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An Experimental Study of Enhanced Cell Killing by Hyperthermia and Bleomycin

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Summary: In vitro cell killing effects from heating and/or bleomycin have been investigated using the KK-47 cell line derived from a human bladder carcinoma and the colony-forming assay system. Percent survivals of the asynchronous cells heated to 40°, 41°, 42° and 43 °C for 30 min were 96.6%, 93.3%, 91.4% and 71.2%, respectively, and those at 43 °C for 2 h were 80.6%, 75.5%, 46.0% and 1.4%, respectively. The survival percentages of the cells treated with bleomycin at the concentrations of 1, 3, 6 and 10 μg per ml at 37 °C for 30 min were 66.0%, 40.4%, 21.9% and 11.9%, respectively. When hyperthermia at 43 °C was combined with bleomycin, a greatly enhanced cell killing effect was obtained by pre-heating and simultaneous heating as compared with the effect of post-heating.

Key words: Cell killing, Hyperthermia, Bleomycin.

Introduction

In recent years, there have been many experimental and clinical investigations of hyperthermia as a method of treatment in malignant diseases. The beneficial effect of hyperthermia has been suggested not only by itself but also in combination with anticancer agents or irradiation.

In the urological field, local hyperthermia treatment has been adopted for bladder cancer using hot water perfusion [3]. Because bleomycin is one of the heat stable anticancer agents, a solution containing bleomycin has been used to perfuse the bladder in the local hyperthermic treatment for bladder cancer [6, 8].

So far, very little is known about the thermal enhancement of cell damage induced by bleomycin in in vitro cultivated bladder cancer cells. In the present study, the lethal effect of 43 °C hyperthermia and/or bleomycin was investigated using a human bladder carcinoma cell line, KK-47, which was established in our department in 1977.

Materials and Methods

Cells and Culture Conditions

KK-47 cells, which had grown in a semi-confluent monolayer 3 days after subcultivation, were dispersed in the medium by gentle trypsinisation and a monocellular suspension was prepared. Five ml of the suspension containing 100, 200, 300, 400 and 500 cells was applied into triplicate tissue culture dishes, 60 x 15 mm (Falcon Co., Cal., USA). They were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cultivation was continued for 12 to 14 days and colonies composed of more than 50 cells were counted macroscopically, and computed for their plating efficiency.

Population Doubling Time

Using the replicated culture method [1], triplicate glass test tubes were seeded with 1×10^4 cells in 1.5 ml of monocellular suspension per tube. The tubes were incubated at 37 °C for 1, 3, 5, 7 and 9 days. After each incubation period, 3.5 ml of 0.1 M citric acid containing 0.05% crystal violet was added into each tube, and shaken for 30 min at 37 °C, and 4 ml of the supernatant was removed after centrifugation. The cell nuclei were counted using a blood cell counting chamber.

Preference of Test Tubes for Hyperthermia Experiments

In order to find the most suitable test tube with the best cell freeing rate from the tube surface by pipetting even after hyperthermia treatment, glass and polyethylene test tubes were studied.

Thermosensitivity of the Cells

The thermosensitivity of the cells at 40°, 41°, 42° and 43 °C were estimated using the following colony-forming method: polyethylene test tubes containing 5 ml of the monocellular suspension, 300 cells per ml, were heated at these temperatures for 30, 60, 90 and 120 min. After heating, the cells were dispersed by pipetting and 1 ml of the cell suspension was transferred into the dishes, and mixed with 4 ml of the fresh medium. The dishes were incubated at 37 °C for 12 to 14 days. From the number of colonies formed, the survival curve of the cells at each temperature was obtained. No heating was used in the control series.

Cell Killing Effects of Bleomycin at 37 °C

The cell killing effect was determined at concentrations of 1, 3, 6 and 10 μg per ml for the exposure periods of 30, 60, 90, 120 and

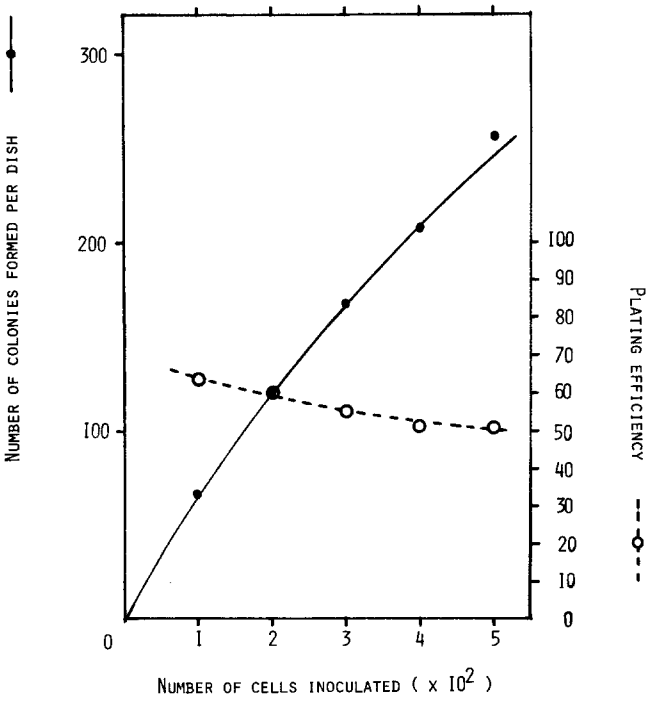


Fig. 1. Colony-forming ability of KK-47 cells cultured in a medium composed of 80% Ham's F12 supplemented with 20% calf serum for 12 to 14 days

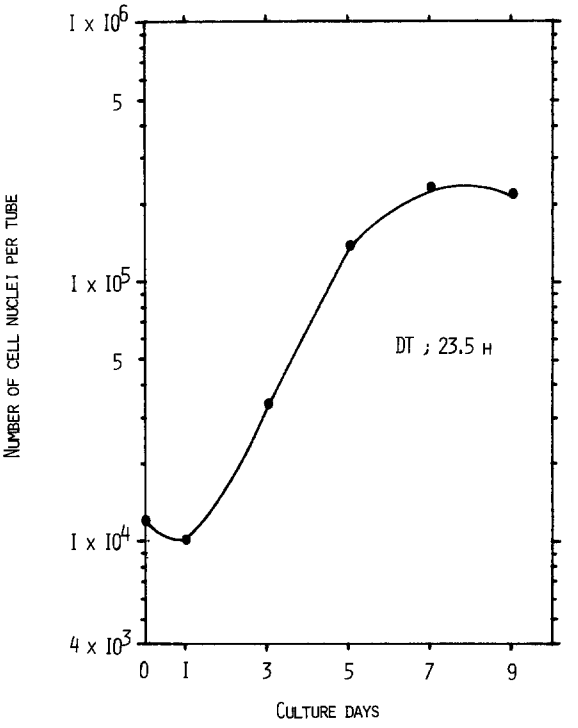


Fig. 2. Cell multiplication of KK-47 cells during 9 days when cells were cultured in a medium composed of 80% Ham's F12 supplemented with 20% calf serum

150 min. Five ml of the monocellular suspension containing 300 cells per ml was applied into polyethylene test tubes, and then each concentration of bleomycin was added. After the bleomycin exposure, the test tubes were washed twice with serum free medium and the treated cells were resuspended with the fresh medium at a concentration of 300 cells per ml. One ml of the resuspension and 4 ml of the fresh medium were dispensed into the triplicate culture dishes and incubated for 12 to 14 days. The survival rate of the cells at each of the concentration levels was determined from the number of colonies formed. No drug was added to the control series.

Cell Killing Effects of the Combination of 43 °C Hyperthermia and Bleomycin

The experiments consisted of five different groups: 1) bleomycin exposure at 37 °C for 30 min (control series), 2) bleomycin exposure at 37 °C for 30 min followed by 43 °C hyperthermia for 30 min without bleomycin (post-heating series), 3) bleomycin exposure at 43 °C for 30 min (simultaneous series), 4) 43 °C hyperthermia for 30 min followed by bleomycin exposure at 37 °C for 30 min (pre-heating series), 5) a sequential treatment of 43 °C hyperthermia for 30 min, bleomycin exposure at 43 °C for 30 min and 43 °C hyperthermia for 30 min (sandwich heating series). In these experimental series, bleomycin was removed by twice repeated centrifugations with serum free medium and the treated cells were resuspended with fresh medium. The cell killing effect was determined in the same manner as mentioned above.

Results

Plating Efficiency

As shown in Fig. 1, the plating efficiency of the cells was 64.4%, 55.7% and 51.8% in the dishes seeded with 100, 300 and 500 cells per dish, respectively. On the basis of the results, the cell density of 300 cells per dish, was adopted for the colony-forming method in the subsequent experiments.

Population Doubling Time

As shown in Fig. 2, the population doubling time of the cells was approximately 23.5 h.

Preference of Test Tubes for Hyperthermia Treatment

Fig. 3 shows the percentage recovery of cells incubated in polyethylene test tubes and glass test tubes at 37 °C for 2 h, 94.9% and 81.4%, respectively. From these results, the polyethylene test tubes were used in the following experiments.

Thermosensitivity of the Cells

According to the survival curves shown in Fig. 4, the survival rates of the cells at 40°, 41°, 42° and 43 °C hyperthermia for 30 min were 96.6%, 93.3%, 91.4% and 71.2%, respectively. After heating for 120 min, the survival rates at 40°, 41°, 42° and 43 °C hyperthermia were 80.6%, 75.5%, 46.0% and 1.4%, respectively.

Survival Rate of the Cells Exposed to Bleomycin (BLM)

The survival curves of the cells as a function of increasing concentrations of bleomycin are shown in Fig. 5. Bleomycin exposure of 1, 3, 6 and 10 μg per ml for 30 min resulted in cell survival rates of 66.0%, 40.4%, 21.9% and 11.9%, respectively. As shown in Figs. 5 and 6, those for 120-minute exposure at the same concentrations of bleomycin were

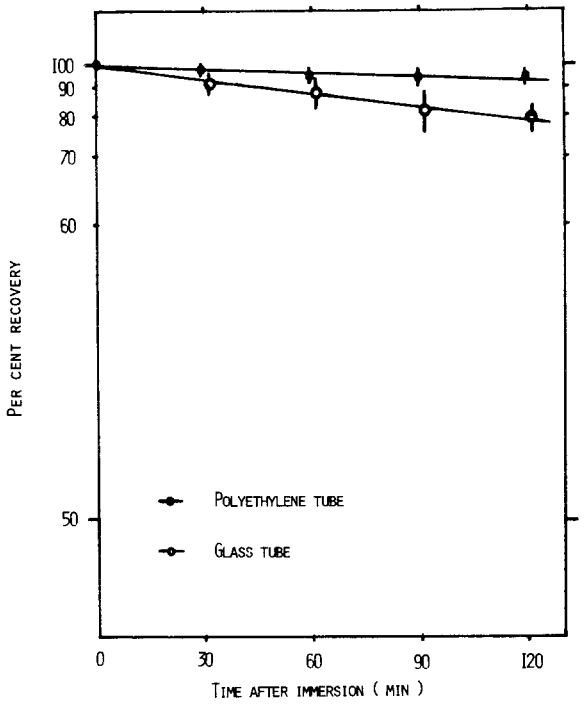


Fig. 3. Percent recoveries of KK-47 cells incubated in the two kinds of test tubes for various lengths of time. The bars indicate standard deviations of the mean for four independent experiments

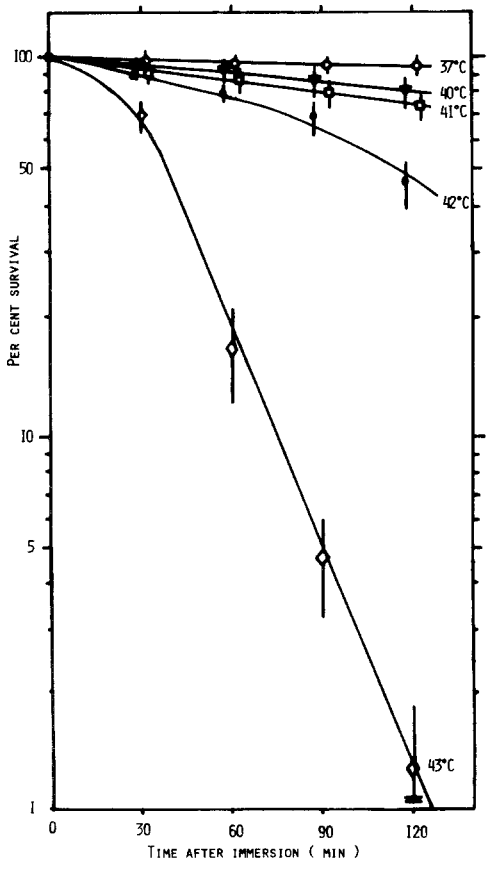


Fig. 4. Survival curves of KK-47 cells heated with different temperatures for various lengths of time

Fig. 5. Survival curves of KK-47 cells in different concentrations and exposure times of bleomycin (BLM)

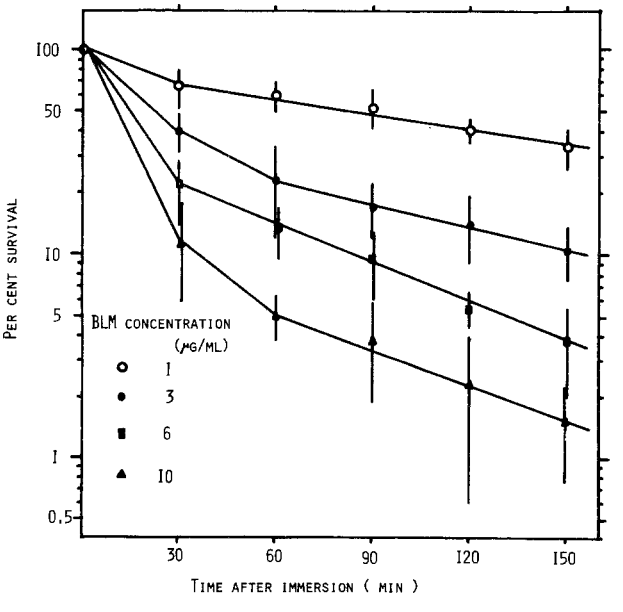
41.5%, 14.1%, 5.5% and 2.3%, respectively. Because BLM produced a bi-phasic survival curve for in vitro cultivated cells, BLM cytotoxicity as a function of increasing exposure times of drug and of increasing concentrations of BLM are illustrated in Figs. 5 and 6.

Cell Killing Effects from the Combination of 43 °C Hyperthermia and Bleomycin

As shown in Fig. 7, the survival rates of the cells exposed to a concentration of 3 μg per ml of bleomycin in the control, post-heating, simultaneous, pre-heating and sandwich series were 40.4%, 25.0%, 19.5%, 21.6% and 5.8%, respectively. At a concentration level of 10 μg per ml of bleomycin, those in the same experimental series were 11.9%, 9.8%, 5.3%, 4.0% and 0.3%, respectively. In both the simultaneous and pre-heating series, there was an increase in the cell killing effect as compared with the post-heating series.

Discussion

Because it is feasible to perfuse hot water containing anticancer drugs into the bladder cavity using a three-way Foley catheter, hyperthermia treatment has been introduced to bladder cancer with relative ease. Some beneficial effects of this clinical application have been reported [6, 8], and these clinical reports suggest that further basic studies are necessary to establish the value of hyperthermia treatment. From past clinical experience, intravesical hyperthermia perfusion therapy above 43 °C was not tolerated by bladder cancer patients without anaesthesia. In in vitro thermostability studies of the cells being heated at 40°, 41°, 42° and 43 °C for 30 to 120 min, the strongest cell killing effect was observed at 43 °C. In addition, the combination of bleomycin and heating for 30 min at 43 °C in vitro, the cell killing effect was significantly higher in the pre-heating and simultaneous heating series than that in the post-heating series. A strong cell killing effect was also obtained in the sandwich heating series. Hahn et al. [2] reported a striking



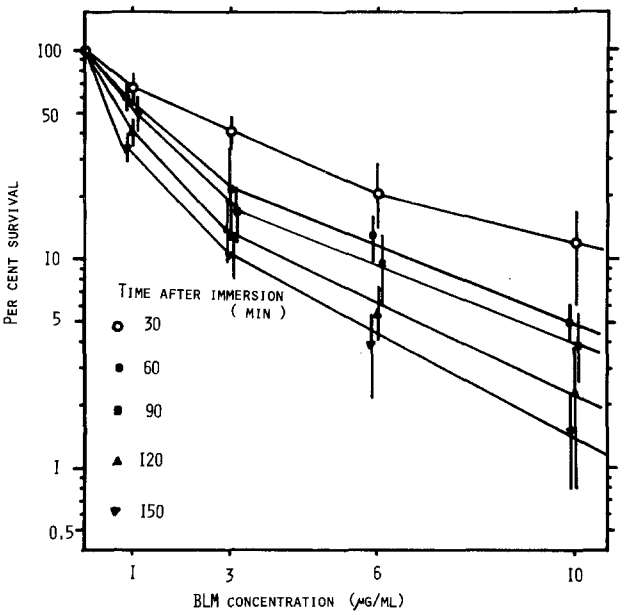


Fig. 6. Survival curves of KK-47 cells in different concentrations and exposure times of BLM

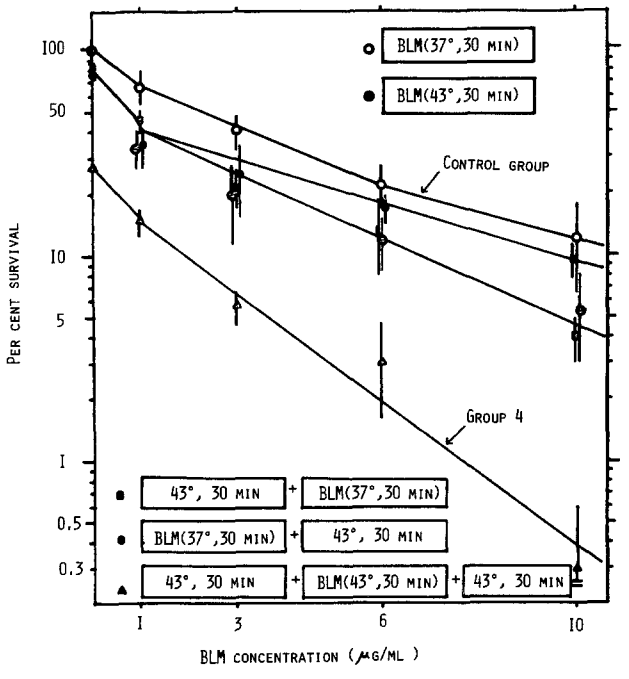


Fig. 7. Survival curves of KK-47 cells by BLM and hyperthermia treatments

synergism in cytotoxicity with the combination of 43 °C hyperthermia and bleomycin in Chinese hamster cells in vitro. In their experiments, the synergistic effect was rather small with the simultaneous combination of 41 °C hyperthermia and bleomycin, and also with 43 °C hyperthermia before or after the bleomycin treatment at 37 °C. Mizuno et al. [7] reported results similar to our observations using mouse mammary carcinoma FM3A cells, that is, the cell killing effect of hyperthermia increased sharply at a temperature from 42 °C to 43 °C and in the combination use of 43 °C hyperthermia and bleomycin, the pre-heating and simultaneous heating showing prominent cell killing as compared with the post-heating. The results mentioned above suggested that 43 °C hyperthermia has a primary sensitising effect on bleomycin cytotoxicity in vitro. In the combination usage of hyperthermia and anticancer drugs, the sequence of the combination played an important role in achieving the most beneficial effect, and also the effect had a close relationship to the degree and duration of heating and the cell killing mechanism in the drugs. Recently, Klein et al. [5] observed using two murine leukaemia cell lines that 41.5 °C hyperthermia for 15 to 60 min followed by an exposure to methotrexate, resulted in almost complete inhibiton of methotrexate cytotoxicity. In the case of reverse sequence of hyperthermia and methotrexate exposure, hyperthermia did not reduce the methotrexate cytotoxicity. Thus, it is suggested that hyperthermia can introduce a modification in the drug sensitivity.

Further studies of thermal cell biology together with research into heating technology would greatly contribute to the progress of clinical benefits from hyperthermia.

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