KOREAN JOURNAL OF ZOOLOGY
Vol. 30, No. 3, 311-323 (1987)

Effects of Environmental pH on the Heat Shock Proteins and Thermosensitivity of Mouse Fibroblasts and SCK Tumor Cells*

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생쥐의 纖維芽細胞와 SCK 腫瘍細胞의 Heat Shock Protein과 熱感受性에 미치는 pH의 영향

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(Received February 19, 1987)

요 약

생쥐의 섬유아세포(MEF)와 종양세포(SCK)를 이용하여 정상세포와 종양세포 사이에 열감수성의 차이가 있는지의 여부 및 환경의 pH가 이 세포들의 열감수성과 heat shock protein(HSP) 합성에 미치는 영향을 생존곡선과 HSP합성 kinetics등을 써서 검토하였다.

MEF와 SCK 세포를 정상 pH(7.4) 또는 산성 pH(6.7)에서 42°C에서 2시간 온열처리 후 3일간에 걸쳐 생존율을 비교해 본 결과, MEF와 SCK세포 사이에 생득적 열감수성의 차이는 없었고 산성 pH에서는 세포의 종류에 관계없이 열감수성이 증감되었다.

온열처리의 결과 유도되는 내열성이 conditioning heat의 크기와 어떤 관계가 있는지를 보기 위해서 45°C에서 5분 또는 20분을 주어본 결과 짧은 conditioning heat를 주었을 때 내열성이 신속히 그리고 높은 수준으로 발생하였고, 이러한 열감수성의 kinetics는 HSP의 합성 kinetics와 잘 일치하였다.

단백질, 특히 HSP 합성에 미치는 pH의 영향을 알아보기 위해서 46°C에서 6분간의 heat shock를 주어 본 바 전반적인 단백질 및 major HSP의 합성양상에는 별로 차이를 보이지 않았다. 그러나 SCK 세포에 43°C에서 30분의 온열처리를 주고 새로 합성되는 HSP_{6P}의 kinetics를 검토해 본 결과 정상 pH에서는 0-5시간에 합성이 일어나나 산성 pH에서는 3-9시간에 합성이 일어나서 몇시간의 합성지연이 관찰되었다.

아울러 HSP_{68}, HSP_{70}, HSP_{87}을 peptide mapping하여 본 결과 HSP_{68}과 HSP_{70}은 유사한 peptide fragment pattern을 보여 amino acid sequence는 유사하고 기능도 같을 것으로 추론되었으나 HSP_{87}은 전혀 다른 pattern을 보였다.

* This work was supported by grant from the Korean Science and Engineering Foundation in 1984.

INTRODUCTION

Hyperthermia, either by itself or in combination with X-rays or cytotoxic agents, has been extensively investigated as a potential modality for cancer therapy. It will be useful for the treatment of cancer to the extent that differential response to heat occurs between normal and tumor tissues. The differential response to heat may be obtained by the variation in intrinsic cellular heat sensitivity or by the difference in the cellular microenvironment.

Some studies demonstrate a selective heat sensitivity of cancer cells (Kase *et al.*, 1975; Tsukeda *et al.*, 1981), whereas others have found no difference (Kachani *et al.*, 1969; Ossovski *et al.*, 1967). On the other hand, during the past few years several lines of evidence have been accumulated which show that much of the tumors exist in an acidic environment, under low oxygen tension, and in a nutritionally deprived state (Dewey *et al.*, 1979) and that these factors modify the thermal response of cultured mammalian cells (Gerweck, 1977; Gerweck *et al.*, 1979). Specifically, it has been examined and demonstrated that "tumor-like" low pH conditions(e.g., pH 6.7) markedly increase the sensitivity of cells to hyperthermia in rodent cell line and human glial cells (Freeman *et al.*, 1980; Gerweck *et al.*, 1981). This pH-sensitizing effect is most apparent at temperatures only moderately lethal at normal pH, and it decreases with increasing temperatures. These facts suggest that treatment temperatures tolerated relatively well under normal physiological conditions may be more lethal under low pH conditions. This seems to provide a good rationale for hyperthermic treatment of tumor surrounded with normal tissues.

Recently, the induction or enhanced synthesis of proteins in response to the environmental stress of a sudden heat shock has been reported in prokaryotic and eukaryotic cells (McAlister *et al.*, 1980; Kelley *et al.*, 1978). These proteins are referred as heat shock proteins (HSPs). The most prominent HSPs in cultured mammalian cells have molecular weights of 81~90K and 67~73K (Welch *et al.*, 1982). Apparently, identical polypeptides can be induced by chemical agents including sulfhydryl reagents (Johnston *et al.*, 1980), amino acid analogues (Kelley *et al.*, 1978) and transition series metals (Levinson, 1980) and by acute infection with several viruses (Collins *et al.*, 1982; Nevins, 1982). These findings suggest that HSPs may have functions essential to cell survival following exposure to certain adverse conditions (Guttman *et al.*, 1980).

The relationship of HSP synthesis to survival after exposure to hyperthermia has been explored in several ways. Li *et al.* (1982) showed that the development of transient thermotolerance following sublethal heat shock correlated with the synthesis of HSPs. Tsukeda *et al.* (1981) suggested that the analysis of HSPs was very useful for identifying the differential heat susceptibilities of cells by comparing the optimal conditions for HSP synthesis. Although the marked effects of low pH environment on the viability of the cells

to heat and on thermotolerance were reported (Gerweck *et al.*, 1983; Goldin *et al.*, 1981), effects of pH on HSP synthesis have not been explored yet. Therefore, we aimed at examining the effect of low pH medium on the protein synthesis, especially the HSPs as a parameter for the levels of thermosensitivity as well as thermotolerance.

MATERIALS AND METHODS

Cell Cultures

SCK tumor cells used in this study were originated from a mammary carcinoma of female A/J mouse which arose spontaneously in 1974 and was adapted to grow both in vivo and in vitro (Kang *et al.*, 1980). The cells were cultured in T-flasks with RPMI 1640 tissue culture medium (Gibco) supplemented with 2 g/*l* sodium bicarbonate, 5 mM N-2-hydroxyethyl-piperazine-N-ethanesulfonic acid (HEPES), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 mg/ml fungizone and were maintained in a CO_2 incubator at 37°C (standard condition). Exponentially growing cells were subcultured with 0.25% trypsin (Difco) and inoculated at an appropriate number of cells every 3 or 4 days. Under these conditions, the population doubling time was about 12 hr during the exponentially growing phase.

Mouse embryonic fibroblasts(MEF), as a normal counterpart of SCK tumor cells, were obtained from 14 day old A/J mouse embryos by mincing with scissors and then digesting with 0.1% trypsin. The resulting cell suspension was centrifuged, and the pellet was then resuspended in RPMI 1640 medium containing 10% fetal calf serum. Cells thus obtained were seeded in 100 mm culture dishes. These cells were subcultured every two days. After third subculture, cells grown as monolayer were used. The population doubling time of MEF cells during the exponentially growing phase was about 24 hr.

Maintenance of pH

pH of the media was adjusted as described by Hahn *et al.* (1983). Normal pH (7.4) and acidic pH (6.7) were obtained by adjusting the amount of sodium bicarbonate dissolved in the media. For normal and acidic media, 2 g and 0.1 g of sodium bicarbonate were added to 1 L medium, respectively. The adjusted pH medium was maintained by continuous gassing with 5% CO_2-air. The pH levels were reproducible from experiment to experiment.

Exposure to Hyperthermia

SCK and MEF cells were seeded at a density of 8×10^5 and 1×10^6 cells in T-flasks, respectively. After 2-day culture, culture flasks inserted in plastic shelves were immersed horizontally in a constant-termerature circulating water bath. Before and after hyperthemia, fresh media adjusted to pH 7.4 or 6.7 were replaced. Following hyperthermia the cells were incubated at 37°C for 4 hr, and then trypsinized, counted, and seeded at a density of 3×10^5 cells per 60 mm tissue culture dishes contaning 4 ml of RPMI 1640 medium supplemented with 10% bovine serum at pH 7.4. Daily, the surviving cells were trypsinized

with 0.25% trypsin and 0.2% EDTA and counted.

Incorporation of ^{35}S-Methionine

For this experiment, SCK and MEF cells were inoculated at a density of 3×10^5 cells per 35 mm Falcon dish and 1×10^6 cells per 60 mm, respectively. The cells were similarly exposed to heat at pH 7.4 or 6.7 at various temperatures and durations as required, followed by incubation for 4 hr at 37°C. Then the medium was discarded and the cells were incubated in pH-adjusted methionine-free RPMI 1640 media containing ^{35}S-methionine (5 μCi/dish for SCK, 15 μCi/dish for MEF) for 1 hr at 37°C. At the end of incubation, the cells were washed with Ca^{2+}, Mg^{2+}-free phosphate buffered saline(PBS) and harvested by adding 200 μl of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (625 mM tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue) to each dish and were scrapped off with a rubber policeman. Samples were stored at -20°C until analyzed.

Evaluation of Thermosensitivity

After heat treatment, MEF and SCK cells in culture flasks were incubated at 37°C in 5% CO_2-air. The number of viable cells was routinely determined by counting the unstained cells with a hemocytometer after the cells were stained with trypan blue. Each experiment was done in duplicate and repeated at least 3 times.

Gel Electrophoresis

Labelled samples were dissociated by heating in boiling water for 3~5 min. Samples having equal cpm were applied to slab gels containing 7% acrylamide using the discontinuous system of Laemmli (1970). Electrophoresis was carried out with a constant current of 20~30 mA for 3~5 hr.

Two-dimensional gel electrophoresis was performed as described by O'Farrell (1976) with minor modifications. Briefly, for the isoelectric focusing 0.32×15 cm cylindrical gels of 4% acrylamide and 0.2% bisacrylamide, containing a mixture of 1.6% of pH 4~6 and 0.4% of pH 3~10 ampholines with Nonidet P-40 were used. Electrophoresis was run for 16~20 hr at 400 V, followed by 1 hr at 800 V. After the run the gels were removed from the tubes and were equilibrated with 50 ml of 2% SDS sample buffer for 90 min. Then, the gels were placed on the top of 7.5% slab gels, overlaid with 1% agarose, and elctrophoresed at 20~30 mA for 8 hr.

Autoradiography and Fluorography

Electrophoresed gels were stained in a solution containing 0.2% Coomassie brilliant blue dissolved in 40% methanol plus 10% acetic acid for 2 hr, and destained in a destaining solution (35% methanol and 7% acetic acid) until the background became clear. Destained gels were dried under gentle vacuum and autoradiograms were prepared by exposing the dried gels on X-ray film.

For fluorography, after electrophoresis or after staining and destaining, the gels were soaked in about 20-fold volume of dimethylsulfoxide (DMSO) for 30 min, repeating the

same procedure twice. The gels were then immersed in 4-fold volume of 20%(w/v) PPO in DMSO for 3 hr and immersed in 20-fold volume of water for 1 hr. Then, the gels were dried and exposed on X-ray film for 12～24 hr at -60°C. After development, X-ray films were scanned in a densitometer (Helena Lab.) at 525 nm.

Peptide Mapping by Partial Proteolysis

Peptide mapping was performed as described by Cleveland *et al.* (1977) using *Staphylococcus aureus* protease V8 (Sigma).

RESULTS

Thermosensitivity of MEF and SCK Cells

Growth profile of unheated and heated MEF and SCK cells at 42°C for 2 hr under normal or acidic pH is shown in Figs. 1 and 2. No difference in the growth profile between MEF and SCK cells was observed at 37°C, being similar in that both cells exhibited a slower proliferation rate without affecting cell viability at acidic pH compared to normal pH. In contrast, sensitizing effect of acidic pH affecting cell viability was significant in the heated cells, irrespective of cell types.

Protein Synthesis at Normal and Acidic pHs

A general pattern of protein synthesis in unheated and heated MEF and SCK cells at 46°C for 6 min is shown in Fig. 3. MEF and SCK cells were found to synthesize the major HSPs, regardless of pH conditions, except that in SCK cells HSP_{110} was not produced at acidic pH to the extent that produced at normal pH. Kinetics of HSP_{68}. synthesis in SCK cells was followed up to 24 hr after hyperthermia at 43°C for 30 min under normal

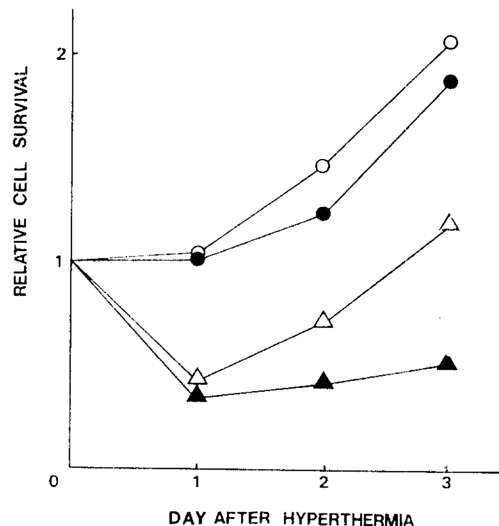

Fig. 1. Relative cell survival for unheated and heated MEF cells at 42°C for 2 hr under normal or acidic pH. ○-○, 37°C at pH 7.4; ●-●, 37°C at pH 6.7; △-△, 42°C at pH 7.4; ▲-▲, 42°C at pH 6.7.

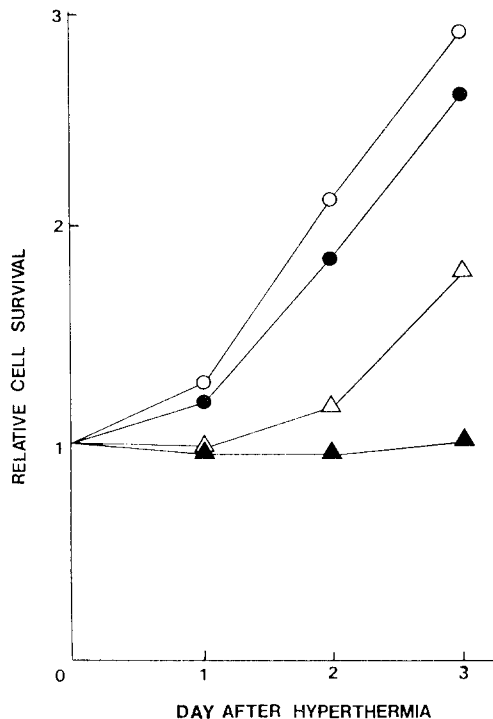

Fig. 2. Relative cell survival for unheated and heated SCK cells at 42°C for 2 hr under normal or acidic pH. ○-○, 37°C at pH 7.4; ●-●, 37°C at pH 6.7; △-△, 42°C at pH 7.4; ▲-▲, 42°C at pH 6.7.

or acidic pH and is shown in Figs. 4 and 5, respectively. As is evident from Fig. 5 acidic pH was found to delay the synthesis of HSP_{68}. Although it was apparent that the protein synthesis, in general, is decreased at acidic pH compared to normal pH, the major HSP synthesis was not influenced to a greater extent by acidic pH except HSP_{68}. For example, de novo synthesis of HSP_{68} occurred at 0~5 hr after hyperthermia in the cells at normal pH, whereas at acidic pH, the synthesis occurred at 3~9 hr after hyperthermia.

Levels of Conditioning Dose and Thermotolerance

Onset of thermotolerance in SCK cells was followed after giving different conditioning heat and is shown in Fig. 6. As is evident from Fig. 6. thermotolerance was developed rapidly and to higher level in the cells which received shorter conditioning heat than those received longer one. In parallel experiments, kinetics of HSP synthesis in the cells which received different conditioning heat was performed as shown in Fig. 7. Kinetics of thermotolerance development correlated well with the kinetics of HSP_{68} synthesis, being slower in the cells which received longer conditioning heat than those received shorter one.

Complexity of Heat-Induced Proteins

In Fig. 8 is shown a two-dimensional electrophoretic pattern of proteins in unheated and heated SCK cells. As is evident from the autoradiograms, a dozen of proteins other than 4

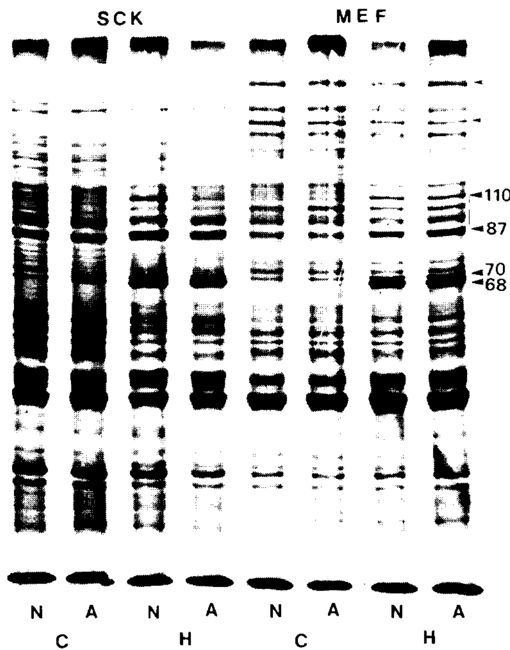

Fig. 3. Major HSPs are apparent in SCK and MEF cells exposed to 46°C for 6 min under normal or acidic pH. C, unheated cell; H, heated cell; N, normal pH; A, acidic pH.

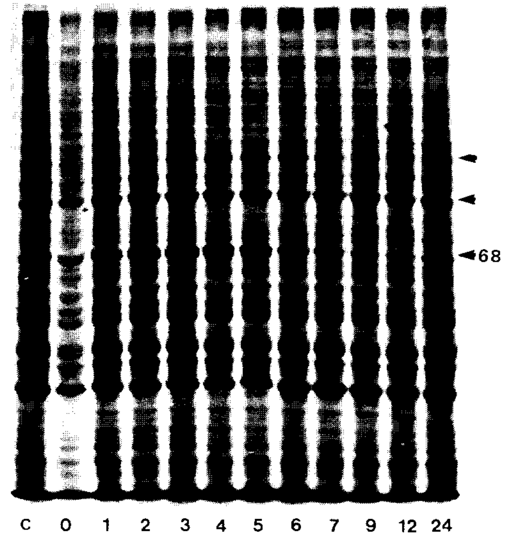

Fig. 4. Rate of major HSP synthesis in SCK cells at different times (0~24 hr) after hyperthermia at 43°C for 30 min under normal pH. De novo synthesis of HSP_{68} appears to occur in 0~5 hr following hyperthermia.

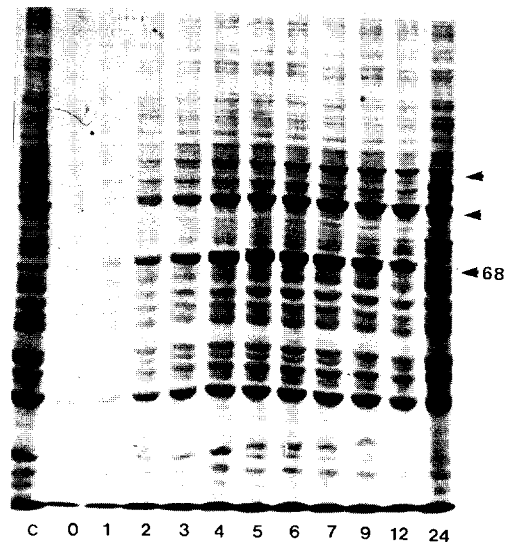

Fig. 5. Rate of major HSP synthesis in SCK cells at different times (0~24 hr) after hyperthermia at 43°C for 30 min under acidic pH. De novo synthesis of HSP_{68} appears to occur in 3~9 hr following hyperthermia.

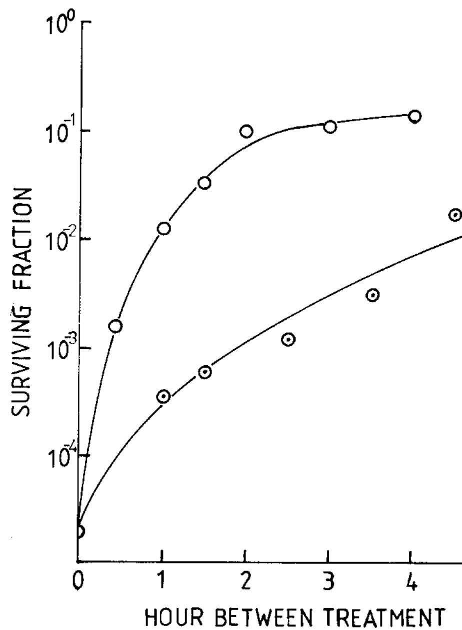

Fig. 6. Onset of thermotolerance in SCK cells. After the conditioning heat of 5 or 20 min at 45°C, cells were incubated at 37°C for 0~4.5 hr before giving a second heat treatment at 45°C for 40 or 25 min, for a total heating time of 45 min. Cell survival was plotted as a function of time between conditioning heat and second heat. ○-○, 5+40min; ●-●, 20+25min.

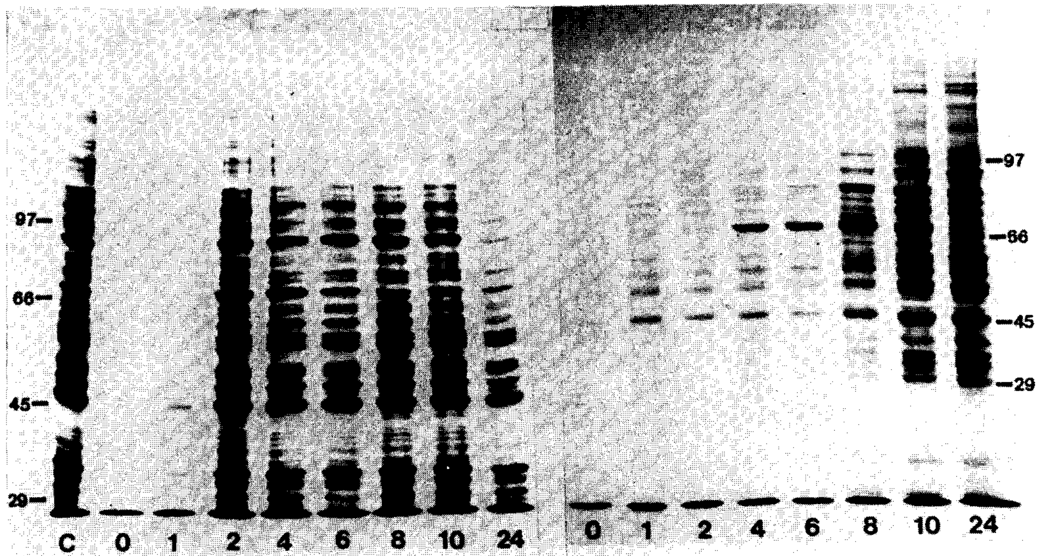

Fig. 7. Fluorograms of SDS-PAGE for ^{35}S-methionine-labelled proteins from SCK cells, heat shocked at 45°C for 5 min (left) or 20 min (right) at different times after heat shock (0~ 24 hr). M_rs are shown $\times 10^{-3}$.

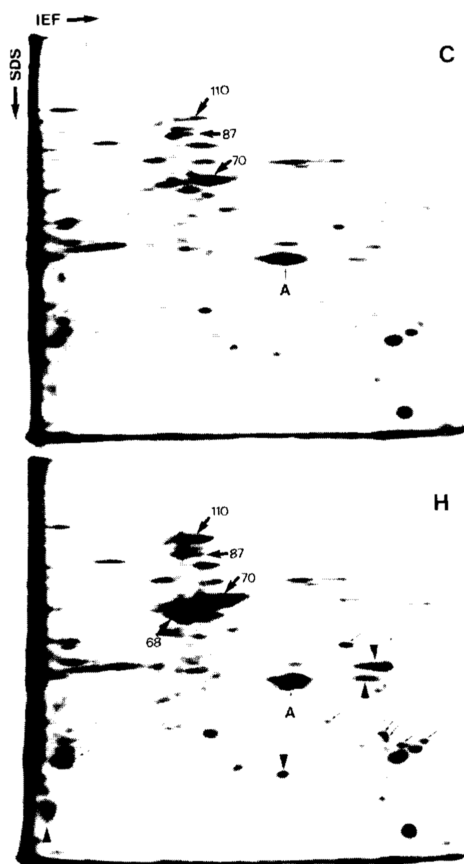

Fig. 8. Two-dimensional pattern of ^{35}S-methionine labelled proteins from unheated and heated SCK cells. C, cells labelled for 2 hr at 37°C; H, cells heat shocked at 42°C for 1hr and transferred to 37°C for 2 hr prior to labelling for 2 hr. Heat-induced proteins other than major HSPs are indicated by ↓ for heavily stimulated and by ↓ for lightly stimulated proteins respectively.

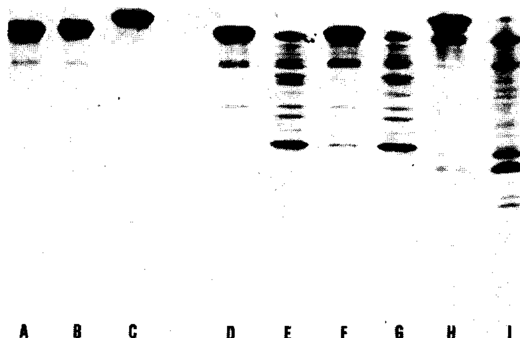

Fig. 9. Partial proteolytic mapping of major HSPs. The HSPs were recovered from acrylamide gels by excision after being located by exposure to X-ray film. The silces were treated with protease V8 and electrophoresed. A, B, and C, untreated HSP_{68}, HSP_{70}, and HSP_{87}, respectively; D and E, HSP_{68} treated with 0.01 and 0.05μg V8; F and G, HSP_{70} treated with 0.01 and 0.05μg V8; H and I, HSP_{87} treated with 0.01 and 0.05μg V8, respectively. Peptide fragment composition of HSP_{68} appears to be similar to HSP_{70} but not to HSP_{87}.

major HSPs showed increased synthesis over the unheated control. The significance of this increased synthesis remains to be ascertained.

Peptide Fragment Composition of HSPs

In order to explore whether the three major HSPs are related to each other by sharing amino acid sequences, we analyzed these HSPs by partial proteolytic mapping using *Staphylococcus aureus* protease V8. The autoradiogram shown in Fig. 9 indicate that a similarity in the partial peptide maps of HSP_{68} and HSP_{70} exists but HSP_{68} and HSP_{87} appear to be different.

DISCUSSION

The present experiment as well as many reports in the literature (Freeman *et al.*, 1980; Goldin and Leeper, 1981; Urano *et al.*, 1980; Vaupel *et al.*, 1981) indicate that low environmental pH at the time of hyperthermic treatment sensitizes mammalian cells to heat killing. Also shown by the present study is that the pH effect is not cell-type dependent, sensitization being similar in both MEF and SCK cells. In this context, it is worth noting that synthesis of HSP is likewise pH dependent, irrespective of cell types, when the cells are exposed to different temperature and duration of heat.

HSP_{68} is observed as a novel protein, while the levels of HSP_{70}, HSP_{87}, and HSP_{110} are enhanced as shown in both MEF and SCK cells. The mRNA of the HSP_{68} is considered to be synthesized de novo upon heat shock, because actinomycin D inhibits the induction of the synthesis of this protein even if it is removed before labelling (unpublished data). These HSPs are equally observable in MEF and SCK cells under acidic as well as normal pH. However, the synthesis of the HSP_{68} in the cells is found to start and end late under acidic pH compared to that under normal pH. The duration of HSP synthesis is 0∼5 hr after hyperthermia in normal medium, whereas that in acidic pH is 3∼9 hr.

The function of HSP is not fully understood. It is suggested, however, that HSP may perform a function essential to cell survival following exposure to heat (Guttman *et al.*, 1980). On the relationship between HSP synthesis and the differential heat susceptibility of normal and malignant cells, Tsukeda *et al.* (1981) reported that the modulation of heat shock protein synthesis after heat treatment reflects exactly the heat surceptibility of the malignant or normal human lung cells. In contrast, Omar *et al.* (1984) showed that the higher intrinsic resistance of the normal cells to killing by heat compared to transformed cells was not directly related to basal HSP levels or to the magnitude of HSP synthesis induced following hyperthermia. The assignment of a heat-protective role is based on the observed correlation between the levels of HSP and heat sensitivity (Li, 1985). For example, a heat treatment that elevates the rate of synthesis of HSP also induces a transient but large increase in heat resistance, the phenomenon of thermotolerance (Field and Anderson, 1982). In support of this view are the results obtained in this study that differential

thermotolerance is induced with different conditioning heat and also that a good correlation is found between the kinetics of thermotolerance induction and HSP synthesis.

In summary, the pH dependency of heat sensitivity of MEF and SCK cells could be, at least in part, correlated with the kinetics of HSP synthesis. A differential kinetics for the synthesis of HSP_{68} under different environmental pH leads possibly to the following interpretation. The pH sensitizes the cells to heat, leading a part of the cells to be lethal and the rest of cells to be sublethal. In other words, the degree of sensitization determines the cells' viability, thus the ability of the cells to synthesize HSP. When heavily sensitized, the cells undergo a lethal damage leading to death. If weakly sensitized, however, the cells undergo to recovery from sublethal damage before resuming HSP synthesis as a response to heat stress. Therefore the kinetics of HSP synthesis in the cells under acidic pH appears late compared to those under normal pH. The delay in the appearance of HSP synthesis corresponds to the difference in the recovery time between the cells in the acidic pH and those in the normal pH.

Because the HSP is known to correlate with the onset of thermotolerance it is likely that the reduction or delay in the HSP synthesis at low pH is related with the reduction or delay in the thermotolerance development. Therefore, the diminished or delayed production of HSP in the cells under acidic condition confers cells to be heat sensitive than those under normal pH. However, as the induction of thermotolerance is not dependent solely on the HSP and the kinetics of HSP synthesis is dependent on the temperature and duration of hyperthermia, further studies are needed to draw a sound conclusion.

ABSTRACT

In order to explore whether differential thermosensitivity exist between the MEF and SCK cells and the effects of environmental pH on the thermosensitivity and heat shock protein (HSP) synthesis, cell survival rate and kinetics of thermotolerance induction as well as HSP synthesis were examined.

Effect of hyperthermia at 42°C for 2 hr on the relative survival rate of MEF and SCK cells under normal or acidic pH revealed that no intrinsic difference in the thermosensitivity exists between MEF and SCK cells and that cellular thermosensitivity is sensitized by acidic pH, regardless of cell type.

Effect of magnitude of conditioning heat on the thermotolerance induction was examined by treating SCK cells with 5 min or 20 min at 45°C. Thermotolerance was induced rapidly as well as to high level with shorter exposure time compared to longer one, and the kinetics of thermotolerance induction was found to correlate with that of HSP synthesis.

Heat shock at 46°C for 6 min at normal or acidic pH exhibited the similar pattern of protein synthesis, including HSPs. However, hyperthermia at 43°C for 30 min brought about different kinetics of HSP_{68} synthesis depending upon environmental pH, revealing a

delay of a couple of hours at acidic pH.

Peptide mapping of three major HSPs indicated that the partial peptide maps of HSP_{68} and HSP_{70} are similar, possibly by sharing amino acid sequences, but those of HSP_{87} are different.

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