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Abstract. Both adriamycin (ADM) and hyperthermia show thermal chemo-enhancement. Tolerance induction against ADM in heated cells has been reported resulting in clinical difficulty of cancer therapy. We investigated thermo-enhancement induced with ADM (0.2 μg/ml) treatment alone or combined with ADM and 42°C hyperthermia in Chinese hamster V79 cells *in vitro*. Intracellular accumulation of hsc70 and hsp72 proteins after hyperthermia or ADM was observed to examine the possible relationship between cell killing effect and their accumulations. Thermosensitivity of V79 cells at 42°C after the simultaneous treatments with ADM showed marked thermo-enhancement within the short-term treatments for less than 1 h, while the combined treatments for longer than 1 h, the cells showed reduced thermosensitivity. Survival from the simultaneous treatments for less than 1 h was reduced markedly less than the single treatment both with ADM or 42°C hyperthermia alone. Thermotolerance was markedly induced in a step-up hyperthermia (42°C 2 h - 44°C). The combined treatments with ADM and 44°C hyperthermia following the 42°C preheating alone does not inhibit thermotolerance development. The combined treatments with ADM and 42°C preheating showed markedly interactive cell killing, but no thermo-enhancement to the following 44°C hyperthermia was shown. The leveling slope of the 44°C heating period - survival curve was drawn. In the Western blot analyses, hsc70 existed constitutively in the V79 cells. Following the 42 or 44°C hyperthermia alone, intracellular accumulation of hsp72 was determined. ADM treatment alone did not induce any accumulation of hsp72. In the simultaneous treatments with ADM and hyperthermia, the accumulation of hsp72 was markedly reduced. The accumulation of hsp72 after the combined treatment with ADM and hyperthermia was not observed as markedly as that after hyperthermia alone.

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Introduction

Adriamycin (14-hydroxydaunorubicin, doxorubicin, NSC-123127 or adriblastin) was adopted for cancer therapy in 1969 (1). This chemical substance, one of the anthracyclines, was isolated from cultured *Streptomyces peucetius var. caesius* by Arcamone *et al*, and showed anti-tumor effect (1). ADM has been adopted for human cancer therapy and treated alone or in the interdisciplinary therapy combined with radiation, hyperthermia and/or a variety of the other anticancer drugs (2). Mechanism of action of ADM has been widely investigated. It was reported that DNA treated with low concentration of ADM resulted in the single strand breaks among a variety of cell lines (3), and that double strand breaks increased in accordance with increase in ADM concentration. It was first considered that ADM intercalates to the adjacent base pairs of the double stranded DNA and results in inhibition of enzymes concerning DNA replication and transcription (4,5). However, it was suggested that another target existed on nucleic membrane that altered the structure function of the membrane (6). Hyperthermia has been shown interactive effect in combined treatments with a variety of anticancer drugs, in which interactive effect of combined treatment with ADM and hyperthermia is reported (7). It was first reported that cells exposed to step-up heating (i.g. 42-44°C) tolerated the determining heating (44°C) (8,9). Hsp70 family, including hsc70 (10) and hsp72 (11) proteins, is transcriptionally induced as stress protein due to transitional or sublethal damage such as hyperthermia (11,12), inhibition of metabolism, heavy metals, virus infection, UV (13) or ionizing radiation (14) and DNA damaging agents (15). Stress protein synthesis is regulated by stress-specific transcription factors, molecular chaperons, that protect cells from stress, taking an important role to maintain homeostasis (16). Since interactive effect of ADM and hyperthermia is

not observed clearly in *in vivo* experiments or clinical results, mechanism of the interaction has been investigated in quantitative colony formation by surviving cells *in vitro* and Western blot analyses of hsp70 family in the present study.

Materials and methods

Cells and culture medium. Chinese hamster V79 cells (17) were colonially cloned and used for all the present experiments, and cultured in the medium MLN-15, 1 l of which contained 730 ml of Eagle's MEM solution (Nissui Pharmaceutical Co., Tokyo, Japan), 20 ml of 2.5% w/v autoclaved lactalbumin hydrolysate solution (Difco Laboratories, Detroit, MI, USA), 100 ml of NCTC-135 solution (Gibco Laboratories, Grand Island, NY, USA) and 150 ml of inactivated new born calf serum (Gibco).

Hyperthermia and the surviving cell colony formation. Hyperthermia was carried out by immersion of culture flasks of the tightened screw tops in a temperature-regulated water bath (Model EPS-47, Toyo Seisakusho Co., Tokyo, Japan) pre-set at 42 or 44°C. The temperature was maintained within the error of ±0.05°C of the pre-set temperatures as measured by a thermistor (Model D116-1251, TaKaRa Thermistor Instruments Co., Yokohama, Japan). Each flask was exposed to hyperthermia or ADM treatment for graded periods (18). The cells were finally cultured in a CO_2 incubator (Tabai-Espec LNA-3 DH, Osaka, Japan) in the flasks for 5-10 days to allow growth for visible colony formation of the surviving cells from the treatment of hyperthermia or ADM. The surviving cell fractions were determined using a colony forming assay (18).

Treatment with ADM. ADM (Kyowa Hakko Co., Tokyo, Japan) was dissolved in the culture medium at the final concentration of 0.2 μg/ml for all the present treatment, that was about a half of the standard clinical dose range (drug weight/human body weight). The adhered cells on the inner face in the bottom of the flasks were exposed with ADM by replacement with 6 ml of the ADM solution in MLN-15 for graded periods, the ADM solution was chased, the adhered cells were rinsed twice and refed with 6 ml of the 37°C culture medium, MLN-15. Thus treated flasks were incubated stationarily for visible colony formation.

Hsc70 and hsp72 assays. After the variety of those isotoxic treatments, the cells were incubated at 37°C for graded interval periods less than 24 h, and the accumulated amounts of hsc70 and hsp72 in the cells were examined by Western blot analysis (19). The adopted monoclonal antibody against hsp72 was C92F3A-5 (StressGen Biotechnologies Corp., Victoria, BC, Canada) while that against hsc70 was BB70 (StressGen Biotechnologies Corp.).

Results

42°C alone, ADM alone or the simultaneously combined treatments. In heating period - survival relation ship of V79 cells at 42°C, the thermosensitivity was moderate. In the initial part of the survival curve, sublethal thermal damage repair

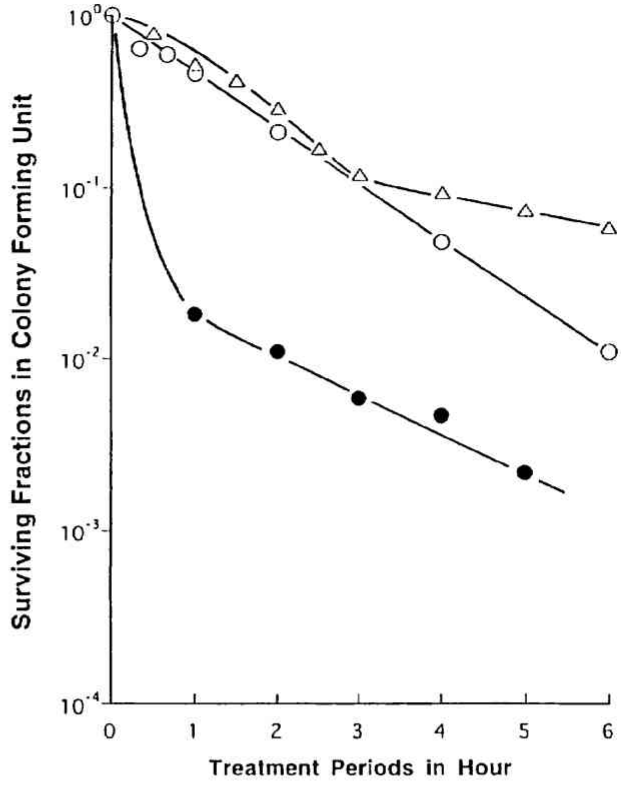

Figure 1. Effect of simultaneous treatment with ADM 0.2 μg/ml on survival of V79 cells from 42°C hyperthermia. Ordinate: log surviving fractions in colony forming unit. Abscissa: treatment periods of ADM, 42°C or ADM at 42°C in hour. Open circles represent surviving fractions from ADM alone. Open circles show exponential regression to the graded treatment periods with ADM but no apparent repair from sublethal chemical damage appeared. Open triangles represent surviving fractions from 42°C hyperthermia alone. The survival curve represents repair from sublethal thermal damage (SLTDR) in shoulder profile followed by gentler slope in exponential regression indicating induction of thermotolerance after 3 h hyperthermia at 42°C. Closed circles represent surviving fractions from simultaneously combined treatments with ADM and 42°C hyperthermia resulting in markedly steeper regression of the surviving fraction for the initial 1 h of the treatment followed by the another exponential regression with reduced lethal sensitivity in the markedly gentler slope for 5 h. The error bars on the symbols represent standard errors of three replicate flasks. Symbols without error bar represent that those bars are within the symbols.

(SLTDR) was shown in the profile of 'shoulder' followed by exponential regression appearing within the 42°C heating period for 1.5-3 h in the survival curve. In the heating period for more than 3 h, surviving fractions further regressed exponentially in markedly gentler slope of the survival curve as thermotolerance developed as shown with open triangles in Fig. 1. The ADM (0.2 μg/ml) treatment period - survival curve at 37°C showed a simple exponential regression as shown with open circles in Fig. 1. Simultaneous treatments with the 42°C hyper-thermia and the ADM resulted in the regression curve of the surviving fraction with markedly steeper slope for the initial 1 h of the treatment, followed by the gentler exponential regression for further 4 h so far as assayed as shown with closed circles in Fig. 1. T_0 values, treatment period required to reduce survivals by 1/e in the compartment of exponential regression, are shown in Table I. In the combined treatment with 42°C hyperthermia and ADM, the lethal sensitivity for the initial 1 h was markedly

Table I. T_0 values of V79 cells in survivals from treatment with 42°C, ADM or 42°C and ADM.

	T_{01} in min (before induction of thermotolerance)	T_{02} in min (after induction of thermotolerance)
42°C hyperthermia	111	183
ADM in 0.2 μg/ml	78	
42°C and ADM	12	118

T_0 value, incubation period required to reduce surviving fraction by 1/e in the exponentially regressing compartment of treatment period - survival curve in colony forming unit.

higher but followed by an exponential regression for the reduced lethal sensitivity for more than 1 h, while treatment with ADM alone showed a constant lethal sensitivity as shown in Fig. 1.

Enhancement effect of 42°C preheating on the lethal chemosensitivity of thermotolerant V79 cells to ADM, and effect of ADM on survival reduction of step-up (42-44°C) thermotolerant V79 cells. The thermotolerance of V79 cells was induced by 42°C preheating for 2 h. Chemosensitivity of the thermotolerant V79 cells to ADM was enhanced rather than the chemosensitivity to ADM alone. However, lethal sensitivity of the thermotolerant V79 cells to simultaneous treatments together with ADM and 44°C hyperthermia (42°C for 2 h alone - 44°C for graded periods in min with ADM) was markedly interactive in the combined treatment for less than the initial 10 min followed by markedly gentle lethal sensitivity, which was similar to those for 44°C lethal thermosensitivity of 42-44°C step-up thermotolerant cells as shown with open triangles in Fig. 2.

Interactive effect of simultaneous treatments together with 42°C and ADM for 2 h on thermo-chemo-enhancement, and effect of ADM on induction of the step-up thermotolerance during the 42°C preheating for 2 h as determined by the 44°C hyperthermia. Surviving fraction from 42°C preheating for 2 h alone in the 42-44°C step-up heating was about 40%. Surviving fraction from ADM treatment alone at 37°C for 2 h was about 20%. Therefore theoretical survival fraction from the combined treatments should be about 8% (0.4x0.2) unless there was any interactive effect. However, the practical surviving fraction from the combined treatments together with ADM and 42°C hyperthermia both for 2 h, was about 1%, that was markedly lower than the theoretically estimated 8%. The resulted surviving fraction was much less than the theoretically estimated as shown on the ordinate of Fig. 3, suggesting the interactive effect between the treatments of ADM and 42°C hyperthermia. Surviving cells from the combined treatments with ADM and 42°C hyperthermia for 2 h were ultimately thermotolerant in their 44°C lethal thermosensitivity to the following 44°C hyperthermia

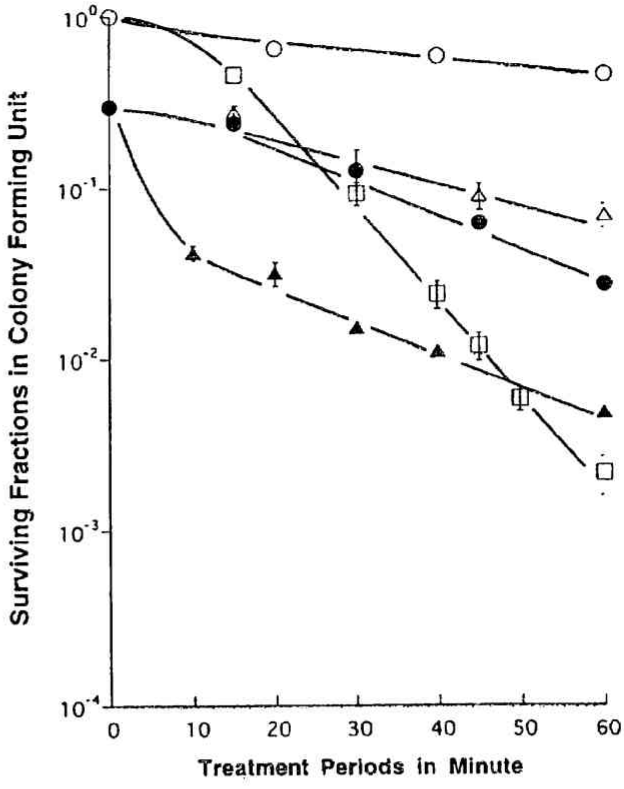

Figure 2. Effect of ADM on survival from 44°C hyperthermia in 42°C preheated thermotolerant V79 cells and effect of 42°C preheating on survival from ADM treatment at 37°C. Ordinate: log surviving fractions in colony forming unit. Abscissa: treatment periods of ADM, 44°C hytperthermia alone or ADM at 44°C in min. Open circles and open squares represent surviving fractions from ADM alone and 44°C hyperthermia alone, respectively. Open triangles represent surviving fractions of 42°C preheated thermotolerant V79 cells, from 44°C hyperthermia for graded periods. Closed circles represent surviving fractions from ADM treatment for graded periods at 37°C after 42°C preheating for 2 h. A closed circle on the ordinate represents a survival from 42°C for 2 h alone. Closed triangles represent surviving fractions from ADM at 44°C for graded periods after 42°C preheating for 2 h, which was interactive in the combined treatment for less than 10 min followed by an exponential regression in reduced lethal sensitivity, and which was similar to those for 44°C lethal thermosensitivity of step-up (42-44°C) thermotolerant cells. The error bars on the symbols represent standard errors of three replicate flasks. Symbols without error bar represent that those bars are within the symbols.

regardless that the ADM was maintained through the 44°C hyperthermia. The marked interactive effect of the treatments together with 42°C hyperthermia and ADM was observed but the surviving cells from the combined simultaneous treatments (ADM and 42°C) showed almost no apparent thermosensitivity to the following 44°C hyperthermia as shown in Fig. 3.

We further investigated the time course of intra-cellular accumulation of hsc70 and hsp72 proteins during the 37°C incubation interval between the treatment and Western blotting.

Western blot analyses after the variety of treatments with ADM, 42°C and 44°C hyperthermia. The 37°C interval periods after the variety of the treatments and the Western blot ranged from 0 to 24 h. Time course of the intracellular accumulation of hsc70 or hsp72 protein was qualitatively analyzed by

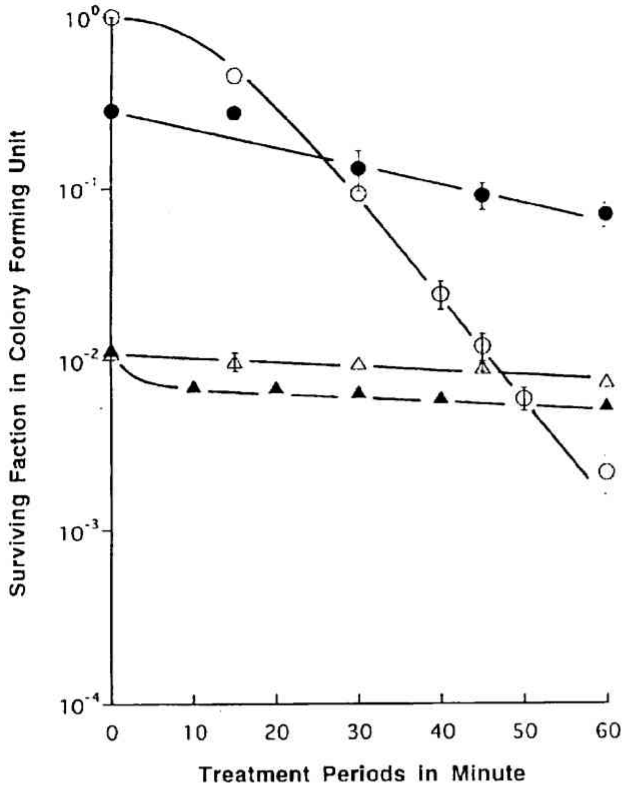

Figure 3. Effect of ADM on induction of thermotolerance or thermoenhancement during 42°C preheating for 2 h in V79 cells. Ordinate: log surviving fractions in colony forming unit. Abscissa: periods of 44°C hyperthermia in min. Open circles represent surviving fractions from 44°C hyperthermia alone. Closed circles represent surviving fractions, of 42°C preheated cells for 2 h, from 44°C hyperthermia for graded periods. Open triangles represent surviving fractions, of pretreated cells together with 42°C and ADM for 2 h, from 44°C hyperthermia for graded periods. Closed triangles represent surviving fractions, of cells pretreated together with 42°C and ADM for 2 h, from the treatment together with 44°C hyperthermia and ADM for graded periods. Closed circle on the ordinate represents surviving fraction from 42°C alone for 2 h. Semiclosed triangle on the ordinate represents surviving fraction from ADM treatment alone for 2 h. Closed and open triangles on the ordinate represent surviving fractions from ADM and 42°C hyperthermia for 2 h. Surviving fractions of cells pretreated together with 42°C and ADM was markedly decreased as shown by the open or closed triangle on the ordinate indicating the marked interactive cell killing, but the induced thermotolerance was markedly appeared by the further treatment with 44°C hyperthermia regardless of ADM treatment. The error bars on the symbols represent standard errors of three replicate flasks. Symbols without error bar represent that those bars are within the symbols.

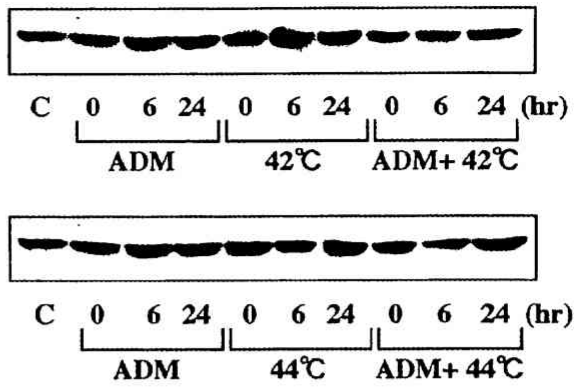

Figure 4. Hsc70 accumulation of V79 cells treated with ADM, 42°C (upper panel) or 44°C (lower panel) hyperthermia, or the combination with ADM and hyperthermia, after the graded periods of incubation intervals. Time course of intracellular accumulation of hsc70 in V79 cells after the treatments with ADM, hyperthermia or the combination were examined by Western blotting as shown. Numerals on horizontal line of the panels represent 37°C incubation intervals in hour between the treatments of ADM, hyperthermia or the combination and the blotting, respectively, and the 'C' on the left of the panels represent the lane for hsc70 of the non-treated control V79 cells. Each treatment period in the upper panel was designed for 3 h for ADM alone, 5 h for 42°C alone and 30 min for the combined treatments (surviving late in C.F.U. of 10%, respectively), while in the lower panel 20 min for 44°C alone and 10 min for the combined treatments (surviving late in C.F.U. of 30%, respectively). Treatment periods in the upper and lower panels were designed to give isotoxic effect of the cell killing, respectively. The incubated cells were examined for hsc70 accumulation by Western blotting, and transferred on the Poly-Screen PVDF membrane 0-24 h after the incubation interval. Stained hsc70 electrophoretic bands were cut and picked up from the membrane and shown. Six and 24 h after the 42°C hyperthermia for 5 h, showed more hsc70 than the other lanes. Accumulation of hsc70, constitutive stress protein probably induced by the miscellaneous stresses, was not reduced by the ADM treatment and slightly enhanced by the hyperthermia at 42 or 44°C but slightly reduced by the combined treatments with ADM and hyperthermia at 42°C.

Western blotting. The qualitative observations suggested as follows; a) accumulation of hsc70 did not appreciably change by the ADM treatment during the 0-24 h interval incubation between ADM treatment and Western blot; b) accumulation of hsc70 increased by the 42°C hyperthermia alone but was not significantly different; c) increase in hsc70 accumulation after the combined treatments with ADM and 42°C hyperthermia was not observed significantly; d) hsc70 accumulation after the 44°C hyperthermia alone increased but was not significant; e) hsc70 accumulation after the combined treatment with ADM and 44°C hyperthermia was observed but not significant; f) the hsp72 after the ADM treatment at 37°C was not appreciably accumulated during the interval incubation periods for 0-24 h; g) the hsp72 was accumulated after the 42°C hyperthermia alone during the interval incubation for 6-24 h but was not appreciably accumulated after the combined treatment with ADM and 42°C hyperthermia; and h) hsp72 was accumulated 6-24 h after the combined treatment with ADM and 44°C hyperthermia, the amount of which was markedly slighter than that after 44°C hyperthermia alone. Western blot finding on hsc70 and hsp72 was no more than qualitative finding, but the lower accumulation of hsc70 was observed after the simultaneous treatments with ADM and 42°C hyperthermia rather than that after 42°C hyperthermia alone and no significant difference was observed between groups of the combined treatments and the control. Hsp72 was markedly accumulated 6-24 h after 42°C or 44°C hyperthermia alone while it was not appreciably observed after ADM treatment alone, but observed after the combined treatment with ADM and 44°C hyperthermia.

Discussion

Survivals in colony forming unit after a variety of treatments. Several results were obtained from the quantitative assay in

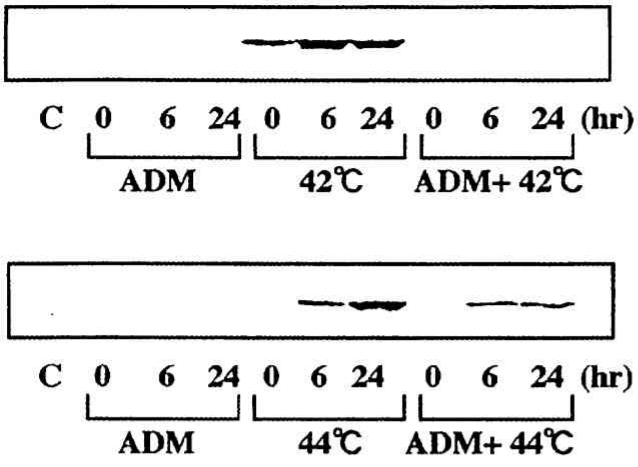

Figure 5. Hsp72 accumulation of V79 cells treated with ADM, 42°C (upper panel) or 44°C (lower panel) hyperthermia, or combined with ADM and hyperthermia, after the graded periods of incubation intervals. Time course of intracellular accumulation of hsp72 in V79 cells after the treatments with ADM, hyperthermia or the combination were examined by Western blotting as shown. Numerals on horizontal line of the panels represent 37°C incubation intervals in hour between the treatments of ADM, hyperthermia or the combination and the blotting, respectively, and the 'C' on the left of the panel represents the lane for hsp72 of the non-treated control V79 cells. Each treatment period was designed 3 h for ADM alone, 5 h for 42°C alone and 30 min for the combined treatments (surviving late in C.F.U. of 10%, respectively), while in the lower panel 20 min for 44°C alone and 10 min for the combined treatments. All the treatment periods were designed to give isotoxic effect of the cell killing. Hsp72 was accumulated 6-24 h after 42°C or 24 h after 44°C hyperthermia alone that was not appreciably accumulated after ADM treatment alone, but accumulated slightly after the combined treatments with 44°C hyperthermia and ADM. In the lanes of control and those after treatment with ADM alone no appreciable accumulation of hsp72 was observed. Hsp72 was accumulated 24 h after 42 or 44°C hyperthermia but was not accumulated after ADM at 37°C, while it was slightly recognized 0, 6 and 24 h after combined treatments with ADM and 44°C for 10 min but was not recognized appreciably 0, 6 and 24 h after combined treatments with ADM and 42°C for 30 min. These blottings indicate ADM treatment did not appreciably induce hsp72 in the present assay system, and it took six or more hours of intervals after hyperthermia to induce hsp72 but ADM combined with hyperthermia efficiently inhibit accumulation of hsp72.

colony formation by the surviving cells from treatments with hyperthermia at 42 or 44°C, ADM and the simultaneous treatments. Interaction in enhanced cell killing by simultaneous treatments with hyperthermia and ADM was yielded as shown in Figs. 1 and 3 and Table I. Initial combined short-treatment with hyperthermia and ADM resulted in markedly enhanced cell killing and Western blot analysis after the combined treatment with hyperthermia and ADM resulted in markedly reduced accumulation of hsp72 rather than that after hyperthermia alone, suggesting inhibition of hsp72 accumulation by ADM, i.e. lower than the theoretically estimated 8.8%. The reduced surviving fraction was much less than the theoretically estimated, suggesting the interactive effect between the treatments of ADM and 42°C hyperthermia. It is elucidated that the simultaneous treatments with ADM and hyperthermia for the initial short period such as 1 h at 42°C or 10 min at 44°C hyperthermia was reasonably indicative to expect marked interactive cell killing.

Enhancement effect of 42°C preheating on the lethal chemosensitivity of the thermotolerant V79 cells to ADM, and effect of ADM on survival reduction of thermotolerant V79 cells from step-up (42-44°C) hyperthermia. Thermotolerance was induced markedly in the V79 cells by the 42°C preheating for 2 h, that was examined by the following 44°C hyperthermia for the graded periods. Chemosensitivity to ADM at 37°C after the 42°C preheating resulted in moderate chemo-enhancement induced by the preheating. Chemosensitivity to ADM at 44°C after the 42°C preheating resulted in the same slope of the survival curve to that of ADM at 37°C. Since the initial 10 min of the treatments together with ADM and the 44°C hyperthermia after the 42°C preheating for 2 h resulted in markedly interactive cell killing, this survival curve (42°C for 2 h - ADM at 44°C) was drawn parallel to and far below another curve (42°C for 2 h - ADM at 37°C) suggesting no appreciable chemical enhancement to ADM by hyperthermia at 44°C except for the initial 10 min of ADM treatment at 44°C after the preheating. Although ADM treatment at 37°C induced no appreciable hsp72 accumulation, but induced marked accumulation of hsp72 after 42 or 44°C hyperthermia, the combined treatments of ADM and hyperthermia yielded no, or very slight, accumulation of hsp72 indicating that ADM did not only induce accumulation of hsp72 but inhibit hsp72 accumulation owing to the hyperthermia. Quantitative time course assay in colony formation of surviving cells from the variety of treatments with ADM and hyperthermia was hardly interpreted by the qualitative Western blot analysis of the intracellular accumulation of hsc or hsp. P53 status of the V79 cells has been reported mutant type (20). The related problems have been reviewed by the authors (21).

Acknowledgments

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