

Enhanced Anticancer Effect of Chemohyperthermia

Teruhisa Shimizu

The First Department of Surgery, Nagasaki University School of Medicine

The enhanced anticancer effect of hyperthermia in combination with bleomycin (BLM) and adriamycin (ADM) was experimentally investigated by using PC-1 cells (human lung cancer cells), in particular, the timing in contact with anticancer drugs was evaluated.

1) The synergistic anticancer effect was observed in the following order, heating at the same time as ADM treatment > ADM treatment prior to heating > ADM treatment after heating. There was no difference in anticancer effects between the timing of BLM treatment and heating.

2) In analysis of cell cycle, accumulation in a G₂M phase was enhanced in the order: heating at the same time as ADM treatment > ADM treatment prior to heating > ADM treatment after heating. In the case of BLM, the anticancer effect revealed almost a similar enhancement.

3) The intracellular uptake of ADM after a two hour exposure was the maximum in the case of heating at the same time as ADM treatment and still remained high levels after six hours. It was consisted with a result of the growth curve and the cell cycle studies. It was presumed that difference in the enhanced anticancer effect between ADM and BLM was in association with the diversity of intracellular uptake.

The interaction of anticancer drugs and heating is different from kind of drugs. The anticancer effect is attributed to the timing of drug exposure in combination with hyperthermia.

Introduction

It is widely accepted that a better cancer therapy is in combination with surgery, irradiation, chemotherapy and immunotherapy. In addition, recent studies focus on anticancer effect of hyperthermia and a clinical use of hyperthermia has become prevalent on the basis of a result of advances in basic study.

It is well known that cancer cells are more sensitive to heating of 41 °C to 43 °C which causes great damage. However, it is limited to the antitumor effect so that main cancer therapy is a combination of radiation with hyperthermia.

A combination therapy with chemotherapy and hyperthermia, which is called chemohyperthermia, is expected to be synergistic for anticancer effect and to become more potent. In fact, there is a matter of great concern about drug selection, administration route, administration period and drug dosis. There are still remaining to be solved.

The purpose of this study is to clarify the effective timing of drugs given including prior to, at the same time or after heating in relation to hyperthermia on the experimental basis.

Material and Methods

Cultured human lung cancer cells (PC-1) provided by Tokyo Medical School were incubated in the admixture of RPMI 1640 (GIBCO Co.) with penicillin G 10000 μ/l and streptomycin 100 mg/l, in part, freeze-dried for storage.

I) Determination of cell counts by heating

To measure a critical temperature of PC-1 cells were adjusted to 1×10⁵/5 ml and 2 ml were taken and packed tightly with silicon-film (Fuji-film), put into the vinyl bag and moved to water bath at 41 °C, 42 °C, 43 °C, 44 °C and 45 °C in a range of 30, 60, 90 and 120 min incubated at 37 °C in 5%CO₂. The cells were counted after 1 week. The survival curve was delineated in comparison with cells with a 1 week culture without any treatment.

II) The growth curve of PC-1 cells on the various conditions

1×10⁵ PC-1 cells were implanted in Petri Dish, 35×10 mm in size (IWAKI Co.), and RPMI 1640 (GIBCO Co.) added 10% FCS (GIBCO Co.) was used as a cell-proliferation culture. PC-1 cells were incubated at 37 °C for 24 hours in 5% CO₂ incubator. Each anticancer drug of 0.1 μg/ml of adriamycin (ADM) and 5.0 μg/ml of bleomycin (BLM) was added and was in contact with heating for 2 hours at the time determined as the following schedule (Fig. 1).

1) Heating alone and independent anticancer drug treat-

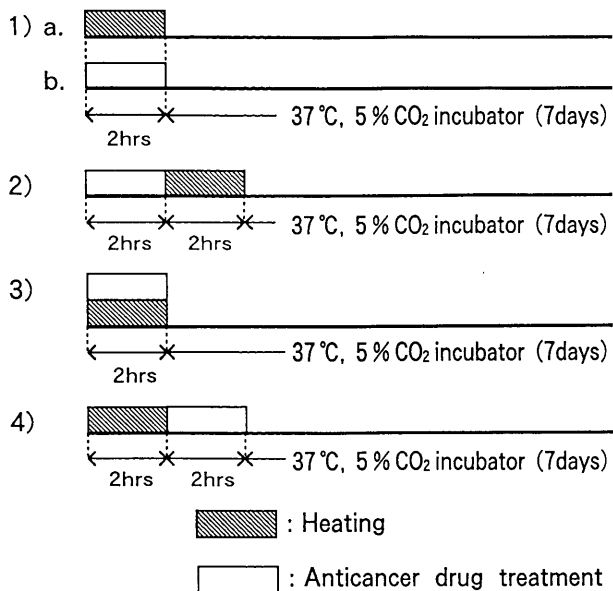


Fig. 1. Schema of various condition

ment

- 2) Anticancer drug treatment prior to Heating
 - 3) Heating at the same time with anticancer drug treatment
 - 4) Heating, followed by anticancer drug treatment
- Furthermore, cells were heated at 43 °C for 2 hours at the same time, prior to or after anticancer drug treatment and

incubated at 37 °C in 5% CO₂, counted after 7 days and the growth curve was constructed.

The drug concentration was selected for almost the same as that of heating alone on the growth curve. The concentrations of adriamycin and bleomycin were 0.1 μg/ml and 5.0 μg/ml, respectively. These concentrations were adjusted on each occasion of the experiment.

III) Changes in DNA and RNA contents during 3 days on the various conditions

8~10x10⁵ PC-1 cells were implanted in Petri Dish (100 x 20 mm IWAKI Co.), heated and treated with anticancer drugs as the above method, fixed with ethanol-acetone. Later changes in nuclear DNA and RNA content were analyzed from the Dot plot display and the histogram by using Flow cytometry (FACS- IV) with a simultaneous acridin-orange staining of cellular DNA and RNA as shown in Fig. 2. Briefly, 1 x 10⁵ PC-1 cells were cultured in the RPMI 1640 culture, containing 10% FCS, incubated at 37 °C for 24 hours in 5% CO₂, were in a 2 hour-contact with 0.1 μg/ml of ADM and/or 5.0 μg/ml of BLM prior to heating, during heating or after heating respectively and heated continuously and simultaneously at 44 °C for 2 hours. Thereafter, formation of the growth curve was achieved. In this study, ADM and BLM were used as the concentration similar to that of heating alone from the growth curve and 0.1 μg/ml of ADM and 5.0 μg/ml of BLM were applied.

— Growth curve —

1 × 10⁵ PC-1 cells/35mm dish

— FCM —

8~10 × 10⁵ PC-1 cells/100mm dish



37 °C, 5 % CO₂ incubator for 24hours



Heating : 44 °C, 2hours

Anticancer drug : ADM 0.1μg/ml, BLM 5.0μg/ml,

- 1) a. Heating alone
- b. Anticancer drug treatment
- 2) Anticancer drug prior to Heating
- 3) Heating at the same time with Anticancer drug
- 4) Heating, followed by Anticancer drug



37 °C, 5 % CO₂ incubator



Fixed with ethanolacetone
→ acridin-orange staining

Growth curve (7days)

DNA, RNA Histogram (3days)
by Flow cytometry (FACS-IV)

Fig. 2. Methods (1)

IV) Intracellular uptake of ADM by heating

To elucidate intracellular distribution of ADM in PC-1 cells, a flow-cytometry technique was used for the analysis in the relationship between heating and ADM uptake. The uptake of ADM was measured in the concentration of 1.0 $\mu\text{g/ml}$ and 5.0 $\mu\text{g/ml}$ of ADM at 4 °C, 15 °C, 37 °C and 43 °C by using the flow cytometry.

The uptake and efflux of ADM were also measured by the flow-cytometry method as shown in Fig. 3. Differences in heating temperatures were evaluated in detail. 1×10^5 ml of PC-1 cells were taken in the 15 ml tube and heated with water bath at 43 °C for 2 hours with a variety of given time, treated in a 2-hour contrast with 1.0 $\mu\text{g/ml}$ of ADM, washed twice with PBS and measured the intracellular ADM content immediately after, 2 hours and 6 hours later by using flowcytometry

- 1) Heating alone or ADM treatment
- 2) ADM treatment prior to Heating
- 3) Heating at the same time with ADM treatment
- 4) ADM treatment after Heating

— Up take, Efflux of ADM —

1×10^6 PC-1 cells / 5ml (15ml tube)

↓
Heating : 43 °C, water bath, 2hours

ADM : 1.0 $\mu\text{g/ml}$, 2hours

- 1) a. Heating alone
- b. ADM treatment alone
- 2) ADM prior to Heating
- 3) Heating at the same time with ADM
- 4) ADM after Heating

↓ washed twice with PBS
Measurement of intracellular ADM content
by Flow cytometry (FACS-IV)

Fig. 3. Methods (2)

Results

1. Critical temperature of PC-1 cells

The survival curve of PC-1 related to heating was shown in Fig. 4. PC-1 cells survived at 41 °C and 42 °C for 120 minutes. In contrast, they failed to survive at 43 °C for 120 minutes. It is defined that a critical temperature of PC-1 cells was in a range of 42 °C and 43 °C.

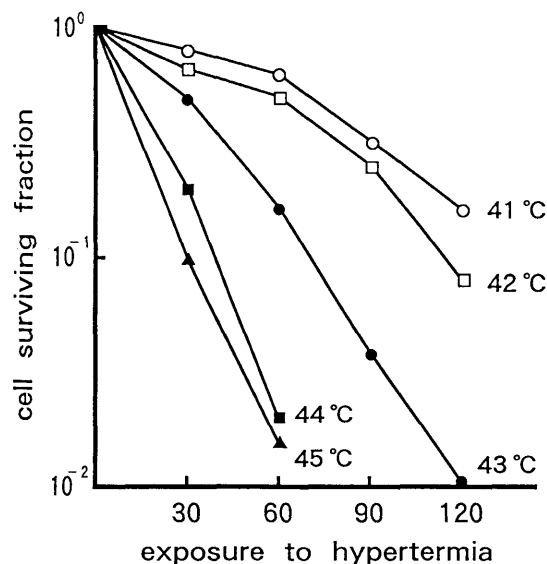


Fig. 4. Survival curves for PC-1 In culture heated at different temperatures, cell number of treatment/ cell number of no treatment

2. Growth curve

The growth curve of PC-1 cells in contact with 0.1 $\mu\text{g/ml}$ of ADM was shown in Fig. 5. The effects of growth inhibition by heating were seen in the following order, Heating at the same time as ADM treatment > ADM treatment prior to Heating > Heating, followed by ADM treatment. The growth inhibition rates were 92.3%, 58.8% and 83.3% respectively as compared with that of heating alone.

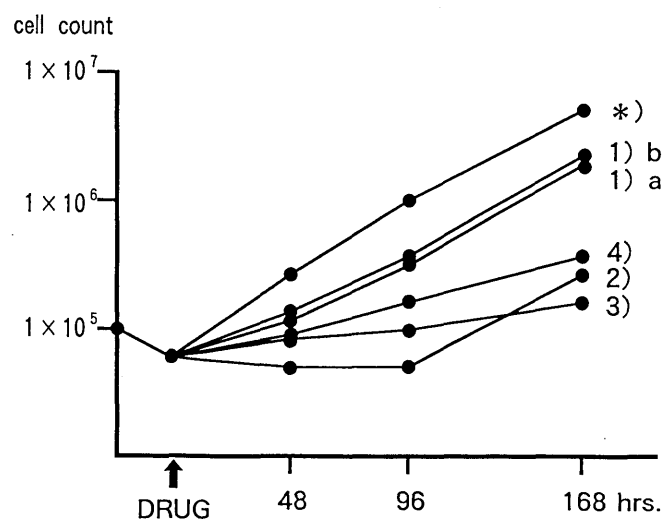


Fig. 5. Growth curve of PC-1 cells in contact with ADM 0.1 $\mu\text{g/ml}$ [*] no treatment, 1) a. Heating alone, 1) b. independent ADM treatment, 2) ADM treatment prior to Heating, 3) Heating at the same time with ADM treatment, 4) Heating, followed by ADM treatment]

On the other hand, the growth curve of PC-1 in contact with 5.0 $\mu\text{g/ml}$ BLM was shown in Fig. 6. The growth inhibition effects were of the magnitude in the following order. Heating at the same time of BLM treatment. Heating, followed by BLM treatment > BLM treatment prior to Heating. The inhibition rates were 98.5% and 98.2%, respectively as compared with heating alone. The inhibition effects of BLM were more potent than those of ADM.

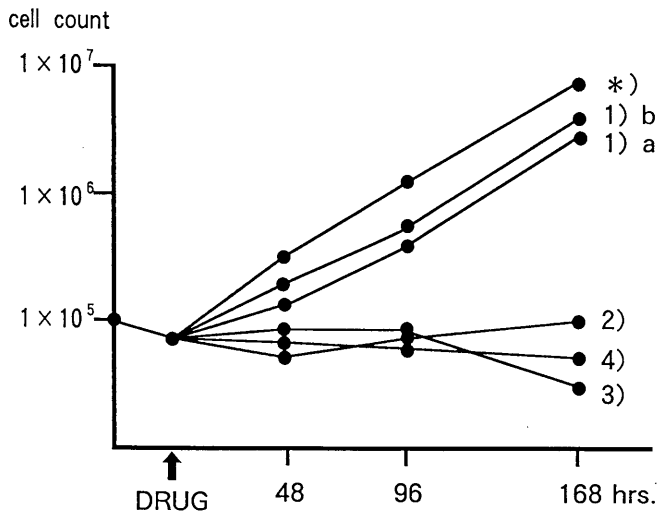


Fig. 6. Growth curve of PC-1 cells in contact with BLM 5.0 $\mu\text{g/ml}$ [*] no treatment, 1) a. Heating alone, 1) b. independent BLM treatment, 2) BLM treatment prior to Heating, 3) Heating at the same time with BLM treatment, 4) Heating, followed by BLM treatment]

3. Changes in cellular DNA and RNA contents by flowcytometry

Fig. 7 showed a Dot plot of DNA and RNA which were measured by flowcytometry and also Fig. 8 displayed a histogram which revealed changes in DNA and RNA contents. The DNA content in cases of heating alone were shown in Fig. 9-a which showed a histogram with slight accumulation in the G_2M phase at 24 hours and with the same 2 phasic peaks of the G_1 phase as that of no-treatment group at 72 hours. Meanwhile, the RNA content revealed a histogram with a sharp monophasic peak as shown on the left in Fig. 9-a. The DNA content in cases of heating at the same time as BLM treatment showed a reduction of the G_1 phase and accumulation of the G_2M phase as shown in Fig. 9-e, whereas the RNA content which represent a transcriptional activity revealed that a peak on the left side shifted to the right side with imbalanced slow growing as shown in Fig. 9-e.

At the same time, a similar curve was shown in cases of heating at the same time of ADM treatment as shown in

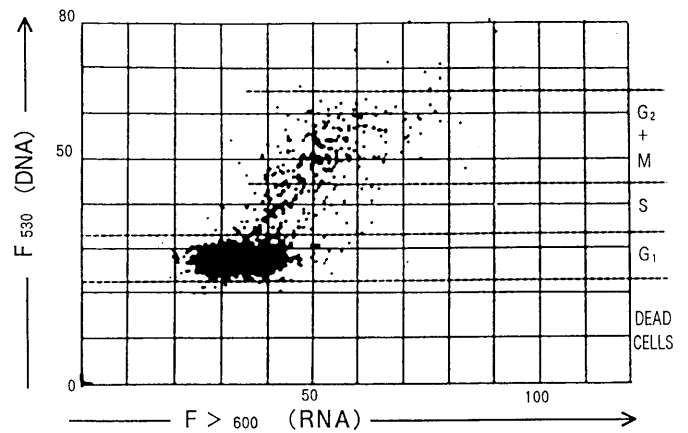


Fig. 7. Dot plot of DNA and RNA by Flow cytometry (FACS IV) Vertical axis: F_{530} (DNA, double helical nucleic acid) Horizontal axis: $F >_{600}$ (RNA, single strand helical nucleic acid)

PC-1

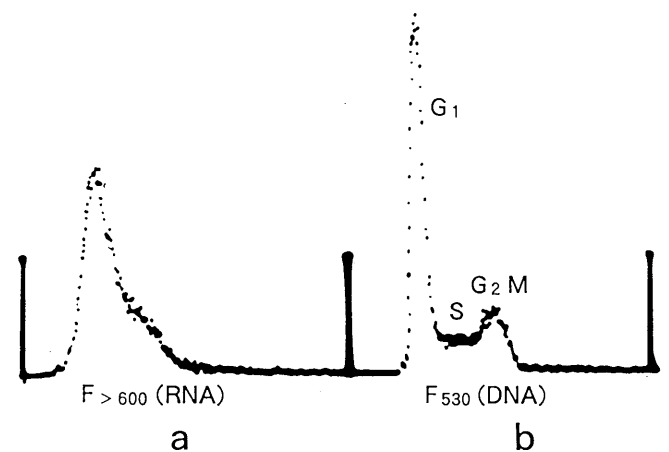


Fig. 8. Histogram in DNA and RNA content by Flow cytometry (FACS-IV) a. $F >_{600}$ (RNA, single strand helical nucleic acid) b. F_{530} (DNA, double helical nucleic acid)

Fig. 9-h. However, a significant accumulation in the G_2M was obtained rather than that in cases of heating at the same time as BLM treatment. Histogram revealed a similar pattern of a reduction in the G_1 phase at 24 hours among the groups of heating, ADM, BLM treatment alone as shown in Fig. 10-A. In cases of heating + BLM, a reduction of the G_1 phase was seen at 72 hours regardless the timing of BLM given as shown in Fig. 10-B. On the other hand, a reduction of the G_1 phase was significant in the order: heating with ADM > ADM prior to heating > ADM after heating in Fig. 10-C. From the standpoint of changes in the G_2M phase, Fig. 11-a showed accumulation at 24 hours, thereafter, a similar histogram with that of non-treatment was obtained. In cases of heating + ADM, the enhanced G_2M phase on histogram was manifest on day 3 in the increasing order: heating + ADM > ADM prior to heating >

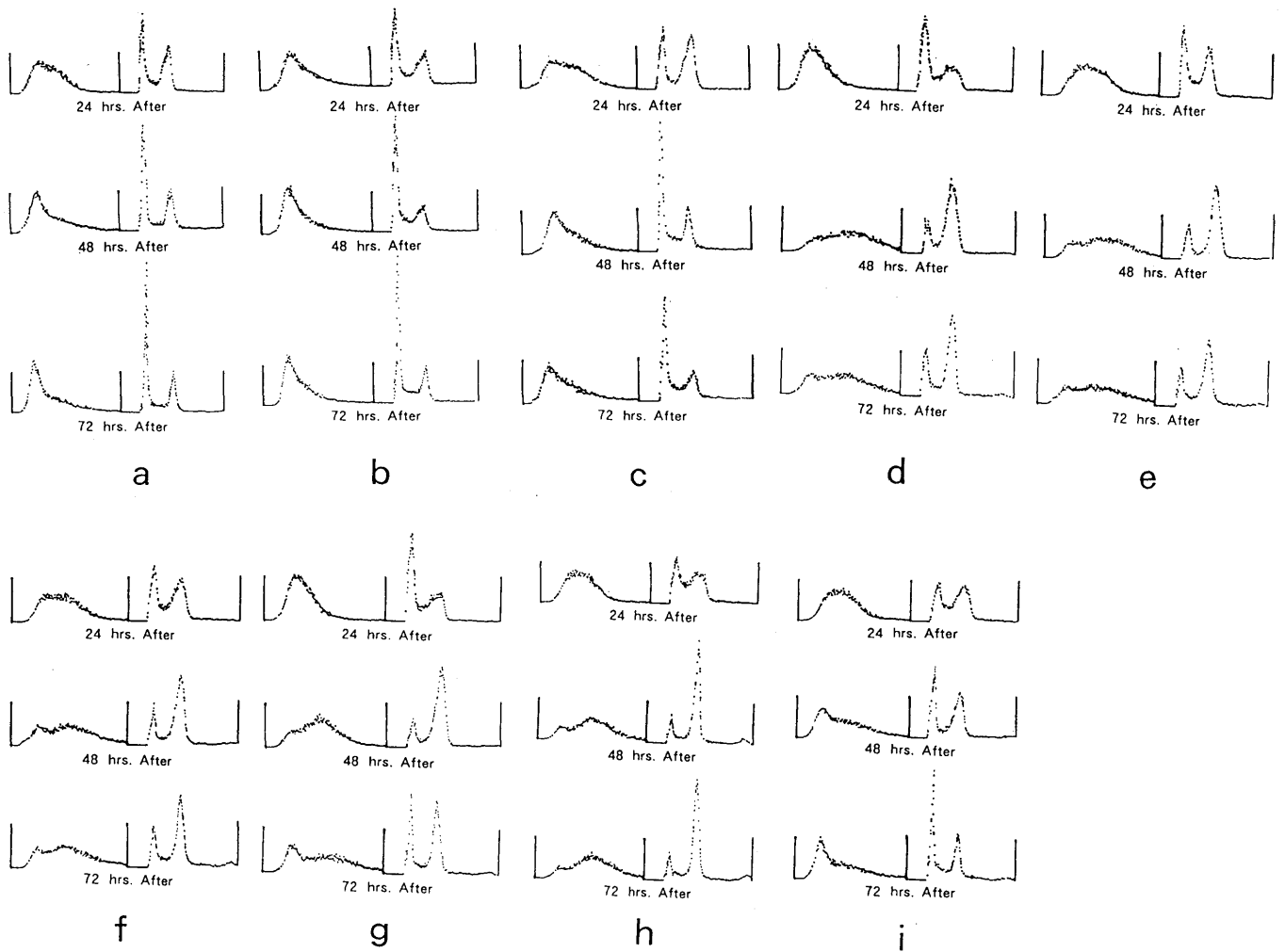


Fig. 9. Histogram in DNA and RNA content in various condition

a. Heating alone, b. BLM treatment alone, c. ADM treatment alone, d. BLM prior to Heating, e. Heating at the same time with BLM, f. BLM after Heating, g. ADM prior to Heating, h. Heating at the same time with ADM, i. ADM after Heating

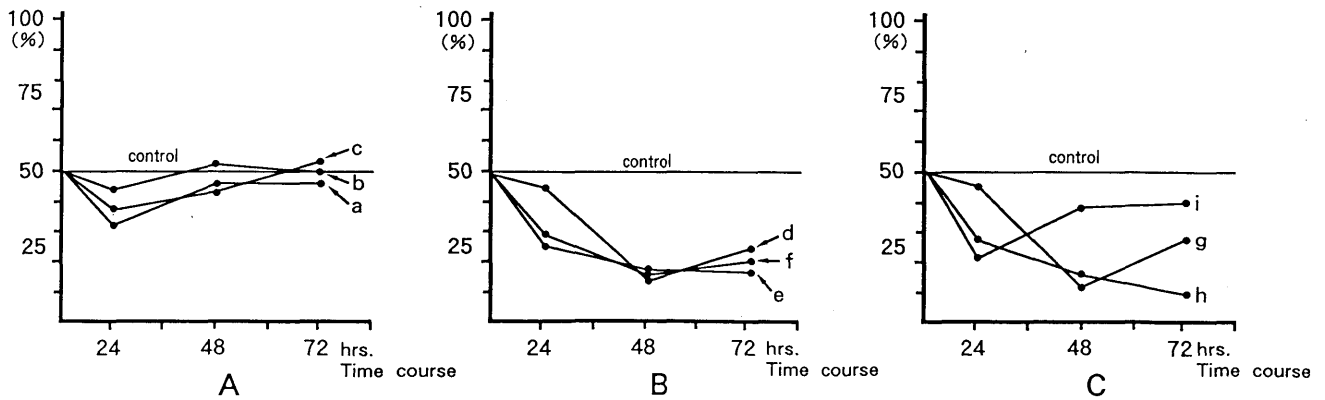


Fig. 10. Changes in the G₁ phase.

a) Heating alone, b) BLM treatment alone, c) ADM treatment alone, d) BLM prior to Heating, e) Heating at the same time with BLM, f) BLM after Heating, g) ADM prior to Heating, h) Heating at the same time with ADM, i) ADM after Heating

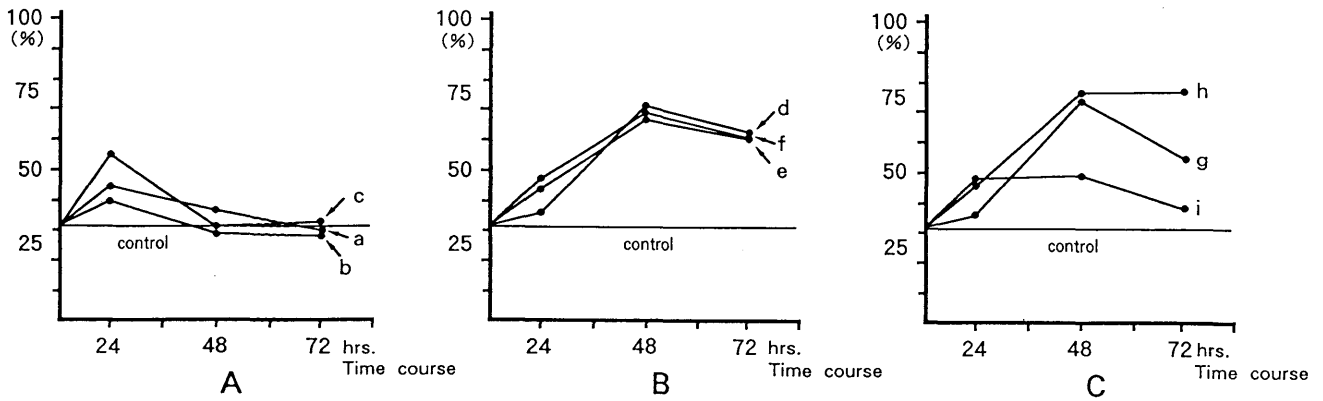


Fig. 11. Changes in the G₂M phase.

a) Heating alone, b) BLM treatment alone, c) ADM treatment alone, d) BLM prior to Heating, e) Heating at the same time with BLM, f) BLM after Heating, g) ADM prior to Heating, h) Heating at the same time with ADM, i) ADM after Heating

ADM after heating. In contrast, an increasing G₂M phase was almost the same among the three groups in cases of heating + BLM.

4. Intracellular distribution of ADM

In analysis of ADM uptake in relation to heating, PC-1 cells were in contact with ADM for 2 hours and the uptake of ADM was measured in accordance with changes in the heating temperature by using flowcytometry. According to an increase in the heating temperature, the uptake of ADM was increased and a rapid increase at 43 °C was seen when compared with that at 37 °C shown as Fig. 12.

The intracellular ADM content 2 hours after ADM treat-

ment, shown as Fig. 13, reached at the maximum in cases of heating at the same time of ADM treatment, followed by ADM treatment prior to heating and ADM treatment after heating. Efflux of ADM after 2 hours remained a high intracellular concentration of ADM following a lapse of 6 hours in cases of heating at the same time of ADM treatment.

Discussion

It is well known that hyperthermia is of a considerable value for malignant tumors. Busch first reported in 1935 that high fever of patients with erysipelas caused a remis-

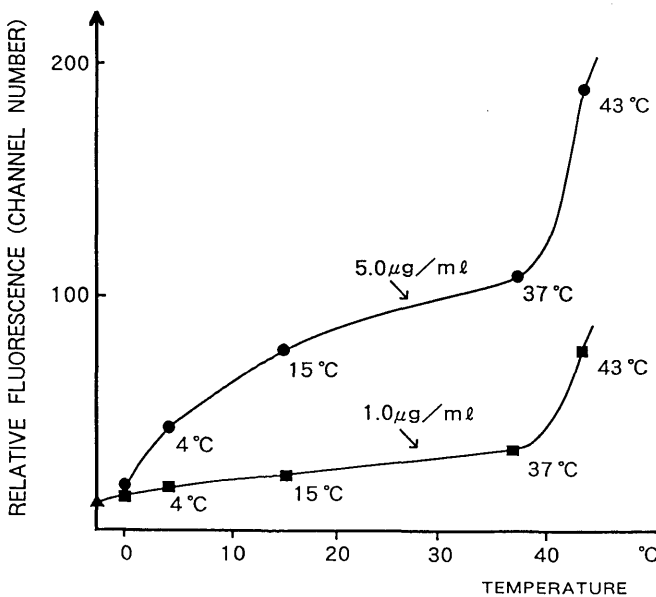


Fig. 12. Hyperthermia and ADM uptake

According to an increase in the heating temperature, the uptake of ADM was increased.

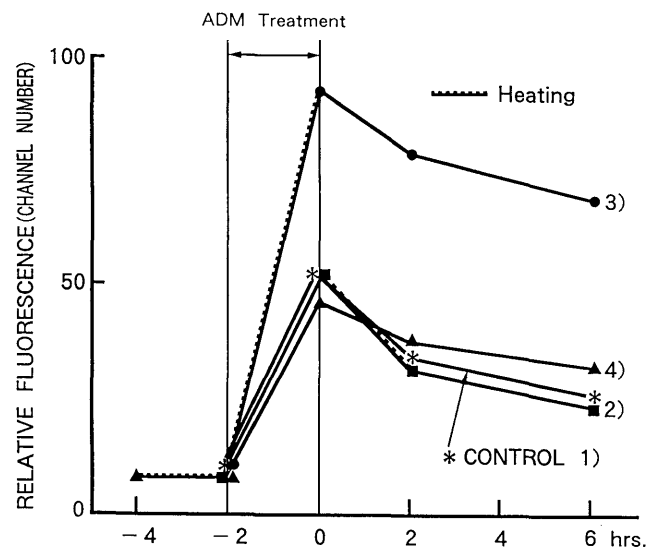


Fig. 13. Intracellular ADM content 2 hours after ADM treatment.

1) control, ADM treatment alone, 2) ADM treatment prior to Heating, 3) ADM treatment at the same time with Heating, 4) ADM treatment after Heating.

sion of sarcomatous tumor as reported by Coley.¹⁾ Warren also confirmed in 1935 that heating at 41.5 to 42°C is effective in eliminating the tumor sizes and also a valid in clinical use. Since 1970, the validity of chemotherapy in combination with hyperthermia has been defined with the use of various cultured cells by many investigators.³⁻⁵⁾ At present, *in vitro* and *in vivo* studies clarified the enhancement of anticancer effects by thermochemotherapy.⁶⁾ And also it was certified that hyperthermia allowed anticancer drugs which was resistant at body temperature to become quite sensitive.⁷⁾ It is known that the use of anticancer drugs in a combination with heating makes the anticancer effects much more effective and the main drugs to enhance the anticancer effects by heating are represented in Table 1.⁸⁾ It is well accepted that the representative drugs to exert an potent cytotoxic effect are bleomycin (BLM)^{5,9-11)} and adriamycin (ADM).^{5,10-12)} The mechanisms of cell killing by heating are explained that the influences of tumor cells on heating cause damage to cells such as the impairment of a DNA, RNA and protein synthesis^{13,14)} selectively impaired cellular respiration¹⁵⁾ relative increase in anaerobic glycolysis due to impairment of cellular respiration¹⁶⁾ and changes in cellular environment with the alteration of pH.¹⁷⁾ In addition, it is said that heating causes greater damage to malignant cells rather than that to normal one. The study concerning the effective timing of heating is now sparse although there are many regarding a combination with anticancer drugs and their selections.

Table 1.

Adriamycin
Bleomycin
Nitrogen mustard
BCNU: 1, 3-bis (2-chloroethyl)-1-nitrosourea
Amphotericin B
Polymixin B
DMSO: dimethyl sulfoxide
DMF: dimethyl formamide
Polyamine
5-thio-D-glucose
Procain
Corynebacterium parvum
misonidazole

In this series, the most effective timing of heating was experimentally investigated in combination with anticancer drugs of ADM and BLM by using PC-1 cells. With respect to a range of heating temperature, much researches have been made and it is defined that an optimal heating in human ranges from 41°C to 43°C and the anticancer effect exert in a range of 42°C to 43°C in spite of a different sensitivity to heating according to cell lines.

Hahn et al⁹⁾ reported that simultaneous effects in combi-

nation with heating and BLM has become inactivated at the heating temperature of at 41°C or below, somewhat activated at 42°C and synergistically activated at 43°C. And also a similar result is reported in case of ADM. In contrast, differences in the time duration of the exposure to drug is not referred.

It is possible that intracellular ADM is able to detect by flowcytometry with a 488 nm laser. Needless to say, the intracellular concentration of ADM is influenced by the drug concentration and the exposure time. However, Kawasaki²²⁾ reported that heating makes it possible to elevate the intracellular concentration of drugs. A rapid increase in intracellular concentration of drugs was experimentally substantiated in this study by using PC-1 cells. Those results clarified that heating enabled the transport of drugs into cells to facilitate vigorously. In addition, it is necessary to assess how long drugs remain in the cells enough evaluate the efficacy of drugs.

In this study, the pharmacokinetics of the uptake and the efflux of ADM and BLM were investigated with the use of PC-1 cells. In this series, the most high and persistent concentrations of drugs by heating was evidenced in the case of heating at the same time as ADM treatment and a high concentration remained even after 6 hours. It was consistent with the growth curve during a 7 day duration of PC-1 cells with heating and ADM treatment.

In analysis of the cell cycle, those results accorded with accumulation of a G₂M phase. It is contemplated that the difference in drug-enhanced effect of hyperthermia is in association with the intracellular uptake of drugs. Imbalanced growth was represented as the alteration of the area in which the RNA content was planimetrically calculated on each histogram. It was apparent that the enhanced drug effect by heating is due to the sequence of imbalanced growth and denegeneration of cells in addition to the impaired repair process. It is generally accepted that heating at the range of 41-43°C may cause apparent damage to malignant cells without normal cells.²³⁾ The reasons are explained that transformation to a malignant cell tends to have a much higher sensitivity to heat at 43°C in mouse prostatic cell line²⁴⁾ and heating induces inhibition of nucleic acid synthesis, inhibition of cell proliferation and arrest of tumor growth.^{25,26)} Recent study focuses on denaturation of chromosomal protein as the main target in cellular heat damage and such injury is not reversible and not selective for malignant cells. In addition, it was confirmed that heating facilitates the uptake of anticancer drug and prevent early efflux from cells in this study.

One should take it into consideration that the critical temperature threshold in hyperthermic damage is around 43°C and the enhanced hyperthermic effect is evident in combination with anticancer drugs on the basis of a result of cell cycle study. Needless to say, it is emphasized that concomitant use to heating and anticancer drugs is a valid to enhance anticancer effects.

In conclusion, a most effective anticancer effect of drugs by heating is predicted in a case of heating with drug at the same time.

Acknowledgment

The author would like to express deep appreciation to Prof. Masao Tomita for his suggestion and revision and to the staff in the First Department of Surgery for their cooperation.

References

- 1) Coley WB, et al: The treatment of malignant tumors by reported inoculation of erysipelas, with a report of ten original cases. *Am. J. Med. Sci.* 105:487-511, 1893.
- 2) Warren LS: Preliminary study of the effect of artificial fever upon hopeless tumor cases. *Am. J. Roentgenol.* 33:75, 1935.
- 3) Palzer RJ and Heiderberger C: Influence of drugs and synchrony on the hyperthermic killing of Hela cells. *Cancer Res.* 33:422-427, 1973.
- 4) Johnson HA, and Pavelec M: Thermal enhancement of thio-TEPA cytotoxicity. *J. Natl. Cancer Inst.* 50:903-908, 1973.
- 5) Hahn GM, Braun J and Har-Keder I: Thermochemotherapy: Synergism between hyperthermia (42-43 °C) and adriamycin or bleomycin in mammalian cell inactivation. *Proc. Natl. Acad. Sci. U. S. A.* 72:937-940, 1975.
- 6) Nakamura H: Strategy of hyperthermochemotherapy. *Jap. J. cancer and chemotherapy.* 10:903-912, 1983.
- 7) Mizuno M: *Cancer, hyperthermia* ed. by Karakawa J. Shinohara Co. Tokyo, 183-199, 1982.
- 8) Okumura H: Medical biologic basis of hyperthermia. *Rad. Biol. Research* 15:32-47, 1980.
- 9) Brawn J and Hahn GM: Enhanced cell killing by bleomycin and 43 ° hyperthermia and the inhibition of recovery from potentially lethal damage. *Cancer Res.* , 35:2921-2927, 1975.
- 10) Mizuno S, Amagi M and Ishida A: Synergistic cell killing by anti-tumor agents and hyperthermia in cultured cells. *Gann*, 71:471-478, 1980.
- 11) Morgan JE and Bleehan NM: Response of EMT6 multicellular tumor spheroids to hyperthermia and cytotoxic drugs. *Br. J. Cancer*, 43:384-391, 1981.
- 12) Hahn GM and Strande DP: Cytotoxic effects of hyperthermia and adriamycin on chinese hamster cells. *J. Natl. Cancer Inst.*, 57:1063-1067, 1976.
- 13) Palzer RJ and Heidelberg C: Influence of drugs and synchrony on the hyperthermic killing of HeLa cells, *Cancer Res.*, 33:422-427, 1973.
- 14) Mondovi B, Agro AF, Rotilio G, et al: The biochemical mechanism of selective heat sensitivity of cancer cells. II. Studies on nucleic acids and protein synthesis. *Europ. J. Cancer*, 5:137-146, 1969.
- 15) Mondovi R and Fanelli AR: The biochemical mechanism of selective heat sensitivity of cancer cells. I. Studies on cellular respiration. *Europ. J. Cancer*, 5:129-136, 1969.
- 16) Cavaliere R, Ciocatto EC, Giovannella BC, et al: Selective heat sensitivity of cancer cell. *Biochemical and clinical studies. Cancer*, 20:1351-1381, 1967.
- 17) Overgaard J: Effect of hyperthermia on malignant cells in vivo. A review and hypothesis. *Cancer*, 39:2637-2646, 1977.
- 18) Hofer KG, Choppin DA and Hofer MG: Effect of hyperthermia on the radiosensitivity of normal and malignant cells in mice. *Cancer*, 38:279-287, 1976.
- 19) Krishan A and Ganapathi R: Laser flow cytometric studies on the intracellular fluorescence of anthracyclines, *Cancer Res.*, 40:3885-3900.
- 20) Tapiero H, Fourcade A, Vaigot P and Farhi JJ: Comparative uptake of adriamycin and daunorubicin in sensitive and resistant friend leukemia cells measured by flow cytometry, *Cytometry* 2:298-302, 1982.
- 21) Krishan A and Ganapathi R: Laser flow cytometry and cancer chemotherapy: Detection of intracellular anthracycline by flow cytometry, *J. Histchem. Cytochem.* 27:1655-1656, 1979.
- 22) Kawasaki S, Sasaki K, Nagaoka S, et al: Increased Accumulation of Adriamycin in Mouse 3T3 Cells with Hyperthermia, *Nipp. Act. Radiol.* 44:727-731, 1984.
- 23) Overgaard J: Ultrastructure of a murine mammary carcinoma exposed to hyperthermia in vivo, *Cancer Res.* 36:983-995, 1976.
- 24) Chen JJ and Heidelberg C: Quantitative studies on the malignant transformation of mouse prostate cells by carcinogenic hydrocarbons in vitro. *Int. J. Cancer* 4:166-178, 1969.
- 25) Ditaël F: *Tumor and temperature Aktuelle probleme bei der Anwendung thermischer Ven fahren in Onkologie und Strahlentherapie.* Munchen. Berlin. Wien Urban Schwarzenberg. 1975.
- 26) Dickson JA and Shah DM: The effects of hyperthermia (42 °C) on the biochemistry and growth of a malignant cell line. *Eur. J. Cancer* 8:561-571, 1972.