Pressure Sensitivity of HSP70 Promoter in Transformed 3T3-L1 Cells

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Mouse 3T3-L1 cells were stably transfected with a plasmid encoding heat shock protein 70 (HSP70) promoter and luciferase gene to probe pressure sensitivity by measuring luminescence. When the cells are exposed in extremely high hydrostatic pressure, the HSP70 promoter can activate expression of luciferase, which induces luminescence. HSP70 promoter activity enhanced by exposure to 40MPa or greater hydrostatic pressure for 30min, although significant morphological changes occurred. In the pressure ranges between 40 to 60 MPa (0.1MPa　1atm), more than 95% of the cells kept alive according to the cytotoxicity assay and respreaded on pressure release and proliferated normally through the cell cycle. At 70MPa-pressure application, HSP70 promoter activity became at maximum and majority of the cells (>76%) damaged or died.

Keywords : 3T3-L1 cells, HSP70, hydrostatic pressure, cell viability, morphological change

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1. Introduction

Despite multicellular organisms have been proved to live in the deep sea at a depth of 11,000m, it remains unclear whether surface or shallow dwelling organisms could adapt to the extremely high hydrostatic pressure environments. Kojima and co-workers reported that the geographical distribution of deep sea organism tube worms were distributed over wider geographical ranges (depth from 82 to 2,000m) and their dispersion was tend to be limited by water depth rather than by horizontal distances (Kojima, 1995). On the other hand, each species of deep sea clams *Calyptogena* was generally narrow and many endemic species were sampled from single sites (Kojima, 1995), although genus *Calyptogena* were widely collected from 6366m depth (*C. phaseoliformis*) (Fujioka, 1992) to 1,100m depth (*C. soyoae*) (Sakai, 1987). From these reports, surface or shallow dwelling organisms could adapt to the extremely high hydrostatic pressure environment up to 60MPa, which would correspond to 6,000 meters depth. The authors, therefore, paid an attention to clarify whether the surface dwelling organisms can alive and induce protective function such as heat shock protein under extremely high hydrostatic pressure exposure.

To address this question, the mouse 3T3-L1 cell was transfected with a plasmid encoding heat shock protein 70 (HSP70) promoter and luciferase genome. The transfected cells produce luciferase protein when the cells are exposed to cellular stresses. In the present study, stress sensitivity of the transfected cells was investigated under extremely high hydrostatic pressure stress, and compared with calcein-AM・EthD-1 cytotoxicity assay. We show that the 3T3-L1 cells induces HSP70 promoter activity over 30MPa and almost all of the cells are kept alive until 60MPa hydrostatic pressure. Intense HSP70 promoter activity was observed at 70MPa high hydrostatic pressure, although 76% of the cells were damaged or died according to the cytotoxicity assay.

2. Materials and methods

2.1. Transfection of 3T3-L1 cell

3T3-L1 cell (American Type Culture Collection) was cultured in a Dulbecco's modified Eagle medium (DMEM; ICN Biomedicals, Inc., Ohio, USA) supplemented with 10%(v/v) fetal bovine serum (FBS; Bio whittaker, Maryland, USA), 50 units/ml penicillin, and 50μg/ml streptomycin (Bio whittaker, Maryland, USA). 3T3-L1 cell was stably transfected with the Picagene basic vector 2 (Wako, Osaka, Japan) which contained the mouse HSP 70 promoter and luciferase encoding gene (Fig. 3). The selection plasmid pMAM2-BSD (Funakoshi, Tokyo, Japan) was co-transfected to confer resistance to Blasticidin S (Funakoshi, Tokyo, Japan). Transfections were performed using Transfectum™ and serum free DMEM generally according to the manufacturer's instruction. Briefly, 3T3-L1 cells were plated at a density of 2×10^4 cells/35mm dish and incubated in 1ml of serum free DMEM supplemented with 50 units/ml penicillin, 50μg/ml streptomycin, 3μg/ml plasmid DNA, and 9μl/ml Transfectum for 1 hr. After 1 hr incubation, 5ml of DMEM containing 10% FBS, 50 units/ml penicillin, and 50μg/ml streptomycin added to the serum free medium and cultured for another 2days. After another 2 days cultivation, the cells were put under selection pressure using DMEM containing 10% FBS, 50 units/ml penicillin, 50μg/ml streptomycin, and 8μg/ml Brasticidin S. Transfected 3T3-L1 cells were obtained after 2 weeks of the cultivation.

2.2. Hydrostatic pressure application

The transfected 3T3-L1 cells were plated a density of $2\sim4\times10^4$ cells/cm^2 on 35mm plastic petri dishes (Falcon Plastic, CA, USA) in DMEM containing 10% FBS, 50 units/ml penicillin, 50μg/ml streptomycin, and 4μg/ml Brasticidin S.

The cells were grown at 37˚C in a humidified atmosphere of 5% CO_2. The cells were replaced in serum free DMEM containing the same concentrations of penicillin, streptomycin, and Brasticidin S and cultured for another 24-60 hr. In serum free condition, 3T3-L1 cells were synchronized with an interphase of cell cycle. The petri dishes were filled with the fresh serum free DMEM and tightly sealed by parafilm (American national can, USA) after air bubbles were removed. The sealed petri dish was placed in a titanium pressurization vessel (inside diameter 62×198 mm; Rigo-sha, Tokyo, Japan) equipped with a hydraulic pump (Tomita, Tokyo, Japan). Hydrostatic pressure was applied to the cells in the pressurization vessel that varied from 5 to 70MPa. Hydrostatic pressure was transmitted to the cells through the flexible sealed film. The given compression speed was 3~5 MPa/sec, and decompression was -15 ~ -20 MPa/sec. The tempera-

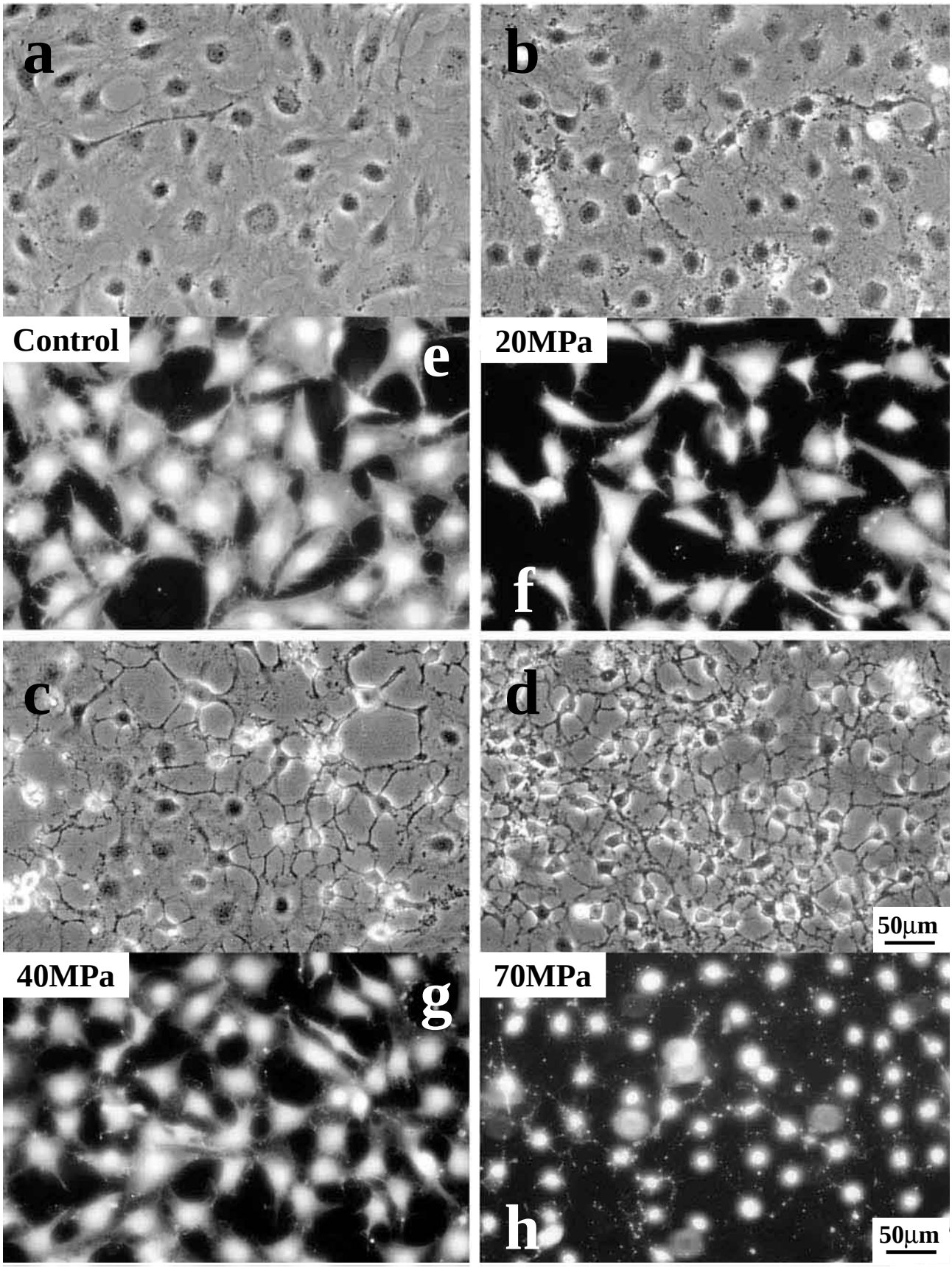

Fig. 1 Rounding cells at high hydrostatic pressure. 3T3-L1 cells at (a) 0.1, (b) 20, (c) 40, and (d) 70MPa hydrostatic pressure immediately after pressurization. Calcein-AM and EthD-1 stained 3T3-L1 cells at (e) 0.1, (f) 20, (g) 40, and (h) 70MPa hydrostatic pressure after 24hr of incubation.

ture was maintained at 37°C with a water bath in which the pressurization vessel was stayed. It was confirmed that we did not change pH of the culture medium in before and after the pressurization.

After the pressurization, each culture medium in the petri dish was exchanged for the fresh serum free DMEM supplemented with the same antibiotics. The cells were maintained at atmospheric pressure in the 5% CO_2 and were incubated for another 24hr. After the incubation, the cells were assayed for luciferase activity and cell viability.

2.3. Luciferase assays

For luciferase analysis, the transfected 3T3-L1 cells were plated a density of 2×10^4 cells/cm^2 on 35mm plastic petri dishes (Falcon Plastic, CA, USA) in DMEM containing 10% FBS, 50 units/ml penicillin, 50μg/ml streptomycin, and 4μg/ml Brasticidin S. The activity of luciferase was determined with the Picagene LT2.0 luminescence kit (Touyou-Inki, Tokyo, Japan) according to the kit procedures. Luminescence from the luciferin-luciferase was detected with a luminometer (NU-600; Microtech-Nichion, Chiba, Japan). The luminescence quantity averaged the independent experiment of 4 times.

2.4. Cell viability assays

The transfected 3T3-L1 cells were incubated with both 2μM calcein-AM and 4μM EthD-1 (live/dead viability/cytotoxicity kit; Molecular probes, Inc., Oregon, USA) in PBS for 37°C. After incubation at 15min., the transfected 3T3-L1 cells were observed a fluorescence microscope under blue excitation light. The live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the intensely green fluorescence of calcein (ex/em ~495nm / ~515nm). EthD-1 enters the cells with damaged membranes and producing a bright red fluorescence in nuclei of dead cells (ex/em ~495nm / ~635nm).

3. Results

3.1. Pressure effects on cell viability

Viability of interphase mouse 3T3-L1 cells was examined when they were exposed to extremely high hydrostatic pressure. The 3T3-L1 cells had spread and grown to $2\sim4\times10^4$ cells/cm^2 (70-100% confluence) at 37°C and 1 atm pressure. The cells were exposed to the hydrostatic pressure for 30 min varied from 10 to 70MPa . Most of the cells were rounded at above 40MPa (Fig. 1). Little or no rounding was observed until 30MPa. After the 24hr of incubation at atmospheric pressure, the shrunk cells re-spread and recovered from the round up shape, except 70MPa (Fig. 1). The cells were partially detached from the bottom of petri dish at 70MPa hydrostatic pressure. The morphological changes seem to depend not only on the pressure level but also on the cell density, because the morphological changes became easy when the cell density was low.

Cell viability was investigated by the double staining with both calcein-AM and EthD-1 as described in materials and methods. Using the double staining technique, living cells emit green fluorescence of calcein by esterase activity. On the other hand, dead cells generate a bright red fluorescence resulted in EthD-1 enters cells with damaged membranes. In these experimental conditions except 70MPa , majority (>95%) of 3T3-L1 cells were kept alive (Fig. 2). At 70MPa application, over 76% of the cells were damaged or died according to the bright red fluorescence of EthD-1 (Fig. 2). It is noted that only a few 3T3-L1 cells recover from the round up shape and become growth after 70MPa-pressure application when the cell density is confluence (data not shown). In addition, these pressure effects also occurred when 3T3-L1 cells were transfected with plasmid vector.

3.2. Effect of pressure on HSP70 promoter activity

Mouse 3T3-L1 cells were transfected with a plasmid vector that contained the mouse HSP70 promoter and

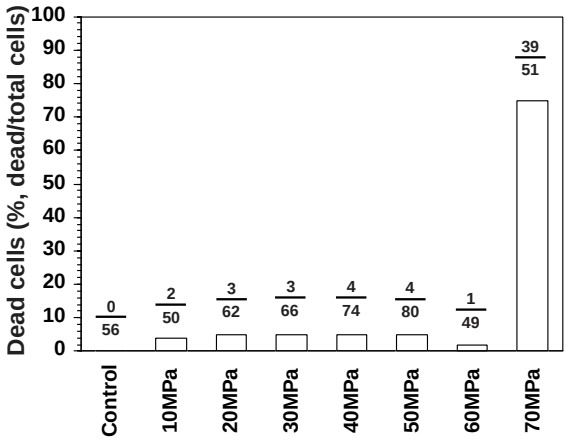

Fig. 2 Relative rate of dead cells. The pressure exposed 3T3-L1 cells were measured using a double staining of calcein-AM and EthD-1 after 24hr of incubation.

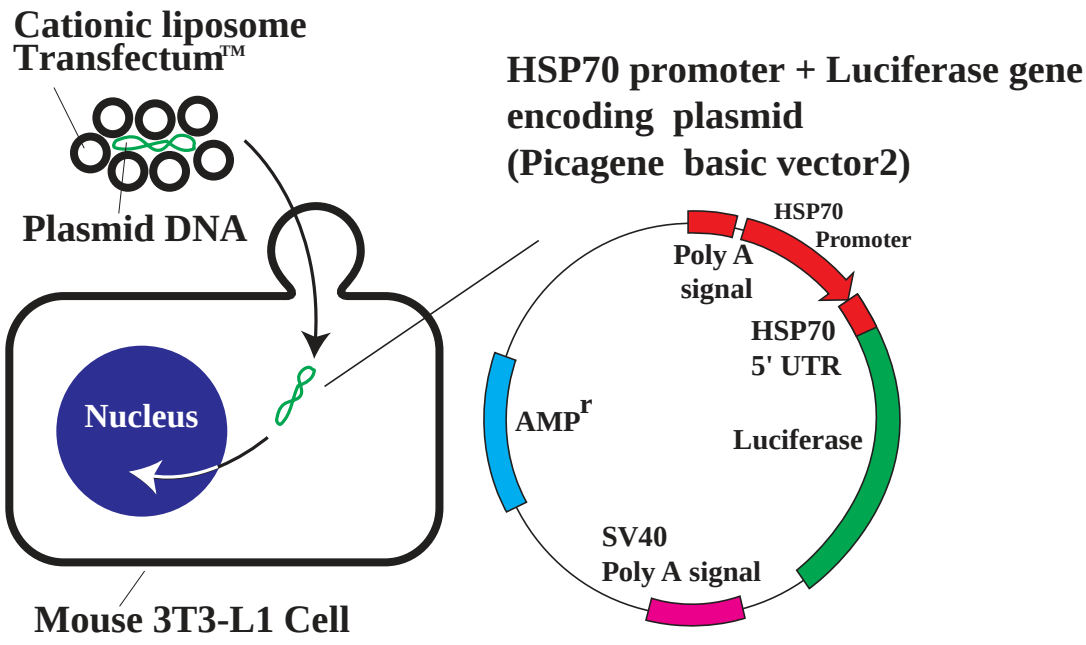

Fig. 3 HSP70 promoter and luciferase encoded plasmid vector.

luciferase gene (Fig. 3). The 3T3-L1 cells had spread and grown to 2×10^4 cells/cm^2 (70-90% subconfluent) at 37°C and 1 atm pressure. The cells were exposed to the hydrostatic pressure for 30 min varied from 5 to 70 MPa. Hydrostatic pressure induced HSP70 promoter activity was detected by luciferase activity after 24 hr of incubation under atmospheric pressure (Fig. 4). Exposure to 40MPa or greater hydrostatic pressure enhanced HSP70 promoter activity ($P<0.01$, vs. control). At 70MPa of hydrostatic pressure exposure, HSP 70 promoter activity was enhanced at maximum and increased about 7 fold over the control ($P<0.001$). In the cells exposed to lower pressure (5-30 MPa), no elevation in luciferase protein levels was observed.

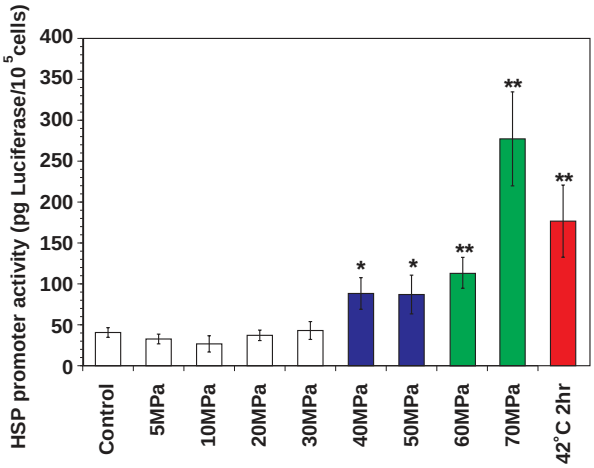

Fig. 4 Hydrostatic pressure induced HSP70 promoter activity. Transfected 3T3-L1 cells were exposed in the hydrostatic pressure for 30 min. The cells were collected after 24 hr of incubation and assayed for luminescence. Values were the means ± S.D. of 4 independent determinations (*$p<0.01$, **$p<0.001$; compared to the control by Student's t-test).

4. Discussion

Several researchers reported that subjecting surface dwelling organisms to 30MPa (0.1MPa=1atm) or greater hydrostatic pressure may induce significant changes in cell morphology (Bourns, 1988; Crenshaw, 1996; Landau, 1960; Landau, 1961). In the pressure range between 30 and 60 MPa, the cells spread back out and normally through the cell cycle when pressure is released (Landau, 1960; Landau, 1961). On the other hand, it has been known for many years that significant changes in the cellular milieu can trigger the protective function against the stresses and the injuries. In chondrocyte like cell lines, 50MPa hydrostatic pressure induced heat shock protein 70, transforming growth factor-β1, tumor necrosis factor-α, and interleukin-6mRNA expressions (Takahashi, 1997; Takahashi, 1998). Although these reports seemed to suggest that the mammalian cells might keep alive until 60MPa hydrostatic pressure exposure, it had been unexpectedly

unknown. Likewise, it has remained unclear whether the protective function such as stress protein synthesis can act over 60MPa hydrostatic pressure exposure. Our present results showed that 3T3-L1 cells could survive, which was associated by activation of HSP70 promoter activity even in the ranges from 40 to 60MPa. At 70MPa, we detected majority (76%) of the cells were died according to EthD-1 red fluorescence although intense HSP70 promoter activity was occurred. Almost all of the exposed 3T3-L1 cells did not spread back out and did not growth after 3 days cultivation.

In the pressure from 10 to 30MPa, HSP promoter activity and cell morphology did not change compared with the control (Fig. 1 and 4). Tissue culturing and breeding of the deep sea organisms at atmospheric pressure could be feasible even if the organisms collected at depths of 3,000m.

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