase (Fig. 3A, right panel). Thus, it was found that the cell cycle progression can be monitored by using this method. So, we examined the effect of high pressure on cell cycle progression (Fig. 3B). MEL cells labeled with BrdUrd were exposed to a pressure of 80 MPa and then cultured at atmospheric pressure. In unlabeled cells at the G1 or G2/M phases during pulse-labeling, no effect of high pressure on cell cycle progression was observed except for an initial time lag (Fig. 3B, windows a, b, and c). On the other hand, the cell cycle progression of BrdUrd-labeled cells was greatly affected by high pressure (Fig. 3 B, windows d, e, and f). Namely, the progression of S phase to G2/M phase was extremely delayed. During culture for 12 h, the cell population in S phase decreased and G2/M cells (seen in window f) were accumulated. These results suggest that the cells in S phase are more sensitive to high pressure than the cells in other phases.

G2 Arrest Induced by High Pressure—To examine whether the accumulation in the G2/M phase of 80 MPa-treated MEL cells is due to G2 arrest or M arrest, the rate of cells in mitosis (mitotic index) was estimated as a function of incubation time after decompression in 80 MPa-treated MEL cells (Fig. 4). The values of mitotic index

remained low up to 12 h but then began to increase. Colcemid, which causes the disappearance of the mitotic spindle, was utilized to induce mitotic arrest. For example, upon exposure to colcemid of pressure-untreated MEL cells for 1 h, the value of mitotic index increased from 2 to 8%. On the other hand, when 80 MPa-treated cells were cultured for various times and then exposed to colcemid, the number of the cells that entered mitosis was low up to 12 h. These results indicate that high-pressure-treated MEL cells arrest in the G2 phase rather than the M phase.

Cell Cycle Progression of G2-Arrested Cells—When the cells arrested in G2 phase by high pressure were further cultured for several hours (3-12 h), these cells reinitiated DNA synthesis without cell division, so that they became polyploid cells (Fig. 5). At 12 h, about half of the G2-arrested cells had initiated DNA synthesis.

Methylxanthines such as caffeine have been utilized to override the G2 checkpoint (*21-23*). So, we examined using flow cytometry whether high-pressure-induced G2 arrest is overridden by caffeine (Fig. 6A). When G2-arrested cells,

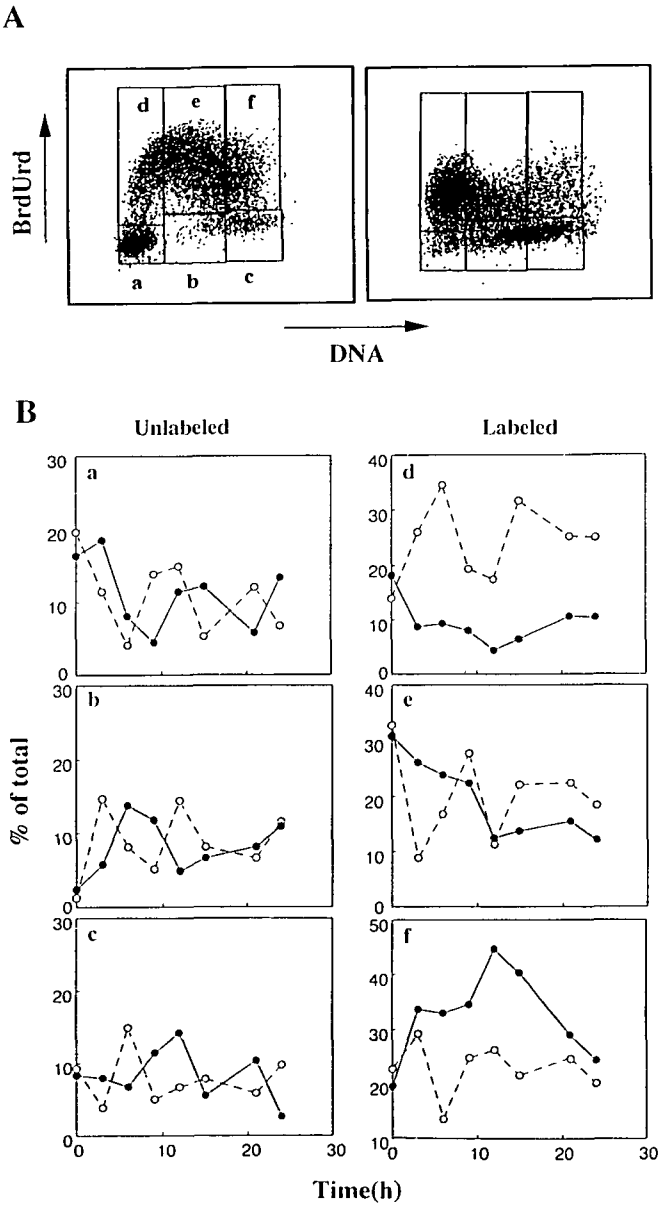


Fig. 3. **Effects of high pressure on cell cycle progression of BrdUrd-labeled MEL cells.** (A) Exponentially growing cells were pulse-labeled with BrdUrd (left panel) and then were cultured for 6 h (right panel). Six analytical windows were set according to the signal intensities of DNA and BrdUrd. (B) BrdUrd-labeled cells were incubated at 0.1 (open circles) or 80 MPa (closed circles) for 30 min at 37°C, and cultured at atmospheric pressure during 24 h. Aliquots were withdrawn at indicated times and used for flow cytometric analysis as described in "MATERIALS AND METHODS." Values (as mean percentage of duplicates) show the rate of cells in each window at the indicated times.

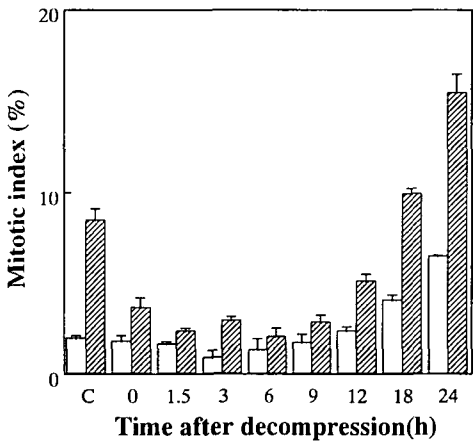


Fig. 4. **Mitotic index of 80 MPa-treated MEL cells.** MEL cells were subjected to a pressure of 80 MPa for 30 min at 37°C. After decompression, cells were cultured at atmospheric pressure. At the indicated times, aliquots were withdrawn from the cell suspension and cultured in the absence (□) or presence (▨) of colcemid (0.08 μg/ml) for 1 h at 37°C. C, high-pressure-untreated MEL cells. Values are the mean±SD of three independent experiments.

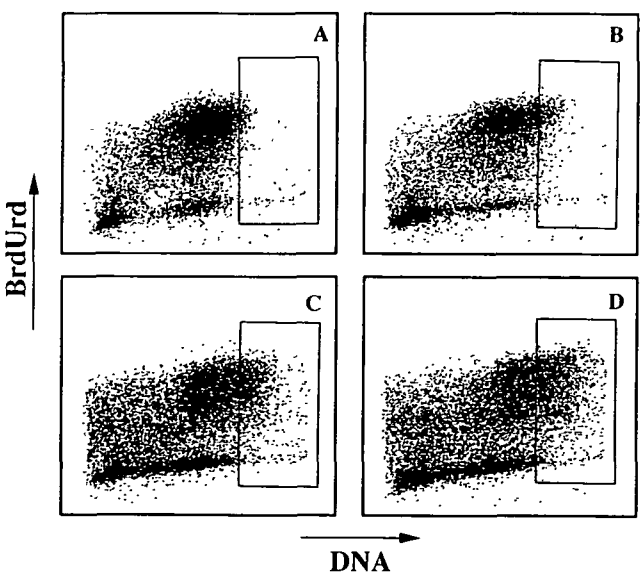
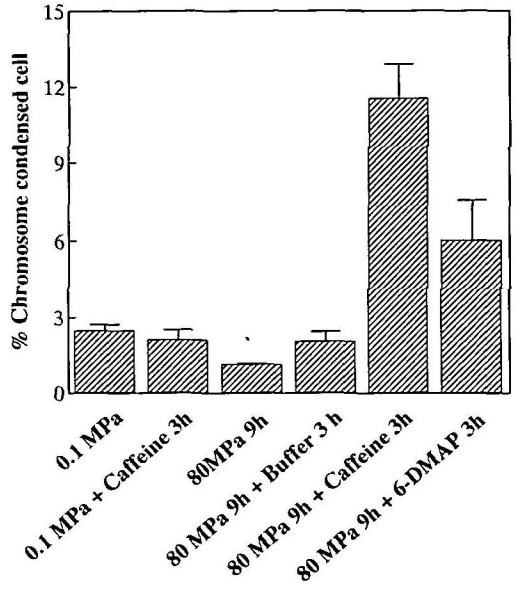


Fig. 5. **Reinitiation of DNA synthesis of G2 arrested cells.** The results of flow cytometric analysis of 80 MPa-treated MEL cells shown in Fig. 3 are represented as a dot plot. G2-arrested cells (A) incubated for 12 h after decompression were cultured for a further 3 (B), 6 (C), or 12 h (D).

A



B

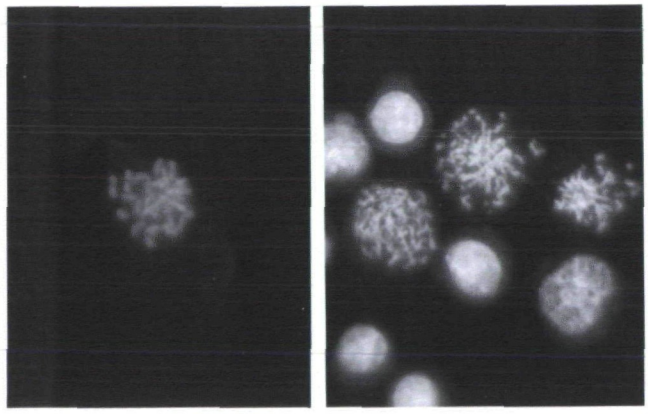


Fig. 6. **Effects of caffeine and 6-DMAP on high-pressure-induced G2 arrest.** (A) 80 MPa-treated MEL cells were cultured for 9 h at 37°C and atmospheric pressure. These cells and pressure-untreated ones (Control) were incubated in the presence or absence of 3 mM caffeine or 1 mM 6-DMAP for 3 h at 37°C. The percentages of cells with condensed chromosomes were estimated by flow cytometry of acid-AO-stained cells. Values are the mean ± SD of three independent experiments. (B) Chromosome spreads of normal mitotic cells (left panel) and caffeine-induced mitotic cells (right panel) were observed by fluorescence microscopy. Cells were swollen in hypotonic solution and fixed with methanol/acetic acid (3 : 1, v/v). These cells were stained with Hoechst 33258.

which appear at 9 h after decompression, were cultured in the presence of 3 mM caffeine, the number of cells with condensed chromosomes was enhanced (Fig. 6A). The condensation of chromosomes can also be induced by 6-DMAP, a protein kinase inhibitor (*23*). In fact, similar phenomena were caused by the 6-DMAP (1 mM) treatment of G2-arrested cells. In addition, when chromosome spreads after caffeine treatment of G2-arrested cells were examined by fluorescence microscopy, chromosome pulverization, which is characteristic of premature mitosis, was observed (Fig. 6B).

DISCUSSION

In the present work, we have demonstrated that the proliferation of MEL cells is suppressed upon exposure to high pressures of 60-110 MPa, but that above 120 MPa the membrane structure of MEL cells is destroyed irrespective of the stage in the cell cycle. Similar results were obtained in Chinese hamster ovary cells and human melanoma cells (Matsumoto *et al.*, unpublished observations). Previously, we showed that the hemolysis of human erythrocytes starts to occur at about 130 MPa (*4*). In such hemolyzed cells, cytoskeletal proteins such as spectrin are partially detached from the membrane (*4*). Furthermore, when Ehrlich ascites tumor cells are subjected to high pressure (up to 150 MPa) *in vitro* and then intraperitoneally inoculated into mice, the proliferation of tumor cells exposed to 130 MPa or more is inhibited (*24*). Taking these data into consideration, it seems likely that membrane damage to mammalian cells is induced by a pressure of 120-130 MPa under our conditions. On the other hand, physiological functions of the cells are affected by lower pressures.

From cell cycle analysis of BrdUrd-labeled MEL cells, it was found that a pressure of 80 MPa induces G2 arrest following S phase delay. It is useful to compare this results with G2 arrest induced by other methods. Ultraviolet and γ-irradiations produce DNA damage such as thymine-thymine dimers and double-strand breaks, respectively, so that they induce G2 arrest (*25-27*). G2 arrest allows the cells to completely repair damaged DNA prior to entry into mitosis. On the other hand, the cause of G2 arrest induced by high pressure may be different from that in the case of radiation. As shown by the fact that the DNA denaturation by heating is suppressed under pressure, in general, high pressure stabilizes DNA double strands (*28*). In this work, we have shown that MEL cells in G1 and G2 phases progress normally through the cell cycle after decompression. Therefore, DNA damage of the type produced by radiation would not be induced by high pressure. However, high pressure may induce the dissociation of associated proteins. For instance, oligomeric proteins such as actin filaments and microtubules are dissociated under high pressure (*5, 6, 29, 30*). The multiprotein structures that assemble in DNA- and protein-synthesizing systems may be significantly perturbed by high pressure. However, we can not neglect the effects of high pressure on DNA polymerase and catabolic processes of MEL cells. Thus, further studies on high-pressure-induced S phase delay are necessary.

The G2 arrest induced by a pressure of 80 MPa is associated with delay of S phase progression. However, there are few data about the effect of high pressure on cell cycle control. On the other hand, G2 arrest induced by DNA damage is well characterized. For instance, anticancer agents and ionizing radiation induce rapid inactivation of $p34^{cdc2}$-cyclin B1 kinase (*27*), which plays a central role in the onset of mitosis (*11, 31-33*). Recent data indicate that such an inactivation of the kinase is associated with the phosphorylation of tyrosine 15 in $p34^{cdc2}$ (*34-36*). Here, it is useful to consider the effect of caffeine on cell cycle progression. Radiation-induced G2 arrest is abolished by caffeine, so that the cells become more susceptible to radiation (*22, 37, 38*). This enhanced lethality may be

associated with premature entry into mitosis. Similar effects of caffeine were observed in the present work. Upon exposure to caffeine, high-pressure-induced G2-arrested cells prematurely enter mitosis. Although the abnormalities in the S phase are different in the case of high pressure and radiation, the responses of these G2-arrested cells to caffeine are similar to each other. Thus, it seems likely that the G2 checkpoint pathway can also be activated by abnormal S phase progression induced by high pressure, as well as DNA damage.

In normal cells, DNA replication is restricted to once per cell cycle. Using 80 MPa-treated cells, however, we have found that the G2-arrested cells reinitiate another round of DNA synthesis during prolonged culture. Such abnormal DNA synthesis is not rare. Anti-tumor drugs such as adriamycin can induce endoreduplication following G2 arrest in cells lacking p21, a cyclin-dependent kinase inhibitor (*39*). Tetraploid cells are formed by heat treatment of temperature-sensitive *cdc2* mutants in fission yeast, which arrest in G2 phase at a restrictive temperature (*40*). Furthermore, K-252a, which may inhibit $p34^{cdc2}$-cyclin B1 kinase activity, can also induce multi-rounds of DNA replication without mitosis (*41*). Thus, abnormal DNA synthesis is brought by uncoupling of S phase and mitosis due to prolonged G2 arrest, destruction of G2 kinase activity, *etc.*

In the present work, we have described the effect of high pressure on the proliferation or cell cycle progression of MEL cells. It is of great interest that G2 arrest due to perturbation of the S phase is induced by high pressure. Some of the G2-arrested cells reinitiate DNA synthesis without mitosis. Application of high pressure to higher eukaryotic cells should provide unique information on the cell cycle at the molecular level.

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