

Sensitization by Wortmannin of Heat- or X-ray Induced Cell Death in Cultured Chinese Hamster V79 Cells

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Here we found that wortmannin sensitized Chinese hamster V79 cells to hyperthermic treatment at 44.0°C as determined either by colony formation assay or by dye exclusion assay. Wortmannin enhanced heat-induced cell death accompanying cleavage of poly (ADP-ribose) polymerases (PARP). Additionally, the induction of heat shock protein HSP70 was suppressed and delayed in wortmannin-treated cells. Heat sensitizing effect of wortmannin was obvious at more than 5 or 10 μ M of final concentrations, while radiosensitization was apparent at 5 μ M. Requirement for high concentration of wortmannin, i.e., order of μ M, suggests a possible role of certain protein kinases, such as DNA-PK and/or ATM among PI3-kinase family. The sensitization was minimal when wortmannin was added at the end of heat treatment. This was similar to the case of X-ray. Since heat-induced cell death and PARP cleavage preceded HSP70 induction phenomenon, the sensitization to the hyperthermic treatment was considered mainly caused by enhanced apoptotic cell death rather than secondary to suppression or delay by wortmannin of HSP70 induction. Further, in the present system radiosensitization by wortmannin was also at least partly mediated through enhancement of apoptotic cell death.

INTRODUCTION

Hyperthermia has cell killing effect either by itself or synergistically with radiation, and is used to treat cancer mostly combined with radiation. One of the biological bases for the

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Abbreviations: DNA-PK; DNA-dependent protein kinase, DNA-PKcs; DNA-PK catalytic subunit, DSB; double-strand break, HSP; heat shock protein, PARP; poly (ADP-ribose) polymerase, SDS-PAGE; sodium dodecyl sulfate-polyacrylamid gel

combined use is that radioresistant S phase cells and hypoxic cells are relatively sensitive to hyperthermia¹). Additionally, hyperthermia has radiosensitizing effect possibly due to inhibition of potentially lethal damage repair²), sublethal damage repair³), DNA polymerases⁴) and rejoining of single- and double-strand breaks⁵).

Wortmannin, originally described as an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), was recently reported to sensitize cells at several micromolar or higher concentration to ionizing radiation and to anticancer drugs, e.g., bleomycin, possibly through inhibition of DNA-dependent protein kinase (DNA-PK) and ataxia-telangiectasia mutated (ATM) kinase having PI3-kinase homologous domain⁶⁻¹¹).

We¹²) and others^{13,14}) recently showed that DNA-PK was inactivated by heat treatment due to heat-lability of Ku subunits, and proposed that heat-induced inactivation of DNA-PK might be a possible mechanism for hyperthermic radiosensitization. Nueda et al¹⁵) recently described that DNA-PKcs-deficient *scid* and Ku86-deficient *xrs-6* fibroblast cell lines were sensitive to heat-induced apoptosis. These results suggest possible involvement of DNA-PKcs and Ku in cellular response process to heat.

We previously reported that Chinese hamster fibroblast V79 cells died after 44°C heat treatment with DNA ladder formation indicating apoptosis¹⁶). In the present study, we examined effects of wortmannin on V79 cells using either colony formation or viability assay with dye exclusion, and by western blotting analysis for poly (ADP-ribose) polymerase (PARP) cleavage and for heat shock protein induction. These results on heat sensitization were also compared with these of wortmannin sensitization of X-irradiation.

MATERIALS AND METHODS

Cell Culture

Chinese hamster V79 cells¹⁶) were cultured in 25 cm² plastic flasks in alpha-MEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% calf bovine serum (HyClone, Logan, UT, USA) and kept at 37°C in humidified atmosphere with 5% CO₂. Exponentially growing cells were used for all experiments.

Wortmannin treatment

Wortmannin (Sigma, St. Louis, MO, USA) was dissolved at 20 mM in dimethyl sulfoxide (DMSO) and was stored at -40°C. The stock solution was diluted just prior to experiments to give desired final concentration in culture medium. Wortmannin was added at 10 μM final concentration 60 min before heat treatment or irradiation, unless otherwise indicated. For control, equal volume of DMSO was added to the medium. Once added wortmannin was not exchanged during the course of experiments. In clonogenic survival experiments (Figs. 1 to 3), fresh wortmannin was added to the medium after plating.

Heat treatment

Heat treatment was carried out by submerging the culture flasks in a water bath set at

44.0 ± 0.05°C. Immediately after heat treatment, the flasks were put in a 37°C water bath for 3 min.

X-irradiation

Cells were exposed to X-ray at room temperature from an X-ray machine (Model SHT 250-3, Shimadzu Seisakusho Ltd., Kyoto) operated at 200 kV- 20 mA with a filter of 1 mm Al and 0.5 mm Cu. The half-value layer was 1.6 mm Cu and the dose rate was 1.4 Gy/min.

Cell survival assay

Following irradiation or heat treatment, cells were harvested by trypsinization and collected by centrifugation for 5 min at 100×g. Cells were resuspended in fresh medium, counted and plated on 60 mm tissue culture dishes. After incubation for one week, colonies were stained with crystal violet and colonies consisting of more than 50 cells were counted. Surviving fractions were normalized to those for unirradiated or unheated controls with DMSO or appropriate concentrations of wortmannin.

Dye exclusion test

At various times after irradiation or heat treatment, cells were harvested by trypsinization, and resuspended in medium. One hundred ml of cell suspension was mixed with 25 µl of saline containing 1% w/v Erythrosin B and examined under microscope. The numbers of stained (dead) cells and non-stained (live) cells were counted and the viability (%) was calculated as follows;

$$\text{Viability (\%)} = (\text{number of non-stained cells} / \text{total cell number}) \times 100$$

Western blot analysis

Cells (2×10^6) were recovered by trypsinization followed by centrifugation and were resuspended in 150 µl of 1 × SDS gel sample buffer (1% SDS, 3% β-mercaptoethanol, 5% glycerol, 62.5 mM Tris-HCl, pH 6.8) and heated at 100°C for 10 min. After centrifugation at 15,000 rpm for 15 min, 15 or 30 µl of the supernatants (containing lysates of 2 or 4 × 10⁵ cells) were applied on to SDS-PAGE. Slab Gels made of 7.5, 10 and 12% polyacrylamide were used for analysis of Ku86, HSP72/73 and PARP 23 kDa fragments, respectively. Separated protein bands were transferred onto polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA, USA) using AE-6678 semidry transfer system (ATTO, Tokyo, Japan). After blocking with 5% skimmed milk in 0.05% Tween-20 in tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.6, 0.9% NaCl) for 60 min at room temperature, the membrane was incubated overnight at 4°C in 5% skimmed milk T-TBS containing 1:300 dilution of rabbit anti-Ku86 primary antibody prepared in our laboratory, 1:100 mouse anti-HSP72/73 (Oncogene Research Products, Cambridge, MA, USA) or 1:4000 goat anti-PARP (R&D systems Inc., Minneapolis, MN, USA). Following rinse with 5 changes of T-TBS at room temperature, the membrane was incubated at room temperature with 5% skimmed milk T-TBS

containing horseradish peroxidase-conjugated secondary antibody (DAKO Japan, Kyoto, Japan) against rabbit immunoglobulin (at 1:3000 dilution), against mouse immunoglobulin (at 1:3000 dilution) or against goat immunoglobulin (at 1:4000 dilution). The membrane was rinsed at room temperature in T-TBS and in TBS and was then developed using ECL plus kit (Amersham International plc, Buckinghamshire, UK).

RESULTS

Wortmannin sensitized V79 cells to heat treatment or X-irradiation

In Figs. 1 to 3, effects of wortmannin on the colony forming ability of Chinese hamster fibroblast V79 cells after heat treatment or X-irradiation were examined. Treatment with 10 μM wortmannin decreased the clonogenic survival of V79 cells after 44.0°C heat treatment (Fig. 1A). Radiosensitizing effect of wortmannin was reported with various cell lines⁶⁻¹¹⁾ excluding a special case, i.e., exponentially growing human osteosarcoma MG-63 cells were not sensitized although they were sensitized in plateau phase¹⁷⁾. However, the present system clearly showed radiosensitizing effect in exponentially growing V79 cells (Fig. 1B). Sensitization was apparent when wortmannin was added before the heat and the effect was minimal when added at the end of heat treatment (Fig. 2). The heat sensitizing effect of wortmannin required 5 μM or higher concentrations (Fig. 3A), similarly to radiosensitizing effect (Fig. 3B). The plating efficiency of unheated or unirradiated control cells was not significantly affected by wortmannin up to 20 μM (data not shown).

Wortmannin enhanced heat-induced cell death

To examine whether wortmannin enhanced cell death induced by heat treatment, we

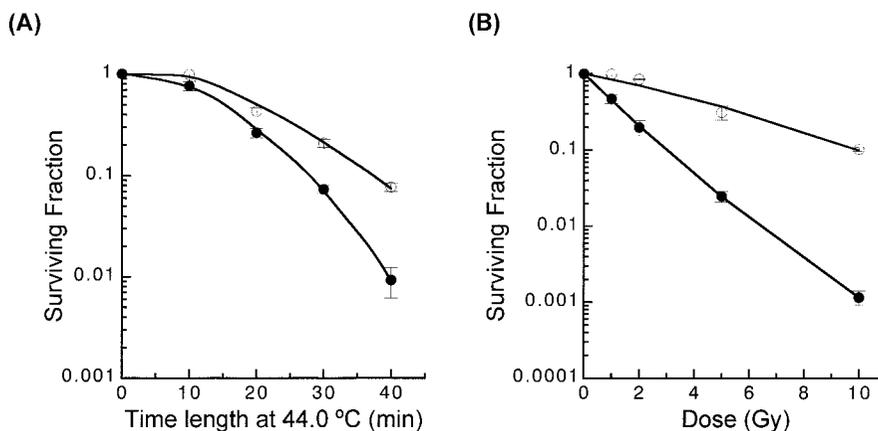


Fig. 1. Sensitization of heat treatment or X-irradiation by wortmannin. Surviving fractions by colony formation of cells after 44.0°C-treatment (A) or X-irradiation (B) with or without 10 μM of wortmannin (○ and ●, respectively). Error bars represent standard errors of the means (SEM), which were obtained from two to four independent experiments.

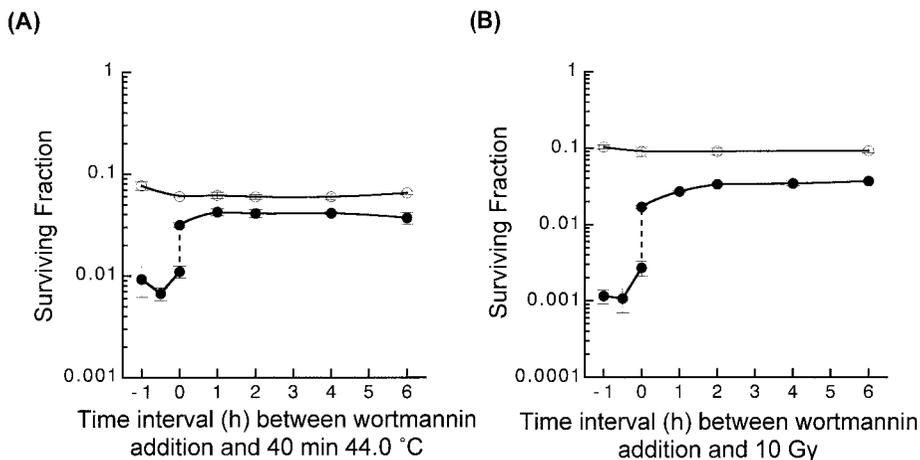


Fig. 2. Effect of wortmannin added at various timing. Wortmannin at 10 μM (●) or DMSO as control (○) was added to medium at various timings before or after 44.0°C-treatment for 40 min (A) or 10 Gy irradiation (B). Surviving fractions of cells were determined by colony formation assay and error bars represent standard errors of the means (SEM) of two or three independent experiments.

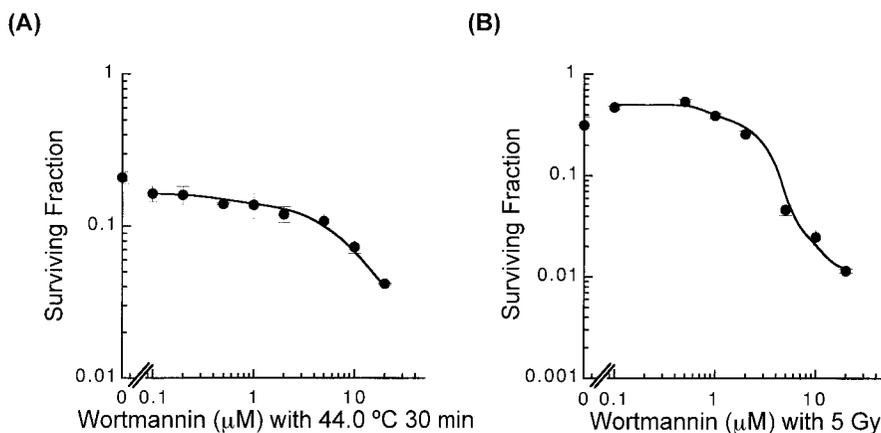


Fig. 3. Concentration dependency of wortmannin on the cellular sensitivity to heat treatment (A) or X-irradiation (B). Wortmannin was added at indicated final concentrations 60 min before heat treatment at 44.0°C for 30 min (A) or X-irradiation of 5 Gy (B). The surviving fractions were determined by colony formation assay and error bars represent standard errors of the means (SEM) obtained from two to five independent experiments.

monitored cellular viability by dye exclusion test (Fig. 4A). In the presence of wortmannin, the viability of the cells began to decrease shortly after 44.0°C – 40 min treatment to the level of 62% at 2 h after the treatment and was 18% at 48 h, while the viability reduced gradually to 64% at 48 h without wortmannin. The reduction of viability was slower in X-irradiation of 10 Gy than in 40 min heat treatment, but it enhanced by wortmannin, i.e., the viability reduced to 20% or 37% with or without wortmannin at 72 h (Fig. 4B). Wortmannin treatment

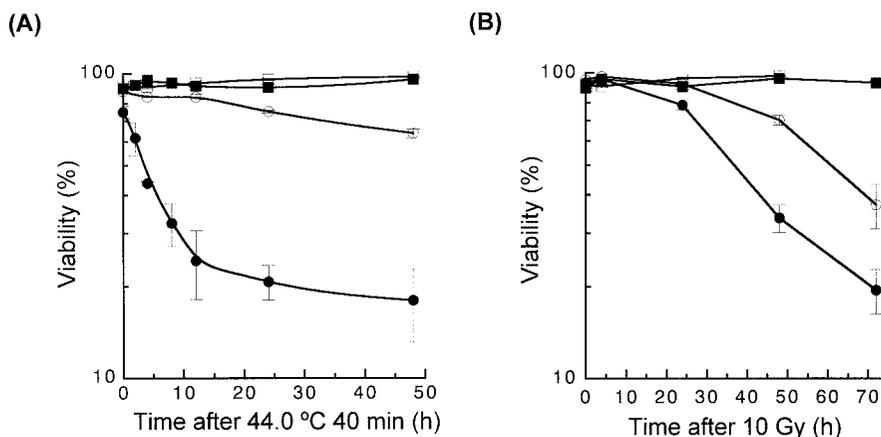


Fig. 4. The effect of prior addition of wortmannin on cellular viability after heat treatment (A) or X-irradiation (B). Viability was determined by dye exclusion test using Erythrosin B at various times after 40 min 44.0°C heat treatment (A) or X-irradiation of 10 Gy (B) with (●) or without (□) 10 μM wortmannin. Squares are for unheated or unirradiated controls. Circles are for heated or irradiated groups.

alone did not change the viability of unheated or unirradiated cells up to 72 h.

Wortmannin enhanced PARP cleavage and apoptotic cell death

PARP, poly (ADP-ribose) polymerase, is one of characteristic substrates of caspases activated during apoptosis^{18,19}. PARP fragment of 23 kDa (Δ PARP) visualized by western blotting was found and increased with post treatment time in 44.0°C – 60 min heated cells, but not in unheated cells and wortmannin enhanced heat-induced Δ PARP production, whereas

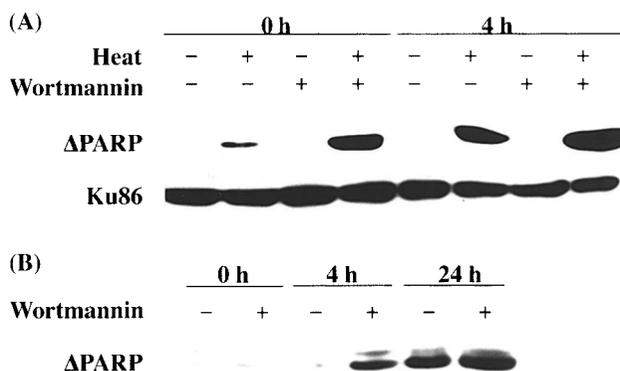


Fig. 5. Enhancement of PARP cleavage by wortmannin after either heat treatment (A) or X-irradiation (B). Cells were harvested at 0 or 4 h after 60 min heat treatment at 44.0°C (A) or at 0, 4 or 24 h after X-irradiation of 20 Gy (B) with or without prior addition of 10 μM wortmannin, and then subjected to western analysis using affinity purified polyclonal antibodies. The particular portions of western blotting profile for cleaved fragments of PARP (Δ PARP 23 kDa) as product of caspase actions and Ku86 as an unchanged marker after heat treatment are enlarged and separately shown.

Δ PARP was not detected in the cells treated with wortmannin alone (Fig. 5A). These results supported that wortmannin enhanced heat-induced apoptotic cell death. Ku86 in the same preparations is shown as a control (Fig. 5A). In the cells irradiated with 20 Gy X-rays, Δ PARP was detected at 4 and 24 h later with wortmannin and only 24 h later without wortmannin (Fig. 5B).

Wortmannin delayed heat-induced HSP70 expression

The induction of HSPs after heat treatment is considered as a possible mechanism that recovers and/or protects cells from heat damage²⁰. In V79 cells without wortmannin treatment, the induction of HSP70 was detected by the western analysis by 4 h after 44.0°C – 60 min treatment (Fig. 6). However in wortmannin treated cells, the induction of HSP70 was delayed and began to appear only 8 h after heat treatment. These results suggested that wortmannin treatment suppressed or delayed heat shock induction of HSP70.

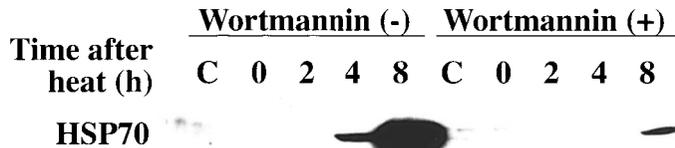


Fig. 6. The effect of wortmannin on HSP70 induction. Cells were harvested at 0, 2, 4 and 8 h after 60 min heat treatment at 44.0°C with or without 10 μ M wortmannin and subjected to western analysis using mouse monoclonal antibodies against HSP70. The particular portions for HSP70 bands are enlarged to show change with induction time. Induced bands at 4 or 8 h after heat treatment are clearly reduced in wortmannin added groups. C indicates unheated control.

DISCUSSION

Here we demonstrated that wortmannin sensitized V79 cells to heat treatment (Figs. 1 to 3) in enhancing cell death (Fig. 4A) and PARP cleavage (Fig. 5A). We also found that wortmannin suppressed and delayed HSP70 induction by heat (Fig. 6). Further, sensitization by wortmannin of radiation induced cell kill was at least partly mediated through enhancement of apoptotic cell death other than well established inhibition of DNA double-strand break repair.

We added wortmannin at various timings and found that prior or concomitant addition to heat was most effective compared with post addition (Fig. 2A). This was similar to the case of X-ray (Fig. 2B). We showed that heat sensitizing effect of wortmannin was apparent at 5 μ M or higher concentrations (Fig. 3A).

Wortmannin has been shown to inhibit various protein kinases, such as PI3-kinase, DNA-PK, ATM, ataxia-telangiectasia and rad3 related (ATR) and FRAP/mTOR *in vivo*^{21–23}. PI3-kinase is quite sensitive to wortmannin and is inhibited completely at low nanomolar concentration of wortmannin²¹. Akt/PKB was identified as an important kinase in survival

signaling pathways and was activated through PI3-kinase²⁴⁾ by various growth factors, such as platelet-derived growth factor (PDGF) and nerve growth factor (NGF). Recent studies suggested that Akt/PKB suppressed apoptosis possibly through phosphorylating Bad, Caspase-9, the Forkhead family of transcription factors, and the NF- κ B regulator IKK²⁴⁾. Although the present results (Fig. 3) may suggest presence of some effect at low wortmannin concentrations (0.1, 0.5 and 1 μ M), clear and significant effect of wortmannin at these low concentrations have not been well documented^{6-9,11,22)}. Thus PI3-kinase may be involved in the regulatory process of wortmannin at high concentrations rendering cells more susceptible to apoptosis though Akt/PKB. Although this underlying mechanism probably played a certain role, the major sensitizing effect in the present system was apparent (Fig. 3) only at much higher concentration, i.e., 5 μ M or more.

The concentration of wortmannin for effective heat sensitization (Fig. 3A) was similar to that for radiosensitization (Fig. 3B). At these concentration wortmannin inhibited DNA-PK and/or ATM, i.e., suppressed rejoining of DSB but not SSBs^{7,9)} and also disturbed accumulation of p53²⁵⁾ inducing radioresistant DNA synthesis¹⁰⁾. Considering high concentration of wortmannin required, the heat sensitizing effect might involve DNA-PK and/or ATM, although hyperthermia induces much less DNA DSBs than X-ray^{26,27)}.

ATM has been considered to phosphorylate p53 Ser15 and to upregulate or to increase p53 after DNA damage^{28,29)}. Ohnishi et al³⁰⁾ and Nitta et al³¹⁾ reported heat-induced p53-dependent p21/WAF1/CIP1 induction and G1 arrest. However, Raaphorst et al³²⁾ reported that the heat sensitivity of human AT cell lines was not significantly different from normal human cell lines.

On the other hand, Nueda et al¹⁵⁾ very recently reported that both DNA-PKcs-deficient *scid* and Ku86-deficient *xrs-6* cell lines were respectively more sensitive to heat-induced apoptotic cell death, accompanying release of histone-DNA complex to cytoplasm, than parental cell lines. They further suggested that delayed induction of HSP70 in *scid* cells as a possible reason. Also Ohnishi et al³³⁾ described similar results with *scid* fibroblasts, i.e., increased heat sensitization by colony formation assay, and disturbed p53 accumulation and HSP70 induction.

In the present study, we showed that wortmannin at high concentrations (5 μ M or more) enhanced heat- or radiation-induced apoptotic cell death accompanying PARP cleavage (Fig. 4, 5) and that wortmannin delayed HSP70 induction by heat (Fig. 6). Thus, our results and the other reports^{15,33)} suggest that DNA-PK, inhibitable only high concentrations of wortmannin, may have some significant roles in the suppression of heat- or radiation-induced apoptotic cell death and in the induction of HSP. V79 cells in the present study have mutated and non-functional p53^{16,34)} and heat induced cell death and PARP cleavage preceded HSP induction phenomenon. Therefore, the heat sensitizing effect was probably caused by enhanced apoptotic cell death and not by accompanying suppression or delay of HSP induction. It is also added that our previous study with the V79 cells¹⁶⁾ clearly shown sensitization of heat-induced apoptotic cell death by 2 – 50 μ M H7 an inhibitor of PKC. Further, regarding wortmannin and radiation, the present study have shown the presence of additional sensitization process through enhancement of apoptotic cell death other than classical inhibition of DNA breaks

and colony survival repair. As a conclusion of examinations of the results including other reports and the present study, an important role of DNA-PK is suggested in the sensitization process by wortmannin of heat- or radiation-induced apoptotic cell death although the exact mechanisms remains to be clarified.

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