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Correlation between Thermotolerance and Heat Shock Proteins in SCK Tumor Cells*

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SCK 腫瘍細胞에 있어서 耐熱性과 Heat Shock Protein의 상관관계

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요 약

본 실험에서는 HSP와 thermotolerance 사이에 어떠한 상관관계가 있는지를 알아보기 위하여, 생쥐 SCK 종양세포에서 heat(45°, 46°C)처리가 단백질 합성과 세포의 생존에 미치는 영향을 비교하여 보았다. 그 결과 heat 처리를 받은 세포는 HSP(70K, 87K)를 특이하게 많이 합성했으며, 다음 heat를 받았을 때는 높은 생존율을 보였다. 그리고 이러한 thermotolerance가 생성되고 감퇴되는 kinetics는 HSP가 합성되고 감퇴되는 kinetics와 연관성을 보여 주었다. 이러한 결과로 HSP는 heat shock로부터 세포를 보호하는 데 중요한 역할을 할 수 있다고 생각된다.

아울러, glycerol을 처리하여 HSP의 합성을 봉쇄시켰을 경우에도 열에 대해 저항성을 갖게 되는 실험결과로 미루어, 세포가 갖게 되는 heat resistance에는 (1) HSP의 합성을 초래하지 않는 요인에 의해 유도되는 heat protection과 (2) 열처리 등의 결과 합성되는 HSP에 의해 유도되는 thermotolerance의 두가지 경우가 있을 것으로 추론할 수 있었다.

INTRODUCTION

It has been known for a considerable time that heat induces or enhances the synthesis of a family of proteins, usually referred to as heat shock proteins (HSPs). Furthermore, it has also been demonstrated that agents other than heat can induce the synthesis of HSPs (Johnston *et al.*, 1980; Li and Werb, 1982; Kim *et al.*, 1983). These proteins, perhaps as many as 20 or more, range in molecular weight from less than 20,000 to more than

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100,000. The function of these HSPs is not well understood but they may be essential to cell survival after certain environmental stresses (Ashburner and Bonner, 1979; Loomis and Wheeler, 1980; Guttman *et al.*, 1980).

One of the interesting aspects of thermal biology is the response of heated cells to subsequent exposures at elevated temperatures. The sensitivity of cells to hyperthermia is substantially reduced by prior exposure to elevated temperatures. The development of resistance to subsequent heat treatment has been demonstrated in mammalian cells. The increased resistance to hyperthermia which occurs as a consequence of prior exposure to heat has been termed thermotolerance. Thermotolerance has been divided in 3 phases (Li and Hahn, 1980): the induction, which can be triggered by treatment at all hyperthermic temperatures; the development, which takes place over 2- to 8-hr under appropriate culture conditions (e.g., temperature, pH); and the decay, which leads to complete disappearance of thermotolerance over a 2- to 3-day period (Gerner and Schneider, 1975; Henle *et al.*, 1978; Nielsen and Overgaard, 1979; Li and Hahn, 1980; Majima and Gerweck, 1983). In spite of the accumulated data on the characterization of thermotolerance, the molecular processes underlying its development and decay are still unknown. The crucial role of protein synthesis in the development of thermotolerance has been recognized for some time (Henle and Dethlefsen, 1978). Because the synthesis of new proteins appears during the development of thermotolerance, the experiments have been performed to determine whether or not HSPs play a role in conferring heat resistance on cells. Indeed, it has been reported that in mammalian cells there is a good temporal correlation between the induction of HSPs and the development of thermotolerance (Li and Werb, 1982; Landry *et al.*, 1982).

To evaluate the possible relationship between the kinetics of synthesis and degradation of HSPs and those of development and decay of thermotolerance in mouse tumor cells, the effects of heat treatment on the protein synthesis and cell survival were examined. In parallel experiments, possibilities of acquiring the heat resistance by other than HSP were also examined using glycerol which is known to block HSP synthesis.

MATERIALS AND METHODS

Cells and Culture Conditions

SCK tumor cells used in this study were originated from a mammary carcinoma of a female A/J mouse which arose spontaneously in 1974 and was adapted to grow both in vivo and in vitro by Dr. Clement in the Radiobiology Laboratory, University of Minnesota Medical School (Kang *et al.*, 1980). The SCK tumor cells were grown in cultures as monolayers in RPMI 1640 tissue culture medium supplemented with 10% calf serum and penicillin-streptomycin (100 units/ml) in a CO_2-incubator at 37°C. Experiments were performed at 3×10^5 cells per cm^2.

Heat Treatment

Heating of cells in monolayer was carried out in a constant temperature circulating water bath. The medium was changed immediately before and after heating.

Cell Survival Studies

After the heat treatment, SCK cells were trypsinized with 0.25% trypsin at 37°C for 15 min. The viable cells were counted using the trypan blue exclusion method and plated after appropriate dilutions were made. After 10 days of incubation at 37°C, clones were stained with crystal violet and counted. Plating efficiency was 50~60%.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis

Cells were labeled with 10 μCi of ^{35}S-methionine in methionine-free RPMI 1640 medium per ml for 1 or 2 hr at 37°C. At the end of the labeling period, the medium was removed, cells were washed three times with cold phosphate buffered saline, and they were harvested with lysis buffer containing 2% SDS. The SDS extracts were boiled for 3~5 min and the amounts of protein were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. After the determination of protein concentrations, the extracts were dissolved in sample buffer (5% 2-mercaptoethanol, 0.001% bromophenol blue, 10% glycerol, 2% SDS, and 62.5 mM Tris-HCl pH 6.8), and equal amounts of proteins were then loaded directly onto the polyacrylamide slab gels (7.5%) and electrophoresed (Laemmli, 1970). Carbonic anhydrase(29,000), egg albumin(45,000), bovine serum albumin(66,000), phosphorylase b(97,400), and β-galactosidase(116,000) were used as molecular weight markers.

Autoradiography and Fluorography

After the electrophoresis, gels were dried under gentle vacuum for autoradiography, and autoradiograms were prepared by exposing the dried gels on X-ray film for a few days.

For fluorography, gels were equilibrated with 20 volumes of dimethylsulfoxide (DMSO) and then impregnated with 2,5-diphenyloxazole (PPO) by immersion in 4 volumes of 20% (w/w) PPO in DMSO for 3 hr. The gels were then soaked in 20 volumes of water, dried and exposed to X-ray film at −70°C.

RESULTS

Effects of Heat Treatment on Protein Synthesis

To examine the effects of heat treatment on protein synthesis, cells were heat-shocked at 46°C for 6 min and returned to 37°C for a period of 4 hr and then labeled for 2 hr. When the proteins synthesized after heat shock were compared with those synthesized by unheated cells, there was a noticeable change in the synthesis of particular proteins. Although the rate of protein synthesis was slightly decreased after heat shock, the 70K and 87K proteins were synthesized in greater amounts than other proteins from heat-shocked cells (Fig. 1). The most prominent change was shown in 70K protein band. In heat-shocked cells, two

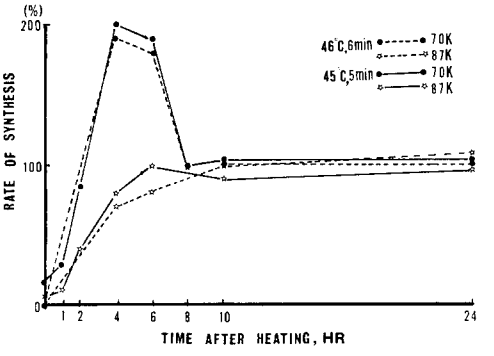

Fig. 1. Kinetics of major heat shock protein synthesis. Rate of synthesis in 1hr was evaluated by the area of corresponding peaks as revealed by densitometric scan of autoradiogram. Rate of synthesis is shown as percent of the controls.

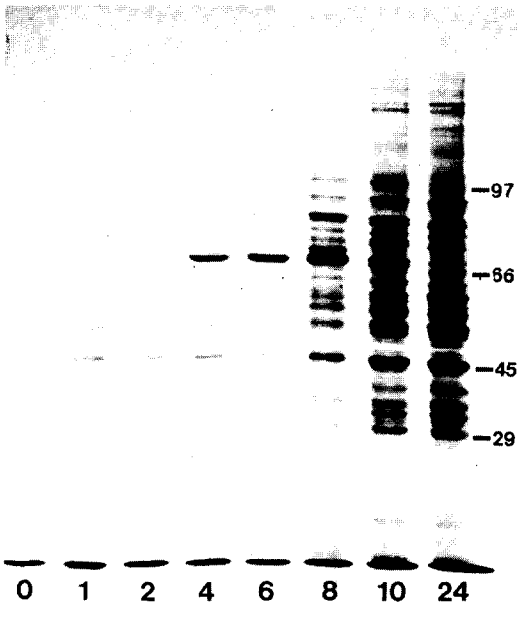

Fig. 2. Fluorogram of SDS-PAGE for lebelled proteins from SCK cells exposed to 46°C for 6min. After the treatment cells were incubated at 37°C for 0~24hr before lebelling for 1hr. Positions of molecular weight marker are shown at right.

bands appeared in 70K region but the lower band was not found in unheated cells.

Induction of Thermotolerance and Heat Shock Protein Synthesis by Initial Treatment at 46°C.

To examine the induction of thermotolerance, cells were heat-shocked at 46°C for 6 min and incubated at 37°C for 0~24 hr before a second treatment at 45°C for 45 min, and cell survival was then assayed. Thermotolerance was markedly well developed by 4~6 hr of incubation at 37°C (Fig. 3). In parallel experiments, the effects of 46°C treatment on protein synthesis were examined. After 6 min of heating, cells were incubated at 37°C for 0~24 hr before labeling with ^{35}S-methionine for 1 hr at 37°C (Fig. 2). Protein synthesis was drastically inhibited by the heat treatment but recovered gradually during the 24-hr

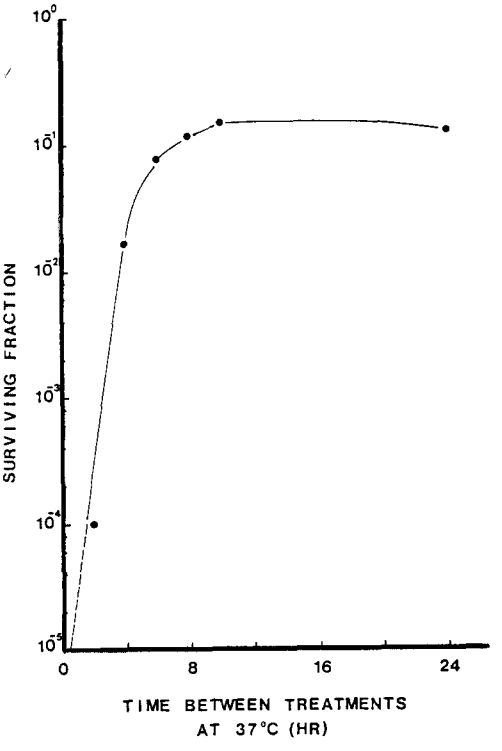

Fig. 3. Induced thermotolerance in SCK cells exposed to 46°C for 6min. After the treatment cells were incubated at 37°C for 0~24hr before giving a second heat treatment at 45°C for 45 min. Cell survival was plotted as a function of time following the initial treatment.

incubation period at 37°C. As the protein synthesis recovered, the 70K and 87K proteins were synthesized in greater amounts than other proteins. Especially the 70K protein was synthesized in far greater amounts than in control cells. Each polypeptide showed different kinetics of synthesis. The rate of synthesis of 70K protein reached to maximum at 4~6 hr after heat shock, and was then decreased. By contrast. the enhanced synthesis of 87K protein was continued longer period than that of 70K protein did. The development of thermotolerance occurred concomitantly with the enhanced synthesis of HSPs.

Decay of Thermotolerance and Degradation of Heat Shock Proteins

To examine the decay of thermotolerance, cells were heat-shocked at 46°C for 6 min and incubated at 37°C for 0~60 hr before a second heat treatment at 45°C for 45 min and cell survival was then assayed (Fig. 5). The rate of decay of thermotolerance was much slower than that of induction. In parallel experiment, the degradation of HSPs was examined (Fig. 4). After the heat shock (46°C for 6 min), cells were incubated at 37°C for 4 hr and labeled with ^{35}S-methionine for 2 hr at 37°C and then incubated at 37°C for 0~54 hr before harvesting. Because the rate of HSPs was close to its maximum after 4 hr incubation (Fig. 4), the labeling was made after incubation at 37°C for 4 hr. The degradation of 70K and 87K proteins correlated well with the decay of thermotolerance.

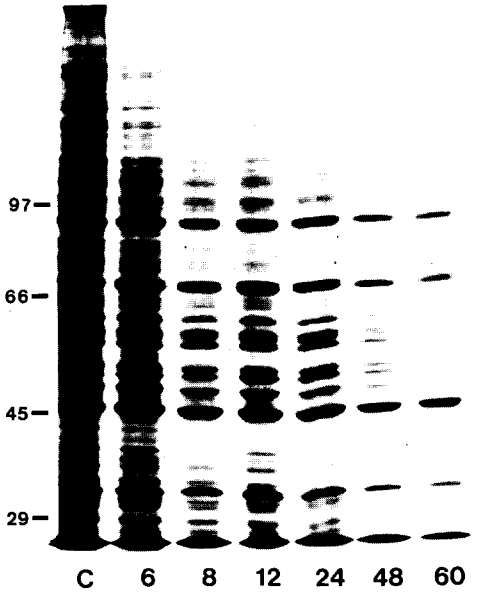

Fig. 4. Fluorogram of SDS-polyacrylamide slab gels showing degradation of ^{35}S-methionine labelled proteins from heat-shocked cells at 46°C for 6 min. After the treatment, cells were incubated at 37°C for 4 hr followed by an additional incubation at 37°C for 0~54 hr before harvesting. C, unheated control; 6~60, total hr of incubation at 37°C after the heat shock. Positions of molecular weight marker are shown at left.

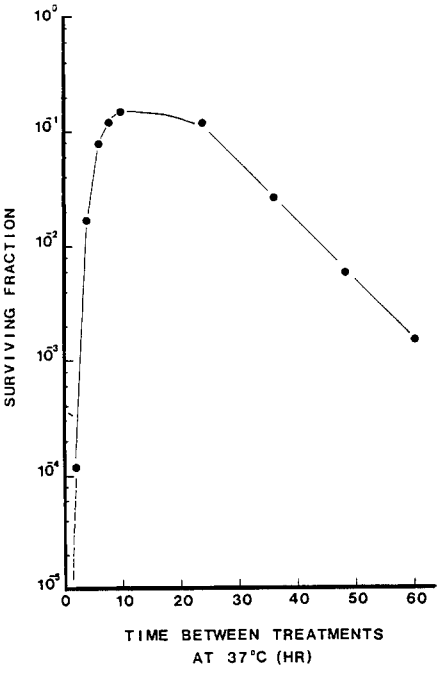

Fig. 5. Decay of thermotolerance in SCK cells. After heat treatment at 46°C for 6 min, cells were incubated at 37°C for 0~60 hr before giving a second treatment at 45°C for 45 min and then the survival was assayed. Cell survival was plotted as a function of time following the initial heat.

Induction of Heat Resistance by Factors Other Than HSPs

Synthesis of HSP was blocked by glycerol treatment as shown in Fig. 6. The cells heated at 42°C for 1 hr without glycerol induced major HSP synthesis, whereas those heated with glycerol induced no HSPs. In order to examine the effects of glycerol treatment on the heat resistance, a second heat treatment at 45°C for 45 min was administered at varying times after the first treatment at 42°C for 1 hr and then the cell survival was evaluated (Fig. 7).

As is evident from Fig. 7, the thermotolerance gradually appeared up to 8 hr after the first heat treatment. An interesting fact to be noted at this point was that even in the cells in which HSP synthesis was blocked by glycerol, quite a similar level of heat resistance to that of heat alone was acquired at 2 hr after the first treatment. Thereafter, however, the levels of heat resistance remained nearly unchanged. Thus, the aquisition of heat resistance at early phase in the processes seemed not to be correlated with the level of HSP

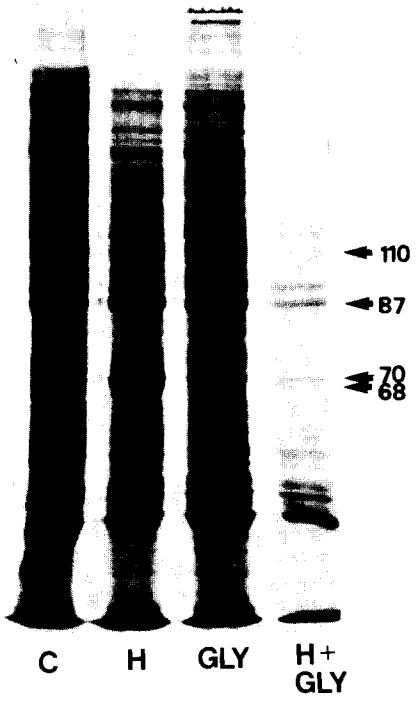

Fig. 6. Autoradiogram of SDS-PAGE for labelled proteins from SCK cells. C, unheated cells; H, heated cells at 42°C for 1hr, incubated and labelled for 2 hr each; GLY, treated cells with 1M glycerol for 80 min, incubated and labelled for 2 hr each; H+GLY, cells pretreated with 1M glycerol for 20 min, followed by heat treatment at 42°C for 1 hr, incubated and labelled for 2 hr each.

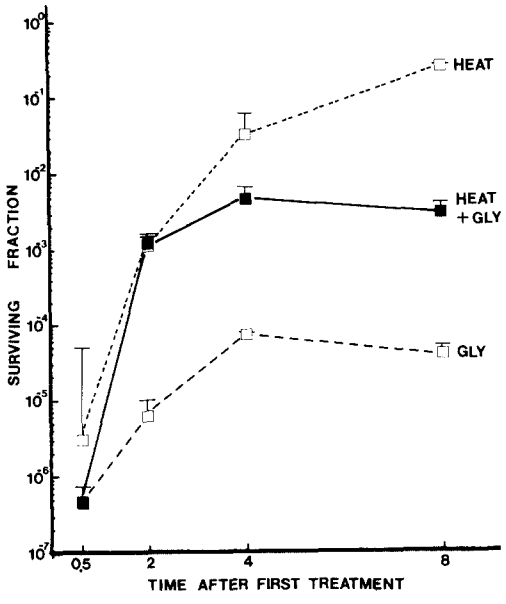

Fig. 7. Induction of thermotolerance in SCK cells by glycerol without HSP synthesis. After the first heat treatment at 42°C for 1hr, cells were incubated for 0.5~8 hr before a second heat treatment at 45°C for 45 min was given. HEAT, heated cells; HEAT+GLY, heated cells in the presence of 1M glycerol with 20 min of pretreatment; GLY, glycerol-treated cells for 20 min.

synthesized. Unknown factors other than HSP might be speculated to play a role in the early induction phase of heat resistance.

DISCUSSION

Several lines of evidence have been presented that thermotolerance and HSP synthesis may be interrelated phenomena in mammalian cells. So it has been suggested that the increased synthesis of HSPs plays a crucial role in the induced thermal resistance of cells (Li and Werb, 1982; Landry *et al.*, 1982). The present study has shown that a number of evidence implicate HSPs were involved in the development of thermotolerance. Heat shock at 45° and 46° enhanced the synthesis of HSPs and induced a transient thermotolerance. Furthermore, the development of thermotolerance occurred concomitantly with the enhanced

synthesis of HSPs. The decay of thermotolerance was also correlated well with the degradation of HSPs.

All these phenomena clearly showed a well-correlated relationship between the kinetics of development and decay of thermotolerance and those of synthesis and degradation of HSPs. Therefore, it can be hypothesized that HSPs may play a role in providing cells with thermotolerance in SCK cells. The HSP of SCK cells showed less conspicuous behavior after heat shock than those of HeLa, HA-1, or MH-7777 cells (Slator *et al.*, 1981; L and Werb, 1982; Landry *et al.*, 1982). The synthesis of 70K and 87K proteins closely conformed to the kinetics of thermotolerance. After the initial heat treatment, the increased or decreased synthesis of each protein at specific times was dependent on the duration and degree of initial heat.

When the proteins induced by heat shock, sodium arsenite, or ethanol were compared, the most prominent protein induced in all cases was the 70K protien (unpublished data). When actinomycin D was treated, only the synthesis of 70K protein was blocked, whereas that of other proteins was without effect. In fluorogram, the 70K region appeared as two bands, but these occasionally looked as if they were one. The lower band (HSP_{68}) showed the most prominent change by heat shock or by other agents.

Considering the temporal correlation between induction of HSP synthesis and that of thermotolerance, the studies on thermotolerance and HSP synthesis in both normal and tumor cells are important in potential application of thermotherapy in the treatment of cancer in human. Tsukeda *et al.* (1981) suggested that the analysis of HSPs is very useful for identifying the differential heat susceptibility of normal and malignant cells. They showed that the malignant and SV40-transformed human lung cells were more heat sensitive and produced maximum 70K protein at lower temperature than normal cells. Although SCK and MEF cells are different in their origin, the heat sensitivity of both cultured cells was compared. However, no significant difference was observed (unpublished data). Omar and Lanks (1984) showed that the higher basal HSP levels in the transformed cells do not confer higher thermal resistance, and suggested that HSPs can not be held responsible for the difference in heat sensitivity between the normal and transformed cells. It is interesting that their suggestion is not in agreement with that of Tsukeda *et al.* (1981).

In any case, a great number of evidence are accumulating that HSPs are involved in thermotolerance, and therefore it can be thought that HSPs play an essential role in protecting cells from heat shock or other environmental stresses.

In connection with the so-called heat conditioning-induced thermotolerance (HTT), heat resistance acquired by protective agents (PTT) is also of great value for elucidating mechanisms of the development of thermotolerance by HSPs.

The present studies have revealed that HSP synthesis was blocked by glycerol treatment (Fig. 6) but heat resistance was obtained in the glycerol-treated cells (Fig. 7). The mechanism by which cells acquire heat resistance is assumed to be the ability of glycerol to stabilize

proteins. Thus, it appears that one of the possible heat-killing mechanisms is the inactivation of a heat-sensitive molecule. It was also shown in the present study that glycerol added during the heat-conditioning period interferes with the appearance of thermotolerance at later period. Therefore, the mechanism for the protective effect of glycerol, at least except early period, is likely to be different from heat-induced thermotolerance.

The mechanism for the glycerol to block the synthesis of HSP can be interpreted as follows, taking into account of a model proposed recently by Ananthan *et al.* (1986). Briefly, in order for HSP genes to be expressed, active HSP specific factor should be accumulated. However, active specific factor is suggested to be labile in noninduced cells. When cells undergo a heat shock or are subjected to other stresses, a fraction of the intracellular proteins is denatured. Such denatured proteins are preferentially degraded and to compete effectively with the active forms of the factor as a substrate of the intracellular proteolytic system. As a result of this competition, the active factor can accumulate and lead to the activation of HSP genes. If glycerol, a macromolecular stabilizer, is added to this system it will stabilize intracellular proteins, preventing them from degradation. When the competing components are stabilized, the active factor will be degraded and thus can not accumulate, resulting in HSP genes in inactive state.

To appreciate the precise function of HSPs in HTT and the mechanism by which glycerol protects cells from heat inactivation, more extensive studies are required.

ABSTRACT

To determine whether the development and decay of thermotolerance are related to HSPs, the effects of heat treatment (45°, 46°C) on protein synthesis and cell survival in mouse tumor cells were examined. The synthesis of specific proteins in heat treated cells was greatly enhanced over that in unheated cells and cell survival was greatly increased, when the cells were challenged by a subsequent heat treatment. The major HSPs were 70K and 87K proteins. The kinetics of development and decay of thermotolerance correlated well with the kinetics of synthesis and degradation of HSPs (Mrs 70K and 87K). These data suggest that HSPs play an essential role in protecting cells from heat shock.

The present study also indicates that heat resistance was induced in the cells when the synthesis of HSPs was blocked by treating glycerol during hyperthermia. These results suggested that the cells might obtain heat resistance by heat protector, inducible by a factor which does not induce HSP synthesis, and by thermotolerance, inducible by HSP which is produced by heat treatment or by other agents.

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