Korean J. Zool. 32:305-313, 1989

A Possible Target for the Heat Inactivation of SCK Tumor Cells

Man-Sik Kang and Joo Young Chung

Dept. of Zoology, College of Natural Science, Seoul National University
Seoul 151-742, Korea

The present investigation aims at inquiring into a possible target for the heat inactivation of SCK tumor cells by comparing the kinetics of cell survival, rate of protein synthesis, and DNA polymerase activity in the presence of heat protector or heat sensitizer.

A possible conclusion to be drawn from the present experiment is that there is no direct correlation between cell death and decrease in the rate of protein synthesis, but that the loss of DNA polymerase β activity correlates quite well with cell inactivation. Thus, protein degradation and/or abnormal protein synthesis causes cell inactivation indirectly, possibly by altering the cellular environment which in turn affects the DNA polymerase β activity. Accordingly, further studies, dealing with the correlation between changes in the cellular environment and DNA polymerase β activity, are needed to get insight into a possible target for the heat inactivation of cells.

KEY WORDS: Target for heat inactivation, Protein degradation, DNA polymerase β

Despite a lot of information is known of the cellular and molecular responses of cells to heat, the primary target(s) responsible for cell death are unknown. Many studies support the hypothesis that the plasma membrane is a critical target for heat cell inactivation. Heat has been reported to induce structural changes in the cell membrane (Bass *et al.*, 1978; Lin *et al.*, 1973; Coss *et al.*, 1978). Cell survival studies combining heat with membrane-active agents such as alcohols (Li *et al.*, 1977) and polyamines (Ben-Hur *et al.*, 1978; Gerner *et al.*, 1980) further indicate that damage to membranes may be a primary target.

In contrast, critical targets other than the plasma membrane have also been suggested for heat inactivation of cells. For example, heat may induce nucleolar changes (Simard and Bernard, 1967; Love *et al.*, 1970), induce chromosomal aberrations (Dewey *et al.*, 1978), and cause alterations in DNA synthesis (Dewey *et al.*, 1980; Henle and Leeper, 1979; Mondovi *et al.*, 1969). In addition, heat inhibits protein synthesis (Dewey *et al.*, 1980; Henle and Leeper, 1979; Mondovi *et al.*, 1969), presumably by blocking the initiation step (Oleinick, 1979), and causes protein denaturation (Rosenberg *et al.*, 1971). Heat also causes an increased association of nonhistone chromosomal proteins with DNA (Tomasovic *et al.*, 1978; Roti Roti and Winward, 1978). The kinetics of this increase has been correlated with the thermodynamics of heat-induced cell inactivation (Roti Roti *et al.*, 1979).

Since our previous study (Kang and Chung, 1989) indicated that the protein degradation was not directly concerned with cell inactivation, a target other than protein degradation has to be predicted. In this connection, it is interesting that polymerase β activity is particularly heat sensitive (Kunkel *et al.*, 1978; Dube *et al.*, 1977), while the activity of the presumed replicative enzyme DNA polymerase α is relatively heat stable (Dube *et al.*, 1977). The polymerase β activity is inhibited

This work was supported by grant from the Korea Research Foundation in 1987.

to a greater extent by tempertures above 37°C than the polymerase α activity (Wawra and Dolejs, 1979; Weniger *et al.*, 1979). In addition, a positive correlation has been reported between cell death and loss of cellular polymerase β activity for cells developing and then losing thermal tolerance during chronic heating (Dewey and Esch, 1982). Furthermore, the level of thermal damage measured by cell lethality correlates well with the level of thermal damage measured by loss of soluble cellular polymerase β activity in Chinese hasmter ovary cells after hyperthermic treatment of 42.5-45.5°C (Mivechi and Dewey, 1984).

Accordingly, in the present investigation we have examined the effect of heat on the cell survival, rate of protein synthesis and DNA polymerase α and β activities in the presence of heat protector or heat sensitizer, aiming at elucidating a possible target for the heat inactivation of cells.

Materials and Methods

Cell Culture

SCK tumor cells, mammary carcinoma origin of A/J mouse, were cultured as described previously (Kang *et al.*, 1980).

Maintenance of Acidic pH

pH of the culture media was adjusted and maintained as described by Hahn and Shiu (1983). Normal (pH 7.4) and acidic (pH 6.7) pHs were obtained by adjusting the amount of sodium bicarbonate in the media. The adjusted pH was maintained by continuous gassing of 5 % CO_2-air. The pH levels were reproducible from experiment to experiment.

Glycerol Medium

1 M glycerol medium in RPMI 1640 medium supplemented with 10 % calf serum was filtered just prior to use.

Heat Treatment

SCK tumor cells appropriately seeded in 60 mm dishes were cultured for 2 days. Prior to heat treatment, the cells were equilibrated with normal, acidic or glycerol medium for 2 hr. Culture dishes thus treated were tightly capped, sealed with parafilm, and inserted in plastic shelves, were immersed horizontally in a constant temperature-circulating waterbath for desired durations.

Cell Survival Studies

Following heating, the cells were incubated for 7-10 days. On termination of the incubation, the cells were fixed and stained with crystal violet and the colonies were counted for surviving fraction.

SDS-PAGE

Cells in a culture dish were labelled with 10 μCi/ml ^{35}S-methionine in methionine-free RPMI 1640 medium for 1-2 hr at 37°C. At the end of this period, the medium was removed and the cells were washed three times with cold phosphate buffered saline and were harvested in lysis buffer which contained 2 % SDS. Labelled samples were dissociated by heating in boiling water for 3-5 min. The amount of protein was determined by the method of Lowry *et al.* (1951). Equal amount or equal radioactivity of protein was directly loaded onto 7.5-10 % SDS-polyacrylamide slab gels by employing the method of Laemmli (1970).

Autoradiography and Fluorography

After the SDS-PAGE, the gels were either autoradiographed or fluorographed routinely as required. The autoradiograms or fluorograms were scanned in a densitometer.

Rate of Protein Synthesis

To examine whether the cell death by heat is correlated with the change in the protein synthesis, the change in the rate of protein synthesis was determined. The cells were cultured in RPMI 1640 medium supplemented with 10 % calf serum for 2 days before heat treatment. Prior to heat treatment, the medium was replaced with either normal, acidic or glycerol medium. After hyperthermia, at varying time intervals the cells in a dish were labelled with 10 μCi of ^{35}S-methionine for 2 hr. The change in the kinetics of the rate of protein synthesis by heat was evaluated by densitometric scan of autoradiogram of SDS-PAGE.

Evaluation of DNA polymerase α and β activities

Determination of DNA polymerase α and β activities was performed as described by Spiro *et al.* (1982) with minor modifications. Following hyperthermic treatment, the cell monolayer from each dish was trypsinized and approximately 2×10^6 cells were used to determine the activities. The cell suspension in an eppendorf tube was permeabilized with 0.1 ml Tris buffer, pH 7.8 containing 0.2 % Triton X-100, 5 mM mercaptoethanol, 20 % glycerol, and 1 mM EDTA for 30 min at 0°C. The cells were then treated with 10 mM N-ethylmaleimide (NEM) for DNA polymerase β activity and distilled water for total DNA polymerase activity. After incubation for 60 min at 0°C, 0.1 ml of solution containing 100 mM Tris buffer (pH 7.8 for total DNA polymerase activity or pH 8.3 for DNA polymerase β activity), 1 mM EDTA, 20 % glycerol, 0.2 % Triton X-100, 1 mM dATP, dCTP, dGTP and ^{3}H-TTP (5 μCi/ml, ICN), 10 mg activated thymus DNA and 200 mM KCl were added. For total DNA polymerase activity KCl was not added. Following 20 min incubation at 37°C, the reaction was stopped by adding 10 ml of 10 % TCA. After washing twice with 10 % TCA, TCA-insoluble fraction was counted to determine the radioactivity per sample. The tubes which received 10 mM NEM and 200 mM KCl represented the activity of DNA polymerase β. The total DNA polymerase activity was determined in parallel with this experiment. The DNA polymerase β activity was then subtracted from the total DNA polymerase activity to obtain the activity of DNA polymerase α.

The change in DNA polymerase activities with increase in heating time at temperatures ranging 42-44°C and under normal, acidic or glycerol condition, and the correlation between cell survival and DNA polymerase activities were processed and plotted with Symphony program (Lotus Development Co.).

Results

Effect of Acidic pH and Glycerol on the Hyperthermic Cell Survival

Survival curves were obtained for the cells exposed to hyperthermia at 41-45°C for the desired durations in normal, acidic or glycerol medium as shown in Figs. 1-3. Comparison of survival curves for glycerol or acidic medium to that for normal medium revealed that glycerol served as a good heat-protector, whereas acidic pH served as a heat-sensitizer. The protective effect of glycerol was found to be more pronounced over the sensitizing effect of acidic pH, and the former effect appeared at all temperature range of 41-45°C but the latter effect except 41 and 45°C.

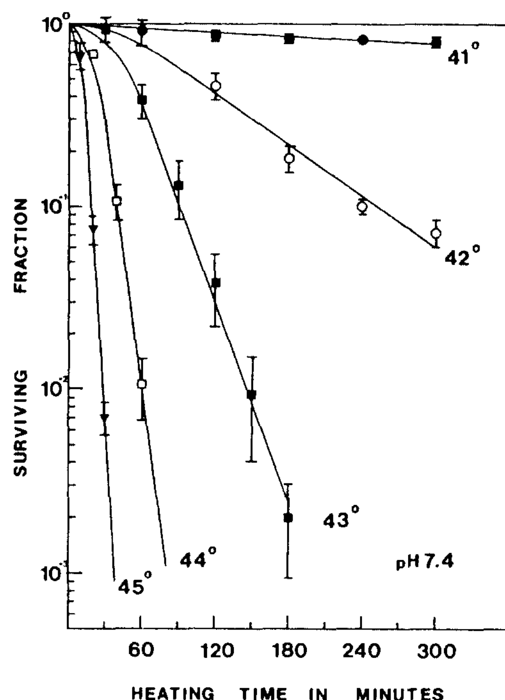

Fig. 1. Effects of hyperthermia at 41-45°C for designated durations on the SCK tumor cell survival at pH 7.4.

Change in the HSP_{70} and 80 kDa Protein Synthesis

Following hyperthermic treatment at varying temperatures for desired durations in normal, acidic or glycerol medium, the cells were labelled with ^{35}S-methionine for 2 hr, followed by SDS-PAGE and autoradiography. The autoradiograms were scanned in a Toyo digital densitometer (DMU-33C). A representative scanning pattern is shown in Fig. 4 to indicate the peaks (a-l)

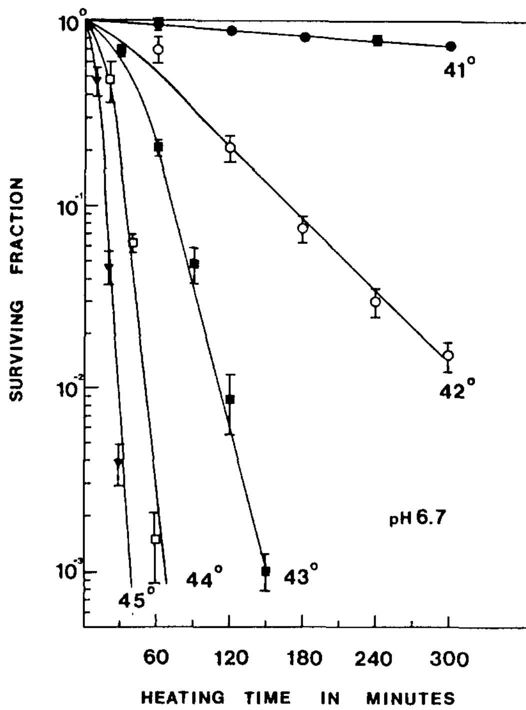

Fig. 2. Effects of hyperthermia at 41-45°C for designated durations on the SCK tumor cell survival at pH 6.7.

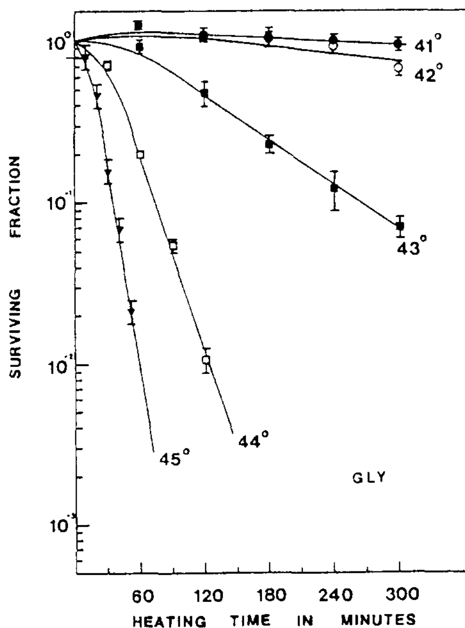

Fig. 3. Effects of hyperthermia at 41-45°C for designated durations on the SCK tumor cell survival in glycerol-treated medium.

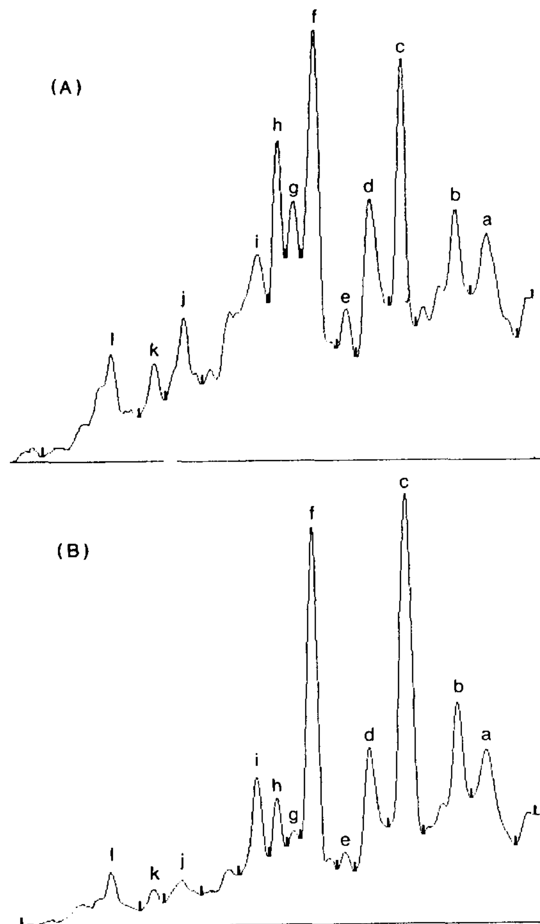

Fig. 4. Rates of synthesis of some proteins as revealed by densitometric scan. A, unheated control; B, heated at 43°C for 30 min.

actually analyzed. Among the peaks examined, only 2 peaks (c and e) were found to be of interest in that they decreased in the order of glycerol, normal and acidic medium and the decreasing patterns of synthesis of HSP_{70} and 80 kDa protein are shown in Figs. 5 and 6, respectively.

Loss of DNA Polymerase α and β Activities by Hyperthermia

The effects of hyperthermic treatment at 42-44°C and in either normal, acidic or glycerol medium on the DNA polymerase α and β activities, as determined by incorporation of ^3H-TTP into activated calf thymus DNA, were investigated.

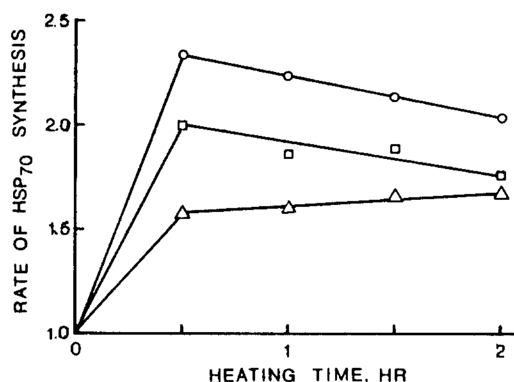

Fig. 5. Decrease of the rate of HSP_{70} synthesis with increase in heating time at 43°C. □-□, pH 7.4; ○-○, pH; △-△, glycerol.

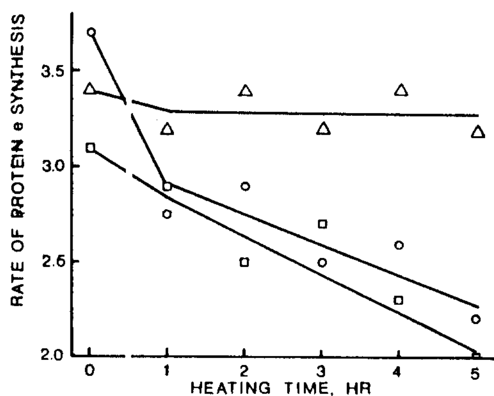

Fig. 6. Decrease of the rate of proteine synthesis with increase in heating time at 43°C. □-□, pH 7.4; ○-○, pH 6.7; △-△, glycerol.

Of these, decrease of DNA polymerase α and β activities with increase in heating duration at 43°C are shown in Figs. 7 and 8, respectively. As is evident in these figures, DNA polymerase α and β activities appeared to be protected or sensitized by glycerol or acidic pH, as are exemplified in the cell survival. The degree of either protection or sensitization was pronounced in DNA polymerase α than in polymerase β. A similar tendency was apparent in the cells exposed to hyperthermia at 42-44°C (dat not shown). In general, the levels of soluble cellular DNA polymerase α and β activities were reduced by heating the cells, but for a given amount of heat, the loss of polymerase β activity was greater than that of polymerase α activity.

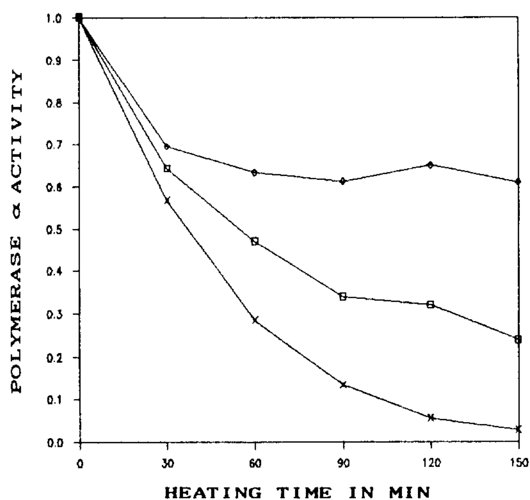

Fig. 7. Effect of heating at 43°C on DNA polymerase α activity as expressed in terms of fraction of the control. □-□, pH 7.4; ○-○, pH 6.7; △-△, glycerol.

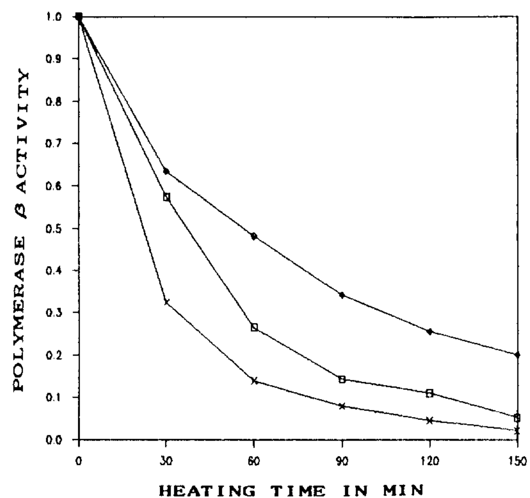

Fig. 8. Effect of heating at 43°C on DNA polymerase β activity as expressed in terms of fraction of the control. □-□, pH 7.4; ○-○, pH 6.7; △-△, glycerol.

Correlation between Cell Survival and DNA Polymerase α and β Activities

To examine whether the loss of polymerase activities are correlated with the cell killing, survival is plotted versus polymerase α or β activities for the temperature range of 42-44°C. Of these,

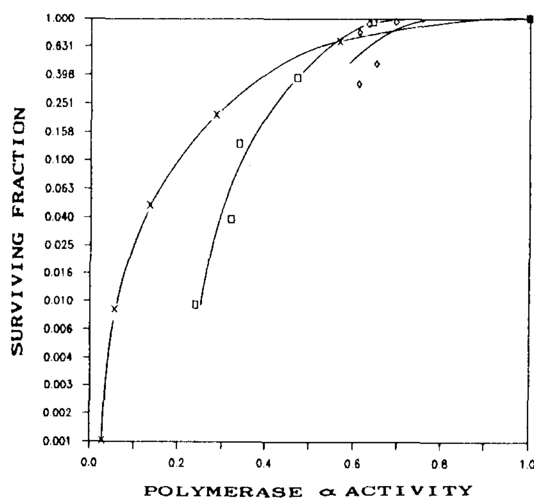

Fig. 9. Correlation between survival and DNA polymerase β activity for cells exposed to 43°C. □-□, pH 7.4; ○-○, pH 6.7; △-△, glycerol.

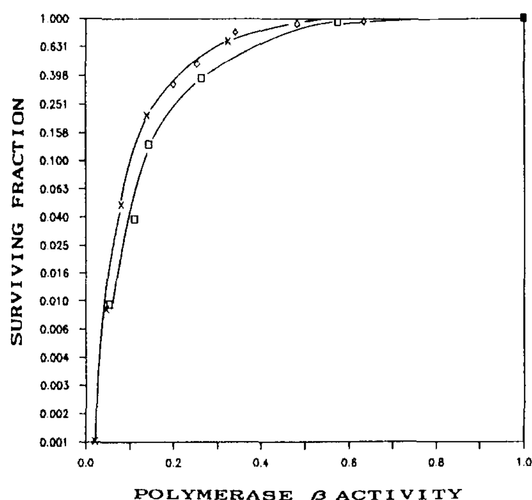

Fig. 10. Correlation between survival and DNA polymerase β activity for cells exposed to 43°C. □-□, pH 7.4; ○-○, pH 6.7; △-△, glycerol.

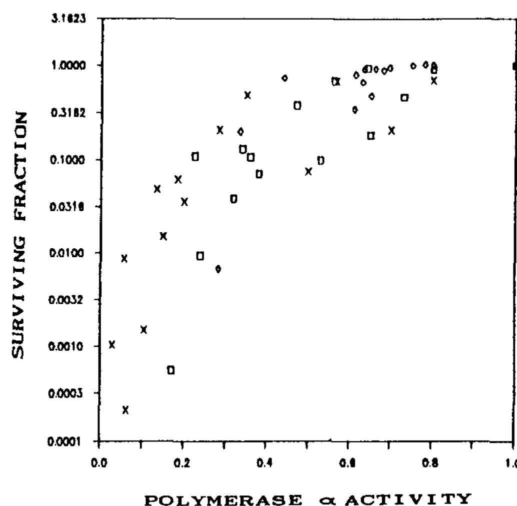

Fig. 11. Overall correlation between survival and DNA polymerase α activity for cells exposed to 42-44°C in either normal, acidic or glycerol-treated medium.

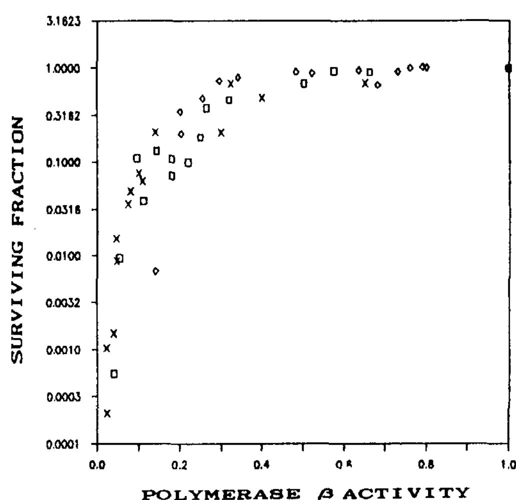

Fig. 12. Overall correlation between survival and DNA polymerase β activity for cells exposed to 42-44°C in either normal, acidic or glycerol-treated medium.

the correlation between surviving fraction versus DNA polymerase α and β activities at 43°C is shown in Figs. 9 and 10, respectively. Comparison of these figures indicates that cell survival is positively correlated with the loss of DNA polymerase β activity but not with that of polymerase α activity. This is illustrated by the close distribution of points for polymerase β after heating at acidic or glycerol medium, whereas for a given survival level, the points for polymerase α spread widely. Collective data for survival versus DNA polymerase α and β activities for the cells heated at normal, acidic or glycerol medium and at temperatures ranging 42-44°C are shown in Figs. 11 and 12. These results strongly suggest that there exists a positive correlation between cell survival and loss of DNA polymerase β activity.

Discussion

Cell survival data revealed that the effect of both heat protector and sensitizer was apparent, the magnitude of the effect being pronounced for protector than for sensitizer. Initially, it was predicted that cell survival following hyperthermia would correspond with the decrease of protein synthesis, at least some of the proteins. However, the rate of synthesis of HSP_{70} or 80 kDa protein was found not to correlate with cell survival following hyperthermia. Therefore, the possibility that cell killing is somehow ascribed to the decrease in the rate of protein synthesis is not likely and the cell inactivation is not affected directly by the decrease in the protein synthesis.

To examine whether cell inactivation is correlated with the loss of soluble cellular DNA polymerase activities, soluble cellular DNA polymerase α and β activities were determined for the cells exposed to hyperthermic treatment at 42-44°C for desired durations and at normal, acidic or glycerol medium. The levels of both polymerase α and β activities were reduced by heating the cells, but for a given amount of heat, the loss of α activity was less than that of β activity (Figs. 7 and 8). For example, in the cells exposed to hyperthermic treatment at 43°C for 60 min, polymerase α activity decreased to a level of 47 % of the control, whereas polymerase β activity decreased to a level of 28 %. Thus, polymerase β activity appeared particularly heat sensitive, while the activity of the presumed replicative enzyme DNA polymerase α appeared relatively heat stable as shown previously (Kunkel *et al.*, 1978; Dube *et al.*, 1977).

The present study indicated that the level of thermal damage measured by cell death correlates well with the level of thermal damage measured by loss of soluble cellular polymerase β activity. specifically, both enhancement of thermal damage by heating at acidic pH and reduction of thermal damage by heating in the presence of glycerol were almost the same for the polymerase β activities but not for polymerase α activities. These results, together with others (Mivechi and Dewey, 1984), suggest that the level of soluble polymerase β activity in the cell serves as an index of thermal damage. Obviously, cell death is not associated with the complete loss of polymerase β activity, because at cell survival levels of about 63 %, polymerase β activities were about 40 % of the controls, and at cell survival of about 10 %, polymerase β activities were about 13 %.

The levels of polymerase α activity also were reduced by heating the cells, but did not correlate with cell lethality when the cells were heat sensitized by acidic pH or were heat protected by glycerol (Figs. 9 and 10). Heat sensitization at acidic pH was greater for reduction of polymerase α activity than for reduction of polymerase β activity, wherase protection from heat damage with glycerol was the least for reduction of polymerase α activity.

When the cells were treated with a membrane active agent, procaine-HCl, they were heat sensitized in terms of loss of β activity in a manner similar to that observed for cell killing, while when the isolated enzyme was treated with procaine, no sensitization to heat was observed (Spiro *et al.*, 1982; 1983). Based on these facts, the results of the present investigation are likely to be explained as following. Heat-induced loss of polymerase activity results both from heat-induced membrane damage, leading to secondary effects on polymerases and from heat denaturation of the polymerases. Thus heat effects of membranes, which could be any of the cellular membranes, can apparently result in changes in environmental conditions within the cell, which can in turn alter the emzyme and/or the direct or secondary effect of heat on the enzyme (Mivechi and Dewey, 1984). The present finding that inactivations of polymerase α and β activities respond differently to the heat modifying effects of low pH and glycerol treatment implies that direct and/or secondary effects of heat on the enzymes also are modified by low pH and glycerol. Thus the differences between the two enzymes in modifications in heat-induced loss of activities, including the good correlation between the two enzyme activities and survival when the cells are heated at acidic pH, or with glycerol, could result from acidic pH and glycerol treatment altering polar and nonpolar interactions, respectively, to different degrees in the two enzyme molecules. Such differ-

ences might be expected because of differences in the molecular structures; i.e., polymerase α has 4-8 subunits (180-450 kDa) and an isoelectric point of pH 7.2 and polymerase β has 1 subunit (40 kDa) and an isoelectric point of pH 8.5 (Hubscher, 1983).

The main conclusions to be drawn from the present study are (1) loss of polymerase β activity correlates well with loss of cell viability for asynchronous SCK tumor cells and (2) loss of polymerase α and β responds differently to heat sensitization and protection. Thus, further studies, dealing with the correlation between changes in the cellular environment and DNA polymerase β activity, are needed to get insight into a possible target for the heat inactivation of cells.

References

Base, H. J., J. L. Moore, and W. T. Coakley, 1978. Lethality in mammalian cells due to hyperthermia under oxic and hypoxic condition. *Int. J. Radiat. Biol.* **33:** 57-67.

Ben-Hur, E., A. Prager, and E. Riklis, 1978. Enhancement of thermal killing by polyamines, *Cancer Res.* **40:** 432-438.

Coss, R. A., W. C. Dewey, and J. R. Bamburg, 1978. The effects of hyperthermia alone and in conjunction with procaine on CHO cells. *J. Cell Biol.* **79:** (Abstract).

Dewey, W. C., and J. L. Esch, 1982. Transient thermal tolerance: Cell killing and DNA polymerase activity. *Radiat. Res.* **92:** 611-614.

Dewey, W. C., M. L. Freeman, G. P. Raaphorst, E. P. Clark, R. S. L. Wong, D. P. Highfield, I. J. Spiro, S. P. Tomasovic, D. L. Denman, and R. A. Coss. Cell biology of hyperthermia and radiation. *In.* Radiation Biology in Cancer Research (R. E. Meyn and H. R. Withers, Eds.) pp. 589-621. Raven Press. New York, 1980.

Dewey, W. C., S. A. Sapareto, and D. A. Betten, 1978. Hyperthermic radiosensitization of synchronous Chinese hamster cells: Relationship between lethality and chromosomal aberration. *Radiat. Res.* **76:** 48-59.

Dube, D. K., G. Seal, and L. A. Boeb, 1977. Differential heat sensitivity of mammalian DNA polymerases. *Biochem. Biophy. Res. Commun.* **76:** 483-487.

Gerner, W. W., D. K. Holmes, D. G. Stickney, J. A. Noterman, and D. J. M. Fuller, 1980. Enhancement of hyperthermia-induced cytotoxicity by polyamines. *Cancer Res.* **40:** 432-438.

Hahn, G. M. and E. C. Shiu, 1983. Effect of pH and elevated temperature on the cytotoxicity of some chemotherapeutic agents on Chinese hamster cells in vitro. *Cancer Res.* **43:** 5789-5791.

Henle, K. J. and D. B. Leeper, 1979. Effects of hyperthermia (45C) on macromolecular synthesis in Chinese hamster ovary cells. *Cancer Res.* **39:** 2665-2674.

Hubscher, U., 1983. DNA polymerases in prokaryotes and eukaryotes: Mode of action and biological implications. *Experientia* **39:** 1-126.

Kang, M. S., C. W. Song, and S. H. Levitt, 1980. Role of vascular function in response to tumors *in vivo* to hyperthermia. *Cancer Res.* **40:** 1130-1135.

Kang, M. S. and J. Y. Chung, 1989. SCK tumor cell killing by hyperthermia in the presence of heat--protector and heat-sensitizer. *Korean J. Zool.* **32:** 134-141.

Kunkel, T. A., J. E. Tcheng, and R. R. Meyer, 1978. Purification and properties of DNA polymerase-β from guinea pig liver. *Biochim. Biophys. Acta* **520:** 302-316.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

Li, G. C., G. M. Hahn, and E. C. Shiu, 1977. Cytotoxicity of commonly used solvents at elevated temperatures. *J. Cell. Physiol.* **93:** 331-334.

Lin, P. S., D. F. H. Wallach, and S. Tsai, 1973. Temperature-induced variations in the surface topology of cultured lymphocytes are revealed by scanning elctron microscopy. *Proc. Natl. Acad. Sci.* U.S.A. **70:** 2492-2496.

Love, R, R. Z. Soriano, and R. J. Walsh, 1970. Effect of hyperthermia on normal and neoplastic cells *in vitro*. *Cancer Res.* **30:** 1525-1533.

Lowry, O. H., N. G. Rosenberg, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the folin reagent. *J. Biol. Chem.* **193:** 265-275.

Mivechi, N. F. and W. C. Dewey, 1984. Effect of glycerol and low pH on heat-induced cell killing and loss of cellular DNA polymerase activities in Chinese hamster ovary cells. *Radiat. Res.* **99:** 352-362.

Mondovi, B., A. Finazzi'Agro, Moricca, and A. Rossi Fanelli, 1969. The biochemical mechanism of selective heat sensitivity of cancer cells. II. Studies on nucleic acids and protein synthesis. *Eur. J. Cancer* **5:** 137-146.

Oleinick, N. L., 1979. The initiation and elongation steps in protein synthesis: Relative rates in Chinese hamster ovary cells during and after hyperthermic and hypothermic shocks. *J. Cell. Physiol.* **98:** 185-192.

Rosenberg, B., G. Kemeny, R. C. Switzer, and T. C. Hamilton, 1971. Quantitative evidence for protein de-

naturation as the cause of thermal death. *Nature* **232:** 471-473.

Roti Roti, J. L., K. J. Henle, and R. T. Winward, 1979. The kinetics of increase in chromatin protein content in heated cells: A possible role in cell killing. *Radiat. Res.* **78:** 522-531.

Roti Roti, J. L. and T. Winward. 1978. The effects of hyperthermia on the protein to DNA ratio of isolated HeLa cell chromatin. *Radiat. Res.* **74:** 156-169.

Simard, R. and W. Bernard, 1967. A heat-sensitive cellular function located in the nucleolus. *J. Cell Biol.* **34:** 61-76.

Spiro, I. J., D. L. Denman, and W. C. Dewey, 1982. Effect of hyperthermia on CHO DNA polymerase α and β. *Radiat. Res.* **95:** 68-77.

Tomasovic, S. P., G. N. Turner, and W. C. Dewey, 1978. Effect of hyperthermia on nonhistone proteins isolated with DNA. *Radiat. Res.* **73:** 535-552.

Wawra, E. and I. Dolejs, 1979. Evidences for the function of DNA polymerase-β in unscheduled DNA synthesis. *Nucleic Acids Res.* **7:** 1675-1686.

Weniger, P., E. Wawra, and I. Dolejs, 1979. The action of hyperthermia on DNA repair. *Radiat. Environ. Biophys.* **16:** 135-141.

(Accepted July 20, 1989)

SCK 腫瘍細胞의 熱不活性化의 標的에 관한 연구

강만식 · 정주영 (서울대학교 자연과학대학 동물학과)

본 연구는 열보호제 또는 열증감제의 존재하에서 세포 생존곡선, 단백질 합성률, DNA 중합효소 β의 활성변화를 비교 검토함으로써 SCK 종양세포가 열에 의해서 불활성화될 때의 표적이 무엇인지를 밝혀보기 위해서 수행되었다.

본 실험의 결과로 추정하건대 열에 의한 세포치사는 단백질 합성률의 변화와는 직접적인 연관성이 없으나, DNA 중합효소 β의 활성도와는 밀접한 연관성이 있음을 알 수 있다. 즉, 단백질의 분해 또는 비정상적인 단백질의 합성이 세포의 환경을 변화시키고 이것이 DNA 중합효소 β의 활성에 영향을 미침으로써 간접적으로 세포의 치사를 초래할 것으로 짐작할 수 있다. 따라서, 세포의 열불화성화의 표적을 좀더 분명히 밝히기 위해서는 세포의 환경변화와 DNA 중합효소 β의 활성과의 관계를 추구하는 연구가 수행되어야 할 것으로 사료된다.