

Effect of Hyperthermia with Interferon on Malignant Cell Growth and Cell Cycle

Tetuya SATOU

*First Department of Surgery
Nagasaki University School of Medicine*

Received for publication, December 26, 1987

ABSTRACT : Combined therapy of hyperthermia with interferon- β (rHuIFN- β) for carcinoma using HeLa cells was evaluated by in vitro study. Inhibitory effect on cell growth curve was observed in the treatment of rHuIFN- β and/or hyperthermia alone. On the other hand, combined treatment much more promoted the inhibitory effect rather than single method.

From the standpoint of the cell cycle on the basis of a result of flow cytometry study, a reduction of the G₁-phase cells population and accumulation of the S-phase cells were significantly noted. Furthermore, the addition of hyperthermia significantly recruited the S-phase cells. When administering rHuIFN- β , the activity of DNA polymerase α was reduced and that of DNA polymerase β was inversely increased. While hyperthermia was added, both the levels of DNA polymerase α and β activities were decreased. It was different patterns in terms of enzymatic activities regarding accumulation of the S-phase cells.

In conclusion, in order to get the cytocidal effect on the HeLa cells, the ideal timing applying combined treatment of HuIFN- β with hyperthermia is that HuIFN- β should be first given, thereafter hyperthermia is applied so as to adjust to the S-phase of the cell cycle which is greatly sensitive to hyperthermia.

INTRODUCTION

The combination treatment of hyperthermia with anticancerous drugs is expecting and promising a long survival of tumor-bearing host. Recently development of interferon (IFN) focused on an attention to the cytocidal and host-mediating actions on cancer cells and it is applied for a combination therapy with hyperthermia.

GROVEMAN¹⁾ in 1984 reported that combined therapy of hyperthermia with IFN produced a great effect in cell-killing of cultured human cancer cells. The same results were reported by others²⁾⁻⁵⁾. However, the mechanism of cytocidal effect of this combined therapy is not yet

made clear.

The aim of this study is to clarify the cytocidal mechanism of combined therapy of IFN with hyperthermia from the standpoint of cell kinetics in cultured cells and also to make clear of proper timing of administrations of IFN and hyperthermia as well as availability of this therapy in view of DNA polymerase activity which is needed for reproduction and repair of DNA synthesis process.

MATERIALS AND METHODS

1. Cells and cell culturing

HeLa (human cervical carcinoma) cells were kindly provided by Dept. Viral. Ricketts., Natl. Inst. Health. All cells were used exponentially

growing cells. Cells were grown as monolayers in plastic flasks at 37°C in a 5% CO₂, growth medium used was RPMI1640 (GIBCO) supplemented with 10% fetal calf serum and antibiotics. Exponentially growing cells were subcultured, utilizing a 0.25% Trypsin, 0.02% EDTA solution.

2. Drugs and Hyperthermia

Human recombinant interferon α , β and γ . rHuIFN- α (Ro22-8181) and rHuIFN- γ (TRP-2) were obtained from TAKEDA YAKUHIN, rHuIFN- β (GKT- β) was obtained from KYOWA HAKKO. rHuIFN- α , β and γ were solved to RPMI1640 with 20% FCS, and then they were stored at -80°C.

The cells were placed in a water bath (Incubator BT-23 Yamato Scientific Co, Ltd) for the assigned length of time. Temperatures were readed with thermometer, the temperature was maintained.

3. Antiproliferative Assays

Cell proliferation was determined to growth curve and colony formation. Exponentially growing cells were plated at 10⁵ cells to 6-Well Multidish (Nunc), after 24 hours, rHuIFN- β was added to Wells continously, Wells were incubated, and then cells were trypsinized and counted.

Colony formation was determined by preparing a uniform single cell suspension and adding 500 cells to each 6-Well Multidish. After 24 hours, heat and/or rHuIFN- β treatment was done, and then Wells were incubated 9 days at 37°C in a 5% CO₂, humidified atmosphere. At appropriate times, 6-Well was fixed with 95% ethanol and stained with Giemsa. Colonies with greater than 50 cells were counted.

4. Cell cycle analysis

Cell cycle distributions were determined by flow cytometry (FCM). Propidium Iodide (PI) staining was done as described by NOMURA⁶⁾. Cells were fixed in 70% ethanol, and the cells were washed with phosphate buffered saline (PBS), pepsinated for 15 minutes at 37°C, disposed of RNase for 20 minutes, and then stained with 5-10ml of PI solution (50mg PI, 0.18 M Tris Buffer, 0.18M NaCl pH 7.5). After 2

hours, cellular DNA content of 10,000 cells was calculated by FCM (FACSIV, Becton Dickinson)

Exponentially growing cells were plated at 10⁶ cells in 10ml medium in 100mm dish, after 24 hours, the cells were treated rHuIFN or hyperthermia, and then the cells were washed and detached. The single cell suspension was stained with PI and cell cycle distributions were determined by FCM.

5. Determination of DNA polymerase activities

Before heating at 43°C, treating with rHuIFN- β or control cells were harvested and the cells (10⁷) were centrifuged and washed twice in PBS. The pellet was resuspended in 0.5 ml of 50mM Tris-HCl (pH7.6) containing 0.1 mM EDTA, 0.2mM dithiothreitol, 10% glycerol, 0.5M KCl.

Cells were minced at two times by sonicator (W-220F, Heat system company), and then centrifugation was conducted in tubes at 6,000 rpm for 20 minutes, the supernate was used as an enzyme source. All procedures were carried out at 0-4°C.

① DNA polymerase α activity

5 μ l of the enzyme source and 20 μ l of polymerase α reaction mixture⁷⁾ were incubated for 30 min at 37°C. The reaction mixture of 20 μ l contained the following: 50mM Tris-HCl (pH7.5), 1mM dithiothreitol, 8mM MgCl₂, 400 μ g/ml of bovine serum albumine, 80 μ g/ml of activated DNA, 50 μ M of all four deoxynucleoside triphosphates, 50 μ M (³H) dTTP, 12% glycerol. The reaction mixture was kept on ice and the reaction was stopped. The radioactive DNA product was collected on a paper disc made of DEAE-cellulose (DE81), the paper disc was washed with 5% Na₂HPO₄, distilled water and alcohol. The samples were placed in liquid scintillation counter (ALOKA, LSC-903) to determine radioactivity per sample. Polymerase β activity was detected by the polymerase β assay due to inhibition of polymerase α by N-ethylmaleimide (NEM, 3.75mM). Polymerase α activity was determined to subtract polymerase β activity.

② DNA polymerase β activity

DNA polymerase β was assayed in 50mM T

MgCl₂, 400 μ g/ml of bovine serum albumine, 80 μ g/ml of activated DNA, 50 μ M of all four deoxynucleoside triphosphates, 50 μ M (³H) dTTP, 12% glycerol, 100mM KCl and 15 μ l NEM (10mM). The β -assay was strictly specific for the polymerase β activity.

③ Measurement of protein concentration

Protein concentrations of assaying crude extracts were determined by Lewry method. Enzyme activities were indicative of cpm/ μ g protein.

RESULTS

1. Effect of hyperthermia on inhibition of cell proliferation and cell cycle

The sensitivity of HeLa cells to hyperthermia was tested by colony forming assay method at 37, 39, 41, 43 and 45°C. As shown in Fig. 1, colony forming rate (lethal rate of living cells) varied with varying temperature and the duration of action. It was confirmed that critical temperature was a range of 41 to 43°C. Therefore, the experiment in this study was made on the condition of 43°C temperature and 1 hour duration.

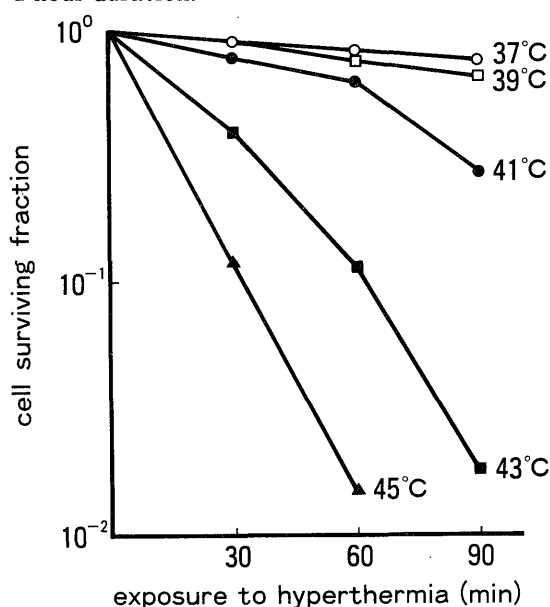


Fig. 1. Survival curves for HeLa cells in culture heated at different temperatures for varying lengths of time, colony number of treatment/colony number of 37°C incubation.

Fig. 2. showed the growth curved and colony formation, compared heated cells at 43°C for 1 hour with control cells at 37°C. As large as 21 % of cytotoxic rate was shown at 43°C for 60 min. However, proliferation was not completely ceased by heating at 43°C and it continued to grow with time in spite of some differences between the control.

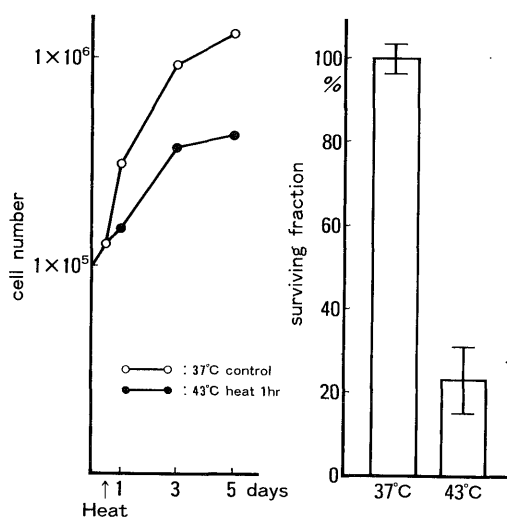


Fig. 2. Cell growth and colony formation after heated at 43°C for 1 hr.

In view of the cell cycle DNA histogram showed a decrease in G₁-phase, slight increase in S-phase and remarkable increase in G₂M-phase at 24 hours, but an increase in G₁-phase and a decrease in G₂M-phase at 48 hours, and later the ratios of G₁, S and G₂M were almost the same as the control at 72 hours as shown in Fig. 3.

2. Effect of rHuIFN on inhibition of cell growth

The inhibitory effect of rHuIFN- α , β , γ on the cell cycle was compared. The growth curves after 5 days administration of HuIFN- α , β , γ was illustrated in Fig. 4. The inhibitory effect of HuIFN- β and γ was significantly pronounced rather than that of HuIFN- α and that of HuIFN- β and γ was almost similar. As a result of observing an alteration of the cell cycle, IFN- α , β administration led to a decrease in G₁-phase cells and an increase in S-

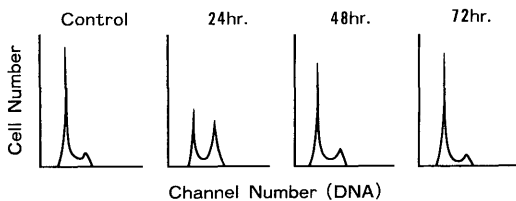


Fig. 3. DNA histograms of cell cycle movement of control and treated cells at 43°C for 1 hr.

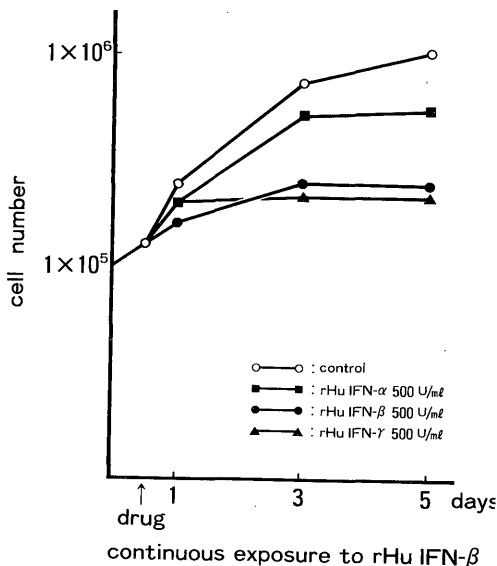


Fig. 4. Effect of rHuIFN- α , β and γ on growth inhibition of HeLa cells. The cells were treated with IFN 500 U/ml for 5 days continuously at 37°C.

phase cells, in particular, IFN- β brought a significant accumulation of S-phase cells. On the other hand, IFN- γ induced an increase in G₁-phase cells and a decrease in S-phase cells. It was different from the action on alteration of the cell cycle between IFN- α , β and γ , and it is characteristic of accumulation of S-phase cells in administering IFN- β (Fig. 5).

Further study was made on a relation between inhibitory effect of cell proliferation and the cell cycle in detail. Fig. 6 showed that continuous administration of rHuIFN- β with a concentration of 250, 500, 1000 U/ml revealed the inhibition of cell proliferation in proportion to a dosis administered. However, these effects were cytostatic at each concentration

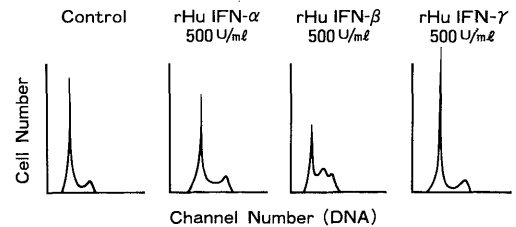


Fig. 5. DNA histograms of cell cycle movement of control and IFN-treated cells for 3 days continuously at 37°C.

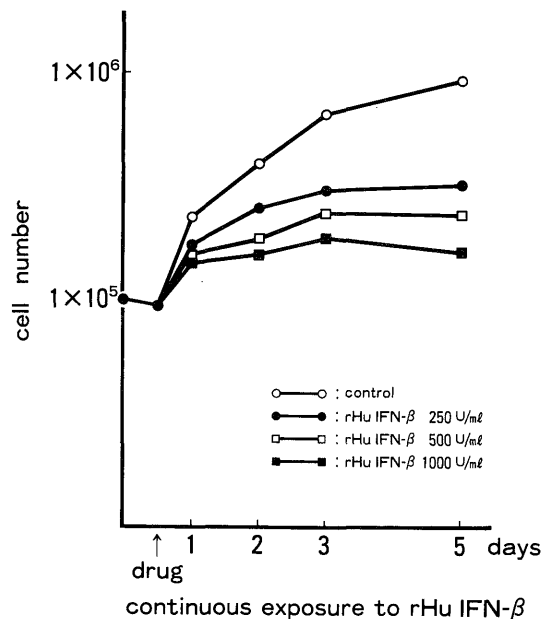


Fig. 6. Effect of rHuIFN- β on cell growth

of IFN- β . And then the effects of IFN- β on the cell cycle were also evaluated as shown in Fig. 7. While 500 U/ml of IFN- β were given, the cell cycle altered into a decrease in G₁-phase, an increase in S-phase and slight accumulation of G₂M-phase on day 3 and this

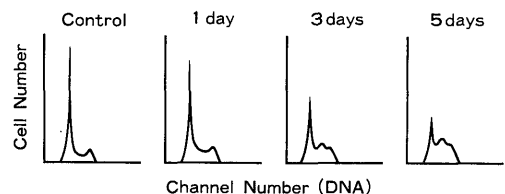


Fig. 7. DNA histograms of cell cycle movement of control and rHuIFN- β 500U/ml-treated cells for 5 days continuously at 37°C.

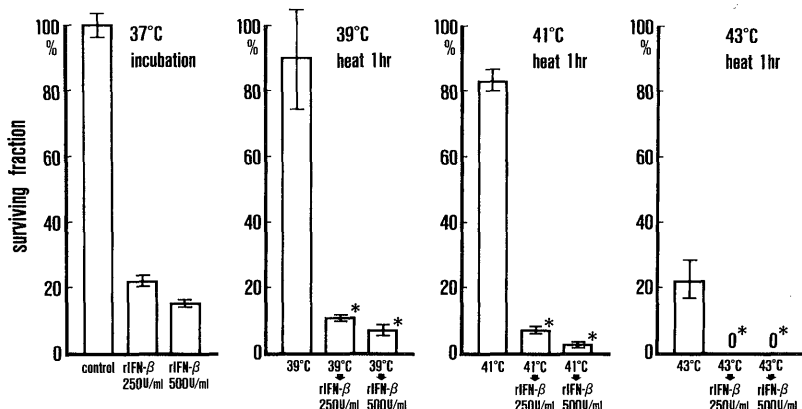


Fig. 8. Synergistic antiproliferative activity of rHuIFN- β and hyperthermia on HeLa cells. After 39, 41 and 43°C heated for 1 hr, the cells were treated with rHuIFN- β 250 or 500 U/ml continuously.

* : synergistic effect of IFN- β and hyperthermia

tendency was most prominent on day 5. An increase in S-phase and G₂M-phase cells as attributed to the concentration and time, although this accumulation was transient and by exclusion of IFN- β , a pattern of DNA histogram returned to be normal.

3. Combined effect of hyperthermia with rHuIFN- β

Combined effect of hyperthermia with rHuIFN- β was evaluated by using colony formation method as shown in Fig. 8. Continuous infusion of HuIFN- β of 250 and 500 U/ml with hyperthermia at 39, 41 and 43°C for 1 hour showed a cytotoxic effect which was synergic regardless a concentration of IFN- β .

4. In order of treatment with IFN- β and hyperthermia

Proper timing of IFN- β administration was assessed on the growth curve (Fig. 9). It was defined by regrowth assay that preceding IFN- β administration to hyperthermia application arose a restart of cell proliferation in fresh medium which was replaced on day 5. In contrast, the regrowth assay method revealed that hyperthermia at 43°C for 1 hour following administration of IFN- β of 500 U/ml for 5 days led to a significant cytotoxic effect.

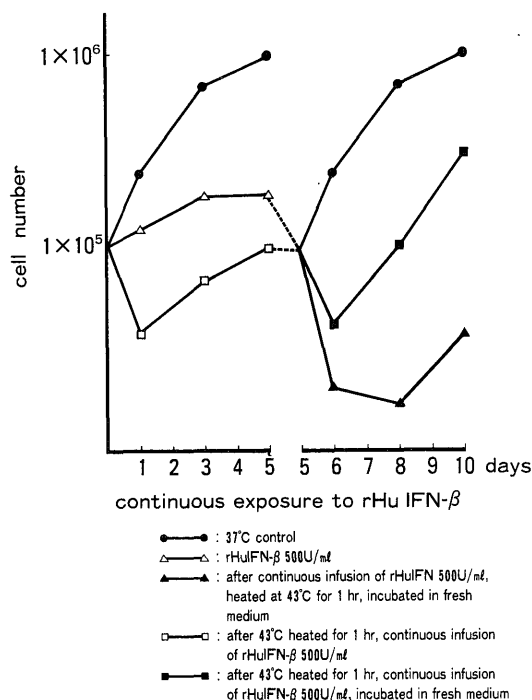


Fig. 9. Regrowth assay of rHuIFN- β and hyperthermia

5. Alteration of the cell cycle during combined therapy of IFN- β with hyperthermia

Fig. 10 shows a result of combined therapy of IFN- β with hyperthermia on DNA histogram. A 5-day administration of IFN- β of 500

U/ml revealed a decrease in G₁-phase cells and an increase in S-phase cells and one day culture in fresh medium resulted in slight increase in G₂M-phase cells and quickly reversed accumulation of S-phase cells as shown in B line in Fig. 10.

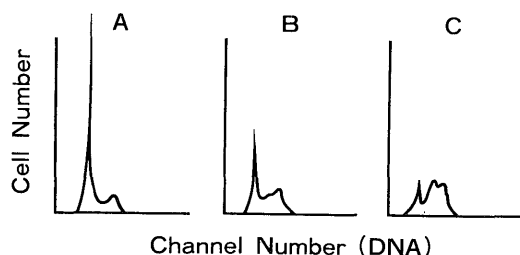


Fig. 10. DNA histograms of cell cycle movement of control and IFN- β combination with hyperthermia.

A : control

B : after continuous infusion of rHuIFN- β 500U/ml for 5 days, incubated in fresh medium

C : after continuous infusion of rHuIFN- β 500U/ml for 5 days, heated at 43°C for 1 hr, incubated in fresh medium

On the other hand, one day culture in fresh medium following a 5-day continuous administration of rHuIFN- β with hyperthermia at 43°C for 1 hour produced a significant reduction of G₁-phase cells and apparent accumulation of S-phase cells as shown in C line in Fig. 10.

6. DNA polymerase activity in combination of rHuIFN- β with hyperthermia

A comparative study between hyperthermia at 43°C for 60 minutes with IFN- β of 500U/ml for 5 days and hyperthermia at 43°C for 60 minutes following continuously administering IFN- β of 500U/ml for 5 days was made by measurement of soluble DNA polymerase α and β activities as shown in Fig. 11.

Hyperthermia induced a decrease in polymerase activity in comparison with the control. In contrast, IFN- β led to a high activity of polymerase β in spite of low activity of polymerase α . Combination of IFN- β with hyperthermia much more reduced DNA polymerase activity. There was statistically significant difference in polymerase activity between IFN- β alone and in combination with hyperthermia despite

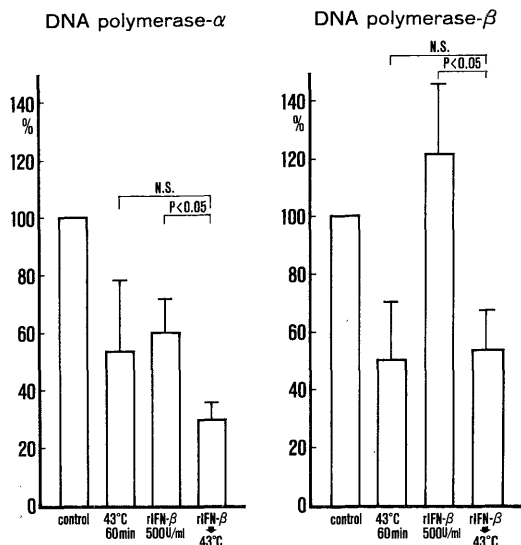


Fig. 11. Activity of DNA polymerase after treatment of HeLa cells with IFN- β and/or hyperthermia.

$$\left(\frac{\text{DNA polymerase activity of treated cells cpm}/\mu\text{g protein}}{\text{DNA polymerase activity of control cells cpm}/\mu\text{g protein}} \times 100 \right)$$

no difference between hyperthermia and combination groups.

DISCUSSION

Since HERON²⁾ reported that the inhibitory action of IFN on cell proliferation was enhanced by heating combination therapy of IFN with hyperthermia has become concentrated on the treatment of carcinomas. GROVEMAN¹⁾ also noted that IFN- α and β revealed effective cell-killing action in human cultured cells of carcinoma of the urinary bladder in combination with mild hyperthermia. FLEISCHMANN³⁾⁴⁾ reported that the inhibitory effect of IFN- α , β and γ on cell proliferation to B-16 melanoma cells of mice was enhanced with increased temperature.

However, the mechanism of inhibitory action of IFN combined with hyperthermia is not yet made clear. In this study, the relation between hyperthermia and cell cycle was studied by using flow cytometry method to clarify the mechanism on inhibition of cell

proliferation. KANO⁸⁾ noted that hyperthermia resulted in induction of block in S and G₂ phases of cancer cells. In this study, an increase in G₂M-phase and a decrease in G₁-phase were noted at 24 hours after, although at 72 hours an increase of G₁-phase and a decrease in G₂M-phase were noted. This result indicates that the inhibitory effect of hyperthermia is transient and temporary. Hyperthermia alone is not sufficient for antigrowth effects on tumor cell proliferation.

On the other hand, CREASEY⁹⁾ analysed IFN action which influenced cell cycle of human melanoma cells with the use of rHuIFN- α . He clarified the results of elongation from G₁ to S phase and increase in S phase. BALKWILL¹⁰⁾¹¹⁾ evidenced an increase in G₂M-phase cells by using MCF-7 cells and HuIFN- β and a prolonged transmission from G₁ to S phases by using fibroblast cells and HuIFN- α and γ . He pointed out from the above results that IFN exerts on different phases of the cell cycle in accordance with the kinds of IFN itself.

In this series, the effects of all kinds of IFN on HeLa cells were evaluated in the analysis of the growth curve and the cell cycle. The inhibitory effect by continuous infusion of 500U/ml rHuIFN- β and γ were still more potent than that of rHuIFN- α . The action of rHuIFN- β was almost the same as that of rHuIFN- γ . It is characteristic of a decrease in G₁-phase and an increase in S-phase cells by IFN- α and β in view of the cell cycle in contrast with IFN- γ which evolves a consequence of an increase in G₁-phase and a decrease in S-phase cells.

As for alteration of the cell cycle for cell damage by heat in combination with the use of IFN, BHUYAN¹²⁾ clarified that hyperthermia is highly sensitive to S-phase cells. It is reasoned that IFN- β is the first choice in combination with hyperthermia because IFN- β is much more stable for heat than IFN- γ .

Inhibition of cell proliferation by combined therapy of IFN- β with hyperthermia is potentiated on the condition of a concentration of 250 to 500U/ml of rHuIFN- β at 39, 41, and 43°C of heating as already reported by others. In this series the effect is recognized on the condition of 39°C and 41°C of low grade

hyperthermia. KUROKI⁵⁾ reported the combination of accumulation in G₂M-phase. Needless to say it is of great benefit to enhance the effects of combined therapy on cell damage. It implies that accumulation of S-phase cells corresponding to retardation of DNA synthesis facilitates cell damage by combined therapy of IFN with hyperthermia.

The measurement of DNA polymerase α and β activities is essential to evaluate a change in the S-phase of the cell cycle. SPIRO¹³⁾ and KAMPINGA¹⁴⁾ evidenced that a reduction of polymerase α and β activities occurred by hyperthermia. On the other hand, LUNDBLAD¹⁵⁾ reported that the use of rHuIFN- β to human glioma cells resulted in accumulation of S-phase cells and reduction of DNA polymerase activity. In this series, it was defined according to Matsukage method⁷⁾ that polymerase activities of HeLa cells exposed to heating of 43°C had become gradually reduced until 45 min and later it showed a stable value keeping a plateau.

Meanwhile, continuous administration of IFN- β depressed DNA polymerase α activity and increased DNA polymerase β activity. It means that repair process of DNA synthesis preserves as it is, and if IFN- β is withdrawn, a pattern of DNA histogram approaches to the control histogram. While hyperthermia is combined with IFN- β , DNA polymerase α activity is much more depressed with a reduction of DNA polymerase β activity. It is suggestive of a depressed ability of DNA repair process with reduced activity of DNA polymerase β . Behavior of cells accumulated in S-phase is influenced by whether DNA polymerase β activity is preserved or not.

It is accepted that the inhibitory action of IFN on cell proliferation not only relates to direct action but to mediate indirectly the immune system of a tumor-bearing host. IFN- α and β stimulate Natural Killer cells and IFN- γ also exerts as macrophage activating factor. Therefore, effectively depressed proliferation of malignant cells can be obtained by stimulation of the immune response of a host by IFN administration and the addition of hyperthermia, providing direct or indirect antitumor effect to the tumor-bearing host. From the result of this study, anti-

tumor effect can be expected by combined therapy of IFN with hyperthermia for the treatment of advanced cancer patients.

ACKNOWLEDGEMENT

I thank Professor M. TOMITA and Dr. Y. TAGAWA, the First Department of Surgery, Nagasaki University School of Medicine for his suggestion and valuable comments. And also thank all the coworkers.

REFERENCE

- 1) GROVEMAN, D. S., BORDEN, E. C., MERRITT, J. A., ROBINS, H. I., STEEVES, R. and BRYAN, G. T. : Augmented antiproliferative effects of interferons at elevated temperatures against human bladder carcinoma cell lines. *Cancer Res.* **44** : 5517 (1984).
- 2) HERON, I. and BERG, K. : The action of interferon are potentiated at elevated temperature. *Nature* **274** : 508 (1978).
- 3) FLEISCHMANN, W. R., FLEISCHMANN, C. M. and GINDHART, T. D. : Effect of hyperthermia on the antiproliferative activities of murine α -, β -, and γ -interferon : Differential enhancement of murine γ -interferon. *Cancer Res.* **46** : 8 (1986).
- 4) FLEISCHMANN, W. R., FLEISCHMANN, C. M. and GINDHART, T. D. : Effect of hyperthermia on combination interferon treatment : Enhancement of the antiproliferative activity against murine B-16 melanoma. *Cancer Res.* **46** : 1722 (1986).
- 5) KUROKI, M., TANAKA, R., HONDO, H., ONDA, K., SUZUKI, Y. and MORIYAMA, M. : Antitumor effect of interferon combined with hyperthermia against experimental brain tumor. *Jpn. J. Hyperthermic Oncol.* **2** : 199 (1986).
- 6) NOMURA, K. : Propidium Iodide and Ethidium Bromide. Flow cytometry p.69 Japanese 1984.
- 7) YAMAGUCHI, M., MATSUKAGE, A. and TAKAHASHI, T. : Chick embryo DNA polymerase γ . *J. Biol. Chem.* **255** : 7002 (1980).
- 8) KANO, E., FURUKAWA, M., *et al.* : Basis of hyperthermia for cancer cells combination with anticancer drugs. *Oncologia* **12** : 114 (1985).
- 9) CREASEY, A. A., BARTHOLOMEW, J. C. and MERIGAN, T. C. : Role of G_0 - G_1 arrest in the inhibition of tumor cell growth by interferon. *Proc. Natl. Acad. Sci. USA.* **77** : 1471 (1980).
- 10) BALKWILL, F., WATLING, D. and PAPADIMITRIOU, T. T. : Inhibition by lymphoblastoid interferon of growth of cells derived from the human breast. *Int. J. Cancer* **22** : 258 (1978).
- 11) BALKWILL, F. R. and BOKHONKO, A. I. : Differential effects of pure human alpha and gamma interferons on fibroblast cell growth and the cell cycle. *Exp. Cell Res.* **155** : 190 (1984).
- 12) BHUYAN, B. K. : Kinetics of cell kill by hyperthermia. *Cancer Res.* **39** : 2277 (1979).
- 13) SPIRO, I. J., DENMAN, D. L. and DEWEY, W. C. : Effect of hyperthermia on CHO DNA polymerase α and β . *Radiation Res.* **89** : 134 (1982).
- 14) KAMPINGA, H. H., JORRITSMA, J. B. M. and KONINGS, A. W. T. : Heat-induced alterations in DNA polymerase activity of HeLa cells and of isolated nuclei. Relation to cell survival. *Int. J. Radiat. Biol.* **47** : 29 (1985).
- 15) LUNDBLAD, D. and LUNDGREN, E. : Changes in DNA-polymerase and thymidine kinase activity during interferon treatment. *Biochem. Biophys. Res. Commun.* **105** : 1569 (1982).