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DEFECT IN THE DEVELOPMENT OF THERMOTOLERANCE IN THE MOUSE TEMPERATURE-SENSITIVE MUTANT ts85 LACKING UBIQUITIN-ACTIVATING ENZYME

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We investigated the effect of heat shock on the development of thermotolerance using mouse FM3A cells and the temperature-sensitive mutant ts85. The shift-up incubation of FM3A from 33 to 39.5° induced thermotolerance to subsequent heating at 44°. In contrast, the similar treatment of ts85 at the nonpermissive temperature of 39.5° could not induce thermotolerance. Furthermore, when ts85 cells were treated at 33° after being heated at 44°, they developed a reduced level of thermotolerance as compared with that developed in FM3A cells. Since ts85 cells are defective in ubiquitin-activating enzyme, these results suggest a role of the ubiquitin-protein conjugation system in the development of thermotolerance.

Key words: Thermotolerance — Hyperthermia — Ubiquitin — ts85 cell

Exposure of cells from various organisms to elevated temperatures induces a transient resistance to subsequent heat exposure. This phenomenon is called thermotolerance. Understanding the mechanism of thermotolerance is of great importance for the development of hyperthermia as a modality of cancer treatment. Despite an extensive characterization of the time and temperature dependence for the induction, development and decay of thermotolerance,[1,2] little is known about the molecular mechanisms of thermotolerance. Heat shock proteins, a family of proteins induced in response to a variety of chemical and environmental stresses, have been suggested to play a role in the state of thermotolerance.[3-5] This is mainly based on the close temporal correlation between the accumulation of heat shock proteins and the development of thermotolerance. There have been, however, some investigations which are contradictory to a direct correlation between the two responses.[6-8]

It has been suggested that there may be a connection between ubiquitin and response to heat shock.[9-11] Ubiquitin, a 76 amino acid residue protein, occurs in eukaryotic cells from yeast to man either free or covalently bound to a variety of protein species including histone H2A.[12-14] Evidence indicates that ubiquitin functions as a signal for attack by proteases specific to ubiquitin-protein conjugates.[9, 13, 14] The ts85 cells are a temperature-sensitive mutant derived from mouse mammary carcinoma FM3A cells.[15] They have been found to be defective in the ubiquitin-dependent protein degradation system and this effect is due to the specific thermolability of the ubiquitin - activating enzyme.[16, 17] Therefore, we investigated using ts85 cells the effect of elevated temperatures on the development of thermotolerance. We report here that ts85 cells, unlike the wild-type FM3A cells, are defective in developing thermotolerance at nonpermissive temperatures.

FM3A, ts85 and tsFT101 cells were supplied by the Japanese Cancer Resources Bank. FM3A cells were grown as a suspension culture at 33° in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS). The ts85 and tsFT101 cells were grown in RPMI 1640 supplemented with 10% FBS at the permissive temperature of 33°. The doubling times of FM3A, ts85 and tsFT101 cells were 17, 18 and 20 hr at 33°, respectively. For heat treatment, cells ($1–3 \times 10^4$ cells/ml of growth medium in plastic tubes) were transferred immediately from 33° to a water bath at an indicated temperature. The exposure time was the total time of immersion of tubes in the water bath. After heat treatment, cells were cultured at 33° in a CO_2

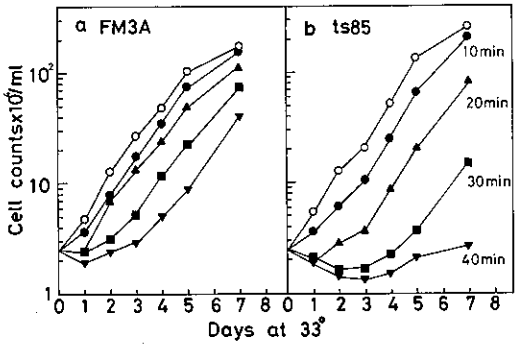

Fig. 1. Growth curves of FM3A (a) and ts85 (b) cells at 33° after exposure to 44° for 0 (○), 10 (●), 20 (▲), 30 (■) and 40 (▼) min.

incubator. The growth of cells was monitored daily by counting aliquots of cell suspensions with a Coulter counter. Each experiment was repeated twice at least.

Figure 1 shows that ts85 cells are more heat-sensitive than the wild type FM3A cells. Both cell lines were heated at 44° for various times from 10 to 40 min and then cultured at 33°. The onset of cell growth was delayed depending on the time of 44° heating. Cell survival was also determined by following clonal growth in a soft agar medium as previously described.[18] The values of the surviving fraction of cells exposed to 44° for 10, 20 and 30 min were 0.70, 0.49 and 0.17 for FM3A cells and 0.39, 0.11 and 0.008 for ts85 cells, respectively. Thus the delay of cell growth was correlated with the decrease in cell survival, and this growth curve assay was employed to assess hyperthermic cell inactivation.

FM3A cells were treated at 39.5° for 3 and 6 hr and subsequently heated at 44° for 45 min. As shown in Fig. 2a, the delay of cell growth was shortened by incubating the cells at 39.5° as compared with the growth delay of cells heated without pretreatment at 39.5°. The treatment at 39.5° for 6 hr alone only slightly decreased the cell count. These results indicated that the treatment of FM3A cells at 39.5° for 3 and 6 hr induced thermotolerance to subsequent heating at 44°. On the other hand, when ts85 cells were similarly treated at 39.5° and heated at 44° for 27 min, the delay of cell growth was not shortened but rather

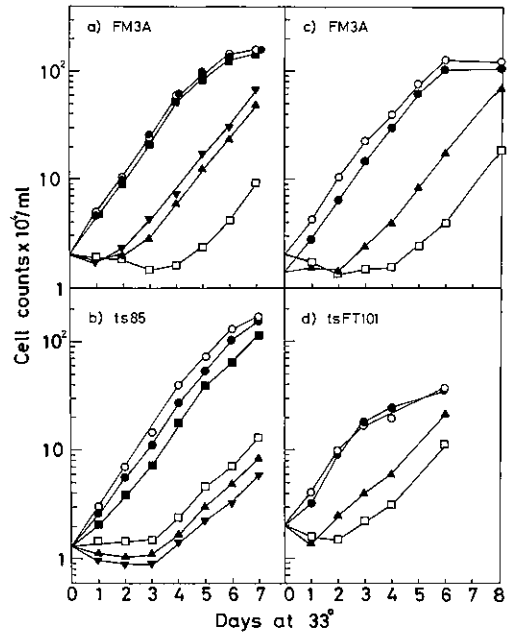

Fig. 2. Growth curves of FM3A, ts85 and tsFT 101 cells exposed to step-up heating from 39.5 to 44°. (a) and (b); FM3A (a) and ts85 (b) cells were treated at 39.5° for 0 (○), 3 (●), and 6 (■) hr. The cells were also treated at 39.5° for 0 (□), 3 (▲) and 6 (▼) hr followed by heating at 44° for 45 (a) and 27 (b) min. (c); FM3A cells (2×10^4 cells/ml) were heated at 44° for 0 (○) and 45 (□) min. The cells (1.4×10^4 cells/ml) were treated at 39.5° for 3 hr and then heated at 44° for 0 (●) and 45 (▲) min. (d); tsFT101 cells were treated at 39.5° for 0 (○) and 3 (●) hr. The cells were also heated at 44° for 30 min with (▲) or without (□) pretreatment at 39.5° for 3 hr.

prolonged (Fig. 2b). Furthermore, proliferation-capable cells were decreased by about 30 and 50% by exposure to 39.5° for 3 and 6 hr, respectively. Therefore, to examine the effect of the decrease in cells on growth delay of thermotolerance-developed cells, FM3A cells of 30% decreased number were treated at 39.5° for 3 hr and heated at 44° for 45 min. As shown in Fig. 2c, a shortened growth delay was evident even in the cells of reduced number. The results indicated that the unshortened growth delay of ts85 cells was due to the failure to develop thermotolerance. The cell line tsFT101, an FM3A cell-derived temperature-sensitive mutant defective in

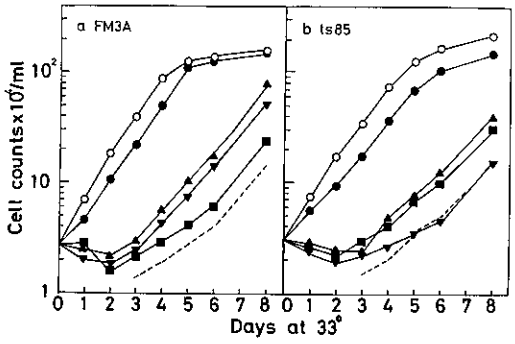

Fig. 3. Growth curves of FM3A (a) and ts85 (b) cells after exposure to fractionated heating. The cells were heated at 44° for 0 (○), 10 (●) and 50 (a) or 30 (b) (■) min. The cells exposed to 44° for 10 min were treated at 33° for 4 (▼) and 8 (▲) hr followed by a 2nd heating at 44° for 50 (a) or 30 (b) min. The dotted lines represent growth curves corresponding to an additive effect of the two heat treatments.

cytokinesis,[19] showed thermotolerance development after the same shift-up heating (Fig. 2 d). We concluded, therefore, that ts85 cells were specifically impaired as regards thermotolerance development at the nonpermissive temperature.

The exposure of cells to 41° for 60 min also induced tolerance in FM3A cells to subsequent heating at 44°, but hardly induced tolerance in ts85 cells (data not shown).

Thermotolerance was also induced and developed by incubating FM3A cells at 33° after heat shock. The cells were initially heated at 44° for 10 min, incubated at 33° and then challenged with a 2nd heating at 44° for 50 min. As shown in Fig. 3a, the initial heating decreased the cell count by about 35%. The growth of cells incubated at 33° for 4 and 8 hr between the two heat treatments was significantly promoted, as compared with the growth of the cells exposed to the 2nd heating alone or the curve (dotted line) which represented the additive effect of the two heat treatments. The ts85 cells were also treated similarly with the fractionated heating. As shown in Fig. 3b, the initial heating decreased the cell count by about 50%. The cells incubated at 33° for 4 hr after heat shock developed no tolerance to the 2nd heating. However, an improved growth curve was observed in the cells incubated for 8 hr at 33°, indicating that ts85 cells have a reduced ability to develop thermotolerance if incubated at the permissive temperature after heat shock.

The ts85 cells are a temperature-sensitive mutant in the ubiquitin-protein conjugation system and this effect is due to the specific thermolability of the ubiquitin-activating enzyme.[16] The ts85 cells fail to degrade abnormal proteins at the nonpermissive temperature of 39.5°, demonstrating that degradation of abnormal proteins is mediated by the ubiquitin-protein conjugation system.[17] We demonstrated in the present study that ts85 cells, in contrast to the wild type FM3A cells, could not develop thermotolerance at nonpermissive temperatures and also had a reduced ability to develop thermotolerance at a permissive temperature after heat shock. These findings suggest that the ubiquitin system may play an essential role in thermotolerance development. It was recently demonstrated that ubiquitin is coded by a multigene family,[20] and the genes were activated and ubiquitin synthesis was elevated upon heat shock.[9, 21] Thermodynamic data indicate that the target for heat-induced cell inactivation is proteins, and heat-denatured proteins aggregate and inactivate cells. Based on these considerations, we speculate that in thermotolerance-developed cells the ubiquitin-protein conjugation system is activated by heat-induced elevated synthesis of ubiquitin to degrade heat-denatured proteins. In ts85 cells, however, the system may not be activated at nonpermissive temperatures even if ubiquitin synthesis is promoted, but when the cells are incubated at a permissive temperature after heat shock, thermotolerance may develop accompanied with recovery of the ubiquitin system. In addition to this model, cells may have alternative mechanisms in which major heat shock proteins (e.g. 70 kD protein) are involved in preventing proteins from forming aggregates.

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REFERENCES

1) Hahn, G. M. "Hyperthermia and Cancer," pp. 41–53 (1982). Plenum Press, New York.
2) Li, G. C. and Mivechi, N. F. Thermotolerance in mammalian systems; a review. *In* "Hyperthermia in Cancer Treatment," Vol. I, ed. L. J. Anghileri and J. Robert, pp. 59–77 (1986). CRC Press, Boca Raton, Florida.
3) Schlesinger, M. J., Ashburer, M. and Tissieres, A. "Heat Shock: From Bacteria to Man" (1982). Cold Spring Harbor Lab., New York.
4) Arkinson, B. G. and Walden, D. B. "Changes in Eukaryotic Gene Expression in Response to Environmental Stress" (1985). Academic Press, New York.
5) Landry, J. Heat shock proteins and cell thermotolerance. *In* "Hyperthermia in Cancer Treatment," Vol. I, ed. L. J. Anghileri and J. Robert, pp. 37–58 (1986). CRC Press, Boca Raton, Florida.
6) Landry, J. and Chretien, P. Relationship between hyperthermia induced heat shock proteins and thermotolerance in Morris hepatoma cells. *Can. J. Biochem. Cell Biol.*, **61**, 428–437 (1983).
7) Widelitz, R. B., Magun, B. E. and Gerner, E. W. Dissociation of 68000 Mr heat shock protein synthesis from thermotolerance expression in rat fibroblasts. *Radiat. Res.*, **99**, 433–437 (1984).
8) Tomasovic, S. P., Steck, P. A. and Heitzman, D. Heat-stress proteins and thermal resistance in rat mammary tumor cells. *Radiat. Res.*, **95**, 399–413 (1983).
9) Finley, D. and Varshavsky, A. The ubiquitin system: functions and mechanisms. *Trends Biochem. Sci.*, **10**, 343–347 (1985).
10) Munro, S. and Pelham, H. What turns on heat shock genes? *Nature*, **317**, 477–478 (1985).
11) Schlesinger, M. J. Heat shock proteins: the search for functions. *J. Cell Biol.*, **103**, 321–325 (1986).
12) Busch, H. and Goldknopf, I. L. Ubiquitin-protein conjugates. *Mol. Cell. Biochem.*, **40**, 173–187 (1981).
13) Hershko, A. and Ciechanover, A. Mechanisms of intracellular protein breakdown. *Annu. Rev. Biochem.*, **51**, 335–364 (1982).
14) Ciechanover, A., Finley, D. and Varshavsky, A. The ubiquitin-mediated proteolytic pathway and mechanisms of energy-dependent intracellular protein degradation. *J. Cell. Biochem.*, **24**, 27–53 (1984).
15) Mita, S., Yasuda, H., Marunouchi, T., Ishiko, S. and Yamada, M. A temperature-sensitive mutant of cultured mouse cells defective in chromosomal condensation. *Exp. Cell Res.*, **126**, 407–416 (1980).
16) Finley, D., Ciechanover, A. and Varshavsky, A. Thermolability of ubiquitin-activating enzyme from mammalian cell cycle mutant ts85. *Cell*, **37**, 43–55 (1984).
17) Ciechanover, A., Finley, D. and Varshavsky, A. Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. *Cell*, **37**, 57–66 (1984).
18) Mizuno, S. Ethanol-induced cell sensitization to bleomycin cytotoxicity and the inhibition of recovery from potentially lethal damage. *Cancer Res.*, **41**, 4111–4114 (1981).
19) Yasumitsu, H., Hanaoka, F., Yasuda, H., Murakami, Y., Enomoto, T. and Yamada, M. Isolation and initial characterization of a temperature-sensitive mutant of mouse FM3A cells defective in cytokinesis. *Cell Struct. Funct.*, **10**, 79–88 (1985).
20) Wiborg, O., Pedersen, M. S., Wind, A., Berglund, L. E., Marcker, K. A. and Vuust, J. The human ubiquitin multigene family: some genes contain multiple directly repeated ubiquitin coding sequences. *EMBO J.*, **4**, 755–759 (1985).
21) Bond, U. and Schlesinger, M. J. Ubiquitin is a heat shock protein in chicken embryo fibroblasts. *Mol. Cell. Biol.*, **5**, 949–956 (1985).