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.

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 dose. 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 μI/l and streptomycin 100 mg/l, in part, freezed for storage.

I) Determination of cell counts by heating

To measure a critical temperature of PC-1 cells were adjusted to 1x10^5/5 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 cultur without any treatment.

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

1x10^5 PC-1 cells were implanted in Petri Dish, 35x10 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-
1. a. 
   b. 37°C, 5% CO₂ incubator (7days) 2hrs

2) 37°C, 5% CO₂ incubator (7days) 2hrs

3) 37°C, 5% CO₂ incubator (7days) 2hrs

4) 37°C, 5% CO₂ incubator (7days) 2hrs

| : Heating |
| : Anticancer drug treatment |

**Fig. 1.** Schema of various conditions

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**

8–10×10⁴ PC-1 cells were implanted in Petri Dish (100 × 20 mm IWAKI Co.), heated and treated with anticancer drugs as the above method, fixed with ethanol-aceton. 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 × 10⁶ 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

↓ 37°C, 5% CO₂ incubator for 24 hours

Heating: 44°C, 2 hours

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

Growth curve (7days)

**FCM**

8~10 × 10⁶ PC-1 cells/100mm dish

↓ Fixed with ethanol-aceton
→ acridin-orange staining

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 μg/ml and 5.0 μg/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 x 10⁶/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 μg/ml of ADM, washed twice with PBS and measured the intracellular ADM content immediately after, 2 hours and 6 hours later by using flow cytometry

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 x 10⁶ PC-1 cells/5ml (15ml tube)↓
Heating : 43°C, water bath, 2hours
ADM : 1.0 μg/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.

2. Growth curve

The growth curve of PC-1 cells in contact with 0.1 μg/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 μg/ml
[ spared, 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 μg/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 μg/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 G1 phase at 24 hours and with the same 2 phasic peaks of the G1 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 G1 phase and accumulation of the G2M 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 > 600 (DNA, double helical nucleic acid)
Horizontal axis: F > 530 (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 G2M 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 G1 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 G1 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 G1 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 G2M 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 G2M phase on histogram was manifest on day 3 in the increasing order: heating + ADM > ADM prior to heating >
T. Shimizu: Enhanced Anticancer Effect of Chemohyperthermia

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 G 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 ºC was seen when compared with that at 37 ºC shown as Fig. 12.

The intracellular ADM content 2 hours after ADM treatment, 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-

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**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

**Fig. 12.** Hyperthermia and ADR 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.9 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 cytocidal effect are bleomycin (BLM)5,8,14 and adriamycin (ADM).4,9,10 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 synthesis15,16 selectively impaired cellular respiration17 relative increase in anaerobic glycolysis due to impairment of cellular respiration18 and changes in cellular environment with the alteration of pH.19 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.

<table>
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<tr>
<th>Drug</th>
<th>Interactions</th>
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<td>Adriamycin</td>
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<td>Bleomycin</td>
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<td>Nitrogen mustard</td>
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<td>BCNU: 1, 3-bis (2-chloroethyl)-1-nitrosourea</td>
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<td>Amphotericin B</td>
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<td>Polymyxin B</td>
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<td>DMSO: dimethyl sulfoxide</td>
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<td>DMF: dimethyl formamide</td>
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<td>Polyamine</td>
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<td>5-thio-D-glucose</td>
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<td>Procaïn</td>
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<td>Corynebacterium parvum</td>
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<td>misonidazole</td>
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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 of heating according to cell lines.

Hahn et al19 reported that simultaneous effects in combination 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, Kawasaki20 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.20 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 line21 and heating induces inhibition of nucleic acid synthesis, inhibition of cell proliferation and arrest of tumor growth.22,23 Recent study focuses on denaturation ofchromosomal 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.

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Preferences