Combination of chemotherapy and mild hyperthermia enhances the anti-tumor effects of cisplatin and adriamycin in human bladder cancer T24 cells *in vitro*

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Abstract. Although the combined effects of hyperthermia and chemotherapy for cancer therapy have been theoretically established, combination therapy with hyperthermia is not widely used clinically, since the application of hyperthermia is complex and maintaining a tumor temperature of 43˚C or above is very difficult. Thus, in the present study, the combined effects of chemotherapy and mild hyperthermia, which is easier to apply than hyperthermia, were examined using a human bladder cancer cell line, T24. T24 cells were incubated at 37-47˚C for 0-5 h, and the survival rate of cells was measured using the MTT method. Cisplatin and adriamycin, which are indicated for human bladder cancer, were used as anti-tumor drugs. In the combination experiment, T24 cells were treated with cisplatin (20 and 200 μg/ml) or adriamycin (4 and 40 μg/ml) at 41˚C for 0-5 h and at 37˚C for 7-12 h. After a total incubation of 12 h, cell survival was measured by the MTT method. i) T24 cells, like cells in general, survived mild hyperthermia at 41˚C, but were killed at over 43˚C. ii) Although a low concentration (20 μg/ml) of cisplatin or of mild hyperthermia at 41˚C did not affect the survival rate of T24 cells, this combination therapy decreased the survival rate of T24 cells with increasing incubation times at 41˚C. The anti-tumor effect of the combination of 20 μg/ml cisplatin and 41˚C for 5 h was the same as that of 10 times the concentration (200 μg/ml) of cisplatin. iii) Adriamycin showed the same effects as cisplatin. The anti-tumor effect of the combination of 4 μg/ml adriamycin and 41˚C for 5 h was identical to the anti-tumor effect of a 10-fold higher concentration (40 μg/ml) of adriamycin. Based on these results, combination with mild hyperthermia may enable a reduction in the dose of anti-tumor drugs. It was suggested that the side effects of chemotherapy could be reduced clinically by combining the drugs with mild hyperthermia. Mild hyperthermia might be a useful and practical heating method which could lead to the increased clinical applications of hyperthermia. We previously reported the preliminary results of M-VAC chemotherapy (methotrexate, vinblastine, doxorubicin and cisplatin) combined with mild hyperthermia, a new therapeutic strategy for advanced or metastatic transitional cell carcinoma of the urothelium. This basic research strongly supports the clinical beneficial results of chemotherapy combined with mild hyperthermia.

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Introduction

Among urological cancers, the incidence of bladder cancer is the highest, and that in men is approximately 2.5 times higher than that in women. Approximately 95% of bladder cancers are urothelial carcinoma. The peak of incidence occurs in the 60-70 year age group, while there are few young patients with this disease under 50 years of age. The response rate of treatment for advanced metastatic transitional cell carcinoma is approximately 40%, in spite of M-VAC chemotherapy (methotrexate, vinblastine, doxorubicin and cisplatin), the gold standard treatment (1,2). Particularly in the case of metastasis to lymph nodes and other organs, the survival rate is low, 20%. There are many reasons for the low response and survival rates. Since elderly patients cannot tolerate anti-tumor drugs, the dose intensity must be decreased (3). Continued administration of anti-cancer drugs becomes difficult due to the physical and mental distress caused by their severe side effects (4).

The efficacy of hyperthermia in combination with chemotherapy has been established experimentally and clinically (5,6). Although chemotherapy is used at all medical institutions, very few institutions employ it in combination with hyperthermia.

The use of hyperthermia is not widespread at many medical facilities, due to the difficulty of heating and maintaining tissues at a temperature of 43˚C or higher. Additionally, the

costs of the devices used for applying clinical hyperthermia along with the associated personnel costs are high.

Thus, the effects of mild hyperthermic heating at a lower temperature of 41˚C were examined in contrast to hyperthermia at temperatures of 43˚C or higher. The temperature used for mild hyperthermia is not sufficiently high enough to kill tumor cells, but it is relatively easy to heat and maintain cells and tissue at 41˚C. In addition, mild hyperthermia is a less severe therapy for cancer patients than standard hyperthermia.

We previously reported that the cytotoxic effects of anti-tumor drugs were increased by combining anti-tumor drugs (adriamycin, vincristine) with mild hyperthermia using the NALM-6 leukemia cell line, and confirmed the effectiveness of mild hyperthermia (7).

In this experiment, using a human bladder cancer cell line, the combined effect of mild hyperthermia with cisplatin and adriamycin was examined. These drugs are the main anti-tumor drugs in the M-VAC chemotherapeutic regimen, which is the typical standard therapy for progressive urothelial cancer.

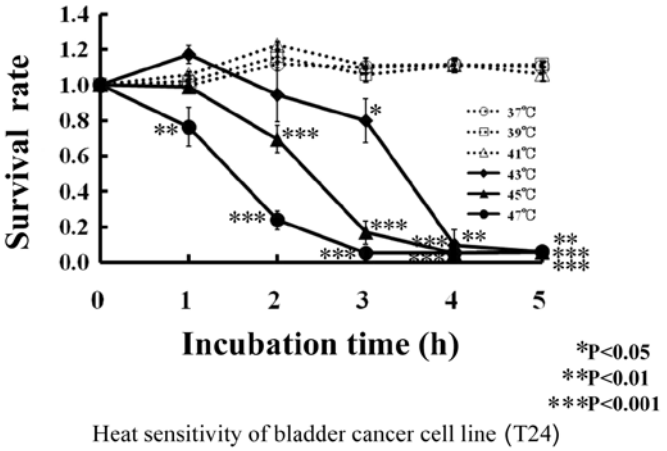

Figure 1. Thermal sensitivity of human bladder cancer cell line, T24s. T24 cells were incubated at 37˚C (--○--), 39˚C (--□--), 41˚C (--△--), 43˚C (♦), 45˚C (▲) or 47˚C (●) for 0, 1, 2, 3, 4 and 5 h, respectively. Viability of T24 cells was measured by the MTT method after incubation. *P<0.05, **P<0.01 and ***P<0.001 compared to 0 h of incubation at 37˚C.

Materials and methods

Cell line. T24 cells, a human bladder cell line, were cultured in RPMI-1640 culture medium including 10% FCS and were used in experiments during the log phase of growth. T24 cells were sown on 96-well plates at $5x10^4$/0.2 ml culture medium/well. These plates were used for experiments after one day in a CO_2 incubator at 37˚C.

Cell survival rate. The cell survival rate was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method (Cell Proliferation Kit; Roche Diagnostics, Mannheim, Germany) (8). After cell treatment, MTT reagent was added (1/10) to the cell reaction medium. Following incubation at 37˚C for 2 h, the same volume of 10% SDS was added to the reaction medium. The absorbance at 550 nm was measured.

Thermal sensitivity of the human bladder cancer T24 cell line. T24 cells in 96-well plates were incubated for 0-5 h at 37, 39, 41, 43, 45 and 47˚C, respectively, with a device capable of maintaining very precise temperatures (temperature accuracy ±0.01˚C; Nihon Kouseikagaku Res. Co., Osaka, Japan). The survival rate of cells was measured by the MTT method.

Response of human bladder cancer T24 cells to anti-tumor drugs. Cisplatin (CDDP) and adriamycin (ADR) are typical carcinostatics for the treatment of bladder cancer with M-VAC chemotherapy. These two anti-tumor drugs were used in combination with mild hyperthermia, and the anti-tumor effect on human bladder cancer T24 cells was examined. In the combination experiment with carcinostatics and mild hyperthermia, human bladder cancer T24 cells were treated with final CDDP concentrations of 20 and 200 μg/ml or ADR concentrations of 4 and 40 μg/ml at 41˚C for 0-5 h. Total incubation time (at both 41 and 37˚C) of all samples was 12 h. After 12 h, the MTT reagent was added to the incubation medium, and the cell survival rate was measured. For example, when samples were treated at 41˚C for 3 h, they were incubated at 37˚C in a CO_2 incubator for 9 h.

Results

Thermal sensitivity of human bladder cancer cells. As shown in Fig. 1, almost all (1.0) the human bladder cancer T24 cells survived at 37, 39 and 41˚C after 5 h of incubation. With treatment at 43˚C, the survival rate of T24 cells was 0.94 after 2 h of incubation, 0.8 after 3 h and 0.06-0.09 after 4-5 h. At 45˚C, the survival rate of T24 cells was 0.69 after 2 h of incubation, 0.17 after 3 h and 0.05 after 4-5 h. At 47˚C, the survival rate of T24 cells decreased linearly to 0.05 after 3 h of incubation.

Human bladder cancer T24 cells were not influenced by the thermal effect of 41˚C, and cell death was noted from 43˚C, just as with cells in general.

Sensitivity of human bladder cancer T24 *cells to anti-tumor drugs*. The sensitivity of T24 cells to CDDP (0, 2 and 20 μg/ml) is shown by the survival rate (y-axis) at 0 h of incubation at 41˚C (12-h incubation at 37˚C) (Fig. 2). The survival rate of T24 cells in CDDP (0 μg/ml) after 12 h of incubation at 37˚C (without mild hyperthermia) was 1.0. Additionally, the survival rate of T24 cells at a low concentration (20 μg/ml) of CDDP was 1.0, and that at a high concentration (200 μg/ml) of CDDP was 0.62 (P<0.01).

The sensitivity of T24 cells to ADR is shown by the survival rate (y-axis) at 0 h of incubation at 41˚C (Fig. 3). Similar to CDDP, the survival rate at a low concentration (4 μg/ml) of ADR was 1.0 and that at a high concentration (40 μg/ml) of ADR decreased the survival to 0.72 (P<0.01).

Combination of anti-tumor drugs and mild hyperthermia. The survival rate of T24 cells with a combination of anti-tumor drugs and mild hyperthermia is shown in Figs. 2 and 3. As shown in Fig. 1, regarding thermal sensitivity, the survival rate of T24 cells was not altered by mild hyperthermia at 41˚C

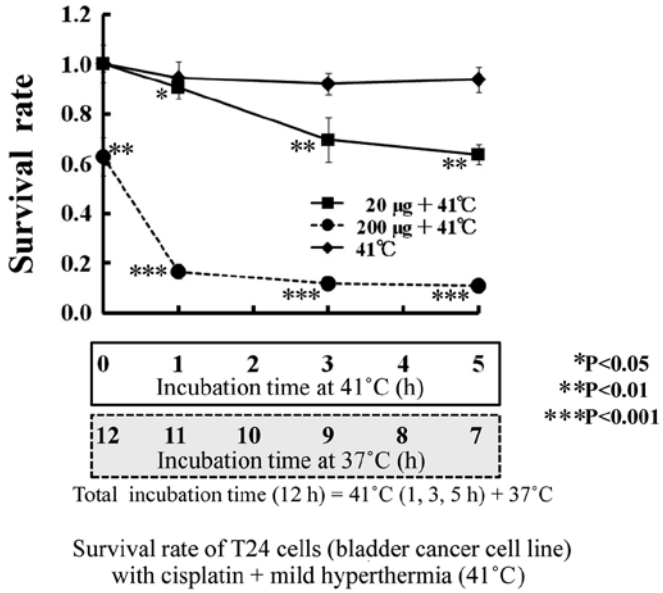

Figure 2. Effects of the anti-tumor drug CDDP in combination with mild hyperthermia at 41˚C on the T24 human bladder cancer cell line. T24 cells were incubated with CDDP [20 μg/ml (■) or 200 μg/ml (--●--)] at 41 and 37˚C, respectively. Cells were incubated at 41˚C (mild hyperthermia) (◆) for 0, 1, 3 and 5 h, and then incubated at 37˚C. Total incubation time at 41 and then 37˚C was 12 h. After incubation for 12 h, the cell viability of the T24 cells was measured by the MTT assay. *P<0.05, **P<0.01 and ***P<0.001 compared to 0 h of incubation at 41˚C.

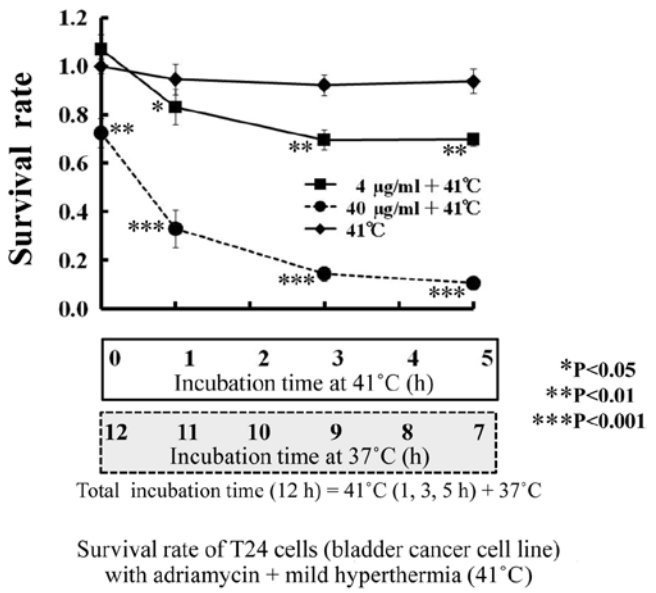

Figure 3. Effects of the anti-tumor drug ADR in combination with mild hyperthermia at 41˚C on the T24 human bladder cancer cell line. T24 cells were incubated with ADR [4 μg/ml (■) or 40 μg/ml (--●--)] at 41 and 37˚C, respectively. Cells were incubated at 41˚C (mild hyperthermia) (◆) for 0, 1, 3 and 5 h, and then incubated at 37˚C. Total incubation time at 41 and then 37˚C was 12 h. After incubation for 12 h, viability of the T24 cells was measured by the MTT assay. *P<0.05, **P<0.01 and ***P<0.001 compared to 0 h of incubation at 41˚C.

for 5 h; nor was it altered by anti-tumor drugs, such as a low CDDP concentration (2 μg/ml) for 12 h (Fig. 2). However, the survival rate decreased with a combination of mild hyperthermia at 41˚C and a low concentration of anti-tumor drugs (CDDP 20 μg/ml or ADR 4 μg/ml).

Mild hyperthermia at 41˚C or a low concentration of anti-tumor drugs CDDP and ADR had no independent influence on the survival of T24 cells. The viability of T24 cells was decreased from 1.0 to 0.90 (P<0.05), 0.70 (P<0.01) and 0.63 (P<0.01) by the combination of mild hyperthermia at 41˚C and 20 μg/ml CDDP for 0, 1, 3 and 5 h, respectively. The viability of T24 cells was 0.62 at a CDDP concentration of 200 μg/ml without mild hyperthermia. Therefore, the viability of T24 cells at a CDDP concentration of 20 μg/ml with mild hyperthermia at 41˚C for 5 h had the same anti-tumor effect as a 10-fold higher ADR concentration (200 μg/ml).

The viability of T24 cells with 200 μg/ml CDDP was decreased from 0.62 to 0.16 (P<0.001) in combination with mild hyperthermia at 41˚C for 1 h, and to 0.11 (P<0.001) at 41˚C for 3 h. The addition of mild hyperthermia at 41˚C for 1 h quadrupled the anti-tumor activity of CDDP (200 μg/ml).

As with CDDP, the viability of T24 cells with 4 μg/ml ADR was decreased from 1.0 to 0.83 (P<0.05), 0.70 (P<0.01) and 0.69 (P<0.01) in combination with mild hyperthermia at 41˚C for 0, 1, 3 and 5 h, respectively. Viability of T24 cells with 40 μg/ml ADR was 0.72. Therefore, the cell viability when 4 μg/ml ADR was combined with mild hyperthermia at 41˚C for 3 h showed the same anti-tumor effect as a 10-fold higher ADR concentration (40 μg/ml).

The viability of T24 cells with 40 μg/ml ADR decreased from 0.72 to 0.33 (P<0.001) when combined with mild hyperthermia at 41˚C after 1 h, and to 0.10 (P<0.001) at 41˚C after 5 h. The addition of mild hyperthermia at 41˚C for 1 h caused a 2.2-fold increase in the anti-tumor activity of 40 μg/ml ADR.

Discussion

Hyperthermia as a cancer therapy requires heating to 43˚C or higher in order to kill tumor cells. However, it is technically difficult to heat and maintain some organs and tissues at that temperature. Mild hyperthermia is easy, safe and cost-effective, and can be practically performed at any medical facility.

We previously reported that mild hyperthermia at 41˚C was effective in combination with chemotherapy, instead of hyperthermia at 43˚C, in an *in vitro* experiment using a leukemia cell line NALM-6 (7). Mild hyperthermia markedly enhanced the anti-tumor effects of anti-tumor drugs.

M-VAC chemotherapy (methotrexate, vinblastine, doxorubicin and cisplatin) is the gold standard therapy for transitional cell carcinoma of the urothelium (1,2). However, M-VAC chemotherapy is associated with severe adverse drug reactions, such as renal damage, bone marrow depression and gastrointestinal toxicity (nausea and vomiting) (3,4). In addition, its response rate is not always high, at about 40%. We examined whether the anti-tumor effect in the leukemic cell line could be extended to transitional cell carcinoma of the urothelium by combining mild hyperthermia with M-VAC chemotherapy in an *in vitro* experiment. In this experiment, the anti-tumor effects of CDDP and ADR, which have the main target action in M-VAC chemotherapy, were examined using the human bladder cancer cell line T24.

The thermal sensitivity of T24 cells was the same as that of cells in general. The survival rate of T24 cells was not

altered by mild hyperthermia at 41˚C, and was decreased by heating at over 43˚C.

CDDP and ADR are well-known anti-tumor drugs in M-VAC chemotherapy for transitional cell carcinoma of the urothelium (1-4). The survival rate of T24 cells was not affected by a low CDDP concentration (20 μg/ml) or mild hyperthermia at 41˚C, independently. However, the survival rate was significantly decreased by a low CDDP concentration (20 μg/ml) in combination with mild hyperthermia at 41˚C according to the incubation time. The anti-tumor effect of a low CDDP concentration (20 μg/ml) and 41˚C for 5 h resulted in the same survival rate as that of a 10-fold higher concentration (200 μg/ml) of CDDP.

As with CDDP, another anti-tumor drug, ADR, showed the same effects. The anti-tumor effect of 4 μg/ml ADR at 41˚C for 3 h achieved the same survival rate as a 10-fold higher ADR concentration (40 μg/ml).

Based on these results, a low concentration of anti-tumor drugs that have no tumor cell killing effect would be expected to have a 10 times greater anti-tumor effect in combination with mild hyperthermia at 41˚C. Given that, at the same concentration of anti-tumor drugs, the anti-tumor effect of 200 μg/ml CDDP increased 4-fold ($P<0.001$) and the effect of 40 μg/ml ADR increased 2.2-fold ($P<0.001$) in combination with mild hyperthermia at 41˚C for 1 h, the increase in tumor cell killing activity of anti-tumor drugs by combination with mild hyperthermia was confirmed.

It is reported that CDDP combines with the DNA strand in cancer cells and inhibits the DNA synthesis and cell division of cancer cells (9). The mechanism of the anti-tumor effect of ADR is the inhibition of both DNA and RNA synthesis after ADR forms a complex with DNA in tumor cells and inhibits both DNA polymerase and RNA polymerase reactions (10,11).

It seems that combination with mild hyperthermia potentiated the cytotoxic effects of these anti-tumor drugs by inducing apoptosis. Kameda *et al* reported that apoptosis was significantly enhanced when mild hyperthermia was combined with an anti-tumor drug (12).

Hyperthermia was found to inhibit the repair of DNA damage by anti-tumor drugs and radiation and increased the anti-tumor activity of chemotherapy and radiotherapy (13). Shioura *et al* (14) and Kano *et al* (15) reported an increase in the cytotoxic effects of combined treatment with low hyperthermia (40˚C) and bleomycin *in vitro*. Ono *et al* also reported that mild hyperthermia increased blood flow and enhanced the uptake of anti-tumor drugs into tumor tissue (16). Furthermore, mild hyperthermia was more effective in promoting heat-mediated suicide-gene (HSP 70) expression than high temperature therapy (17). Mild temperatures below 41˚C showed significantly smaller energies in Arrhenius plots for some anti-tumor drugs than those observed with temperatures above 41˚C (18). Recently, Ahmed *et al* reported mild hyperthermia- and hyperthermia-induced enhancement of drug cytotoxicity in apoptosis (19).

From these reports, it was suggested that mild hyperthermia enhances apoptosis and the anti-tumor effects of chemotherapy through an increase in the uptake of carcinostatics into tumor cells, and inhibits the repair of tumor cell killing by anti-tumor drugs.

These experimental results indicate the possibility that the dose of anti-tumor drugs can be decreased by combination with mild hyperthermia, thus reducing the side effects of the drugs. In poorly effective chemotherapy, a higher response rate is expected by combination with mild hyperthermia.

We found clinically mild hyperthermia to be safe, cost-effective and easy to perform using a far-infrared apparatus (20,21). With the approval of the ethics committee of our University, combination therapy with mild hyperthermia and chemotherapy for progressive bladder cancer was performed with exceptional results (22,23). We previously reported that combination therapy with mild hyperthermia and M-VAC chemotherapy (methotrexate, vinblastine, doxorubicin and cisplatin) reduces gastrointestinal side effects and potentiates the anti-tumor effect, with an excellent response rate of 83% for advanced or metastatic transitional cell carcinoma of the urothelium (24). This basic *in vitro* research strongly supports our clinical results.

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