J. RADIAT. RES., 44, 151–159 (2003) http://jrr.jstage.jst.go.jp

DNA-PK: the Major Target for Wortmannin-mediated Radiosensitization by the Inhibition of DSB Repair via NHEJ Pathway

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Wortmannin/DNA-PK/Chicken knockout cells/SCID cells/Radiosensitization/ATM.

The effect of wortmannin posttreatment was studied in cells derived from different species (hamster, mouse, chicken, and human) with normal and defective DNA-dependent protein kinase (DNA-PK) activity, cells with and without the ataxia telangiectasia (ATM) gene, and cells lacking other regulatory proteins involved in the DNA double-strand break (DSB) repair pathways. Clonogenic assays were used to obtain all results. Wortmannin radiosensitization was observed in Chinese hamster cells (V79-B310H , CHO-K1), mouse mammary carcinoma cells (SR-1), transformed human fibroblast (N2KYSV), chicken B lymphocyte wild-type cells (DT40), and chicken Rad54 knockout cells (Rad54$^{-/-}$). However, mouse mammary carcinoma cells (SX9) with defects in the DNA-PK and chicken DNA-PK catalytic subunit (DNA-PKcs) knockout cells (DNA-PKcs$^{-/-/-}$) failed to exhibit wortmannin radiosensitization. On the other hand, SCID mouse cells (SC3VA2) exposed to wortmannin exhibited significant increases in radiosensitivity, possibly because of some residual function of DNA-PKcs. Moreover, the transformed human cells derived from AT patients (AT2KYSV) and chicken ATM knockout cells (ATM$^{-/-}$) showed pronounced wortmannin radiosensitization. These studies demonstrate confirm that the mechanism underlying wortmannin radiosensitization is the inhibition of DNA-PK, but not of ATM, thereby resulting in the inhibition of DSB repair via nonhomologous endjoining (NHEJ).

INTRODUCTION

Radiotherapy is one of the major therapeutic tools against various types of cancers. Although many tumors could be controlled by radiation, some have shown to be radioresisitant. Efforts have been made to understand the molecular basis that makes the cells radioresistant or radiosensitive. This knowledge will certainly be needed for the discovery of new agents or to modify the structure of existing molecules to improve radiation therapy by enhancing radiosensitivity.

Radiation-induced DNA double-strand breaks (DSBs) are the key type of lesion responsible for cell death[1,2], and cells deficient in DSB repair are killed at a higher rate. This has been clearly shown by the studies of radiosensitive DSB repair–deficient mutants, cells from SCID mice and several other cell lines[3–9]. Basically, two major DSB repair pathways, homologous recombination (HR) and nonhomologous endjoining (NHEJ), are conserved in yeast and mammalian systems for rescuing cells from DNA damage. Therefore any agent that can inhibit the repair of DSB could be a potential radiosensitizer.

Wortmannin, a fungal metabolite, has been shown to have potent inhibitory effects on phoshatidylinositol 3-kinase, an important enzyme involved in intracellular signaling[9–11]. The radiosensitizing effect of wortmannin has been reported recently against various normal and transformed cells of human and murine origin[7,8,12–16]. Although the mechanistic aspect underlying wortmannin radiosensitization is not yet understood completely, it has been attributed to the irreversible inactivation of certain members of the phosphatidylinositol 3-kinase (PI3K) related family. Based on their roles in DNA damage responses, several PI3K, DNA-dependent protein kinase (DNA-PK), ataxia telangietacia mutated (ATM) and the ataxia- and Rad3-related proteins (ATR) have been considered to be potential targets for the radiosensitizing effect of wortmannin. DNA-PK plays a major role in DSB repair via NHEJ and also in V(D)J recombination[17–19]. It is well known that cells defective in any of

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the DNA-PK subunits (i.e., DNA-PKcs, Ku70, and Ku80) are highly sensitive to radiation because they cannot repair DSBs efficiently.

Cells derived from Ataxia telangietasia (AT) patients, an autosomal recessive disorder with such clinical symptoms as neurological disorders, immune system defects, and genetic instability[20] are also known to show innate hypersensitivity to radiation, though they show no obvious DSB repair deficiency. In 1995 it was confirmed that the ATM (ataxia telangietasia mutated) protein, which has shown sizable homology to PI3K kinase[20,22], was responsible for the AT phenotype[21]. However, a recent study on AT cells suggests that the AT phenotype is associated with the defective repair of a certain fraction of DSBs produced after irradiation[23]. Although the role of DNA-PK inhibition by wortmannin for radiosensitization is undoubtedly clear, contradicting results were reported regarding the ATM-mediated pathway. Recently, wortmannin radiosensitization by the inhibition of ATM and DNA-PK activity was also shown in cancer cells[15]. However, Rosenzweig *et al.*[14] demonstrated a partial wortmannin radiosensitization even in cells deficient in either DNA-PK (SCID cells) or ATM, indicating that more than one protein kinases might be involved in wortmannin radiosensitization. Therefore, although wortmannin radiosensitization might be mediated by the specific inhibition of DNA-PK mediated DSB repair via NHEJ, the possibility of the involvement of other kinases cannot be ruled out, and the knowledge accumulated thus far is not conclusive on this point.

Therefore to understand the various possibe mechanisms underlying wortmannin radiosensitization, we used various cells derived from different species (hamster, mouse, chicken and human) in the present study. Moreover, the use of chicken B-lymphocyte DT40 cells and their mutant cells with the distinctive defect in DSB repair pathways, either in HR or NHEJ, facilitated the understanding of wortmannin radiosensitization mediated through DSB repair in these cells[24,25].

MATERIALS AND METHODS

Cells and cell culture conditions

Chinese hamster (V79-B31OH, CHO-K1) cells were obtained from Dr. M. M. Elkind[26]. The SV40-transformed cell line (SC3VA2) was established from the primary SCID mouse lung fibroblasts[27]. SC3VA2 cells and RD13B2 cells (secondary radiation hybrid clone containing fragments of human chromosome 8) were kindly provided by Dr. K. Komatsu (Hiroshima University)[27]. The immortalized transformed SV40 human cell lines, N2KYSV and AT2KYSV, were skin fibroblasts from normal individuals and one ataxia telangiectasia individual (kindly provided by Dr. Y. Ejima of Kyoto University). All these cells described earlier were grown in α-MEM, supplemented with 10% fetal calf serum (FCS, Hyclone, London UK). The cell lines (SR-1 and SX9) were derived from mouse mammary carcinoma FM3A cells (kindly provided by Dr. K. Sato, National Institute of Radiological Sciences, Chiba, Japan). SR-1 cells were used as a wild-type control for X-ray–sensitive mutant cells (SX9)[28]. These cells were cultured in α-MEM supplemented with 10% fetal calf serum, 1% L-glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). All these cells were grown at 37°C in a 5% CO_2 incubator. Chicken B lymphocyte DT40 (wild-type) and its repair gene knockout cells [RAD54$^{-/-}$, KU70$^{-/-}$, KU70$^{-/-}$/RAD54$^{-/-}$[24,25], DNA-PKcs$^{-/-/-}$[29], and ATM$^{-/-}$[30]] were maintained as suspension cultures in α-MEM medium supplemented with mercaptoethanol (10 μM), penicillin (100 μg/ml), streptomycin (100 μg/ml), 10% fetal bovine serum (FBS, Hyclone, London, UK), and 1% chicken serum (Sigma, USA) in a humidified atmosphere of 5% CO_2 in air at 39.5°C.

Irradiation

The cells growing as a monolayer, Chinese hamster cells (V79-B310H and CHO-K1)[26], human cells (N2KYSV and AT2KYSV), and mouse cells (RD13B2 and SC3VA2) were assayed as previously described. In brief, after an overnight growth of initially single cells (attached in 60 mm culture dishes) to yield groups of cells of average multiplicity of about one to three, the cells were exposed to X rays[26]. On the other hand, chicken (DT40 and its repair gene knockout cells) and FM3A mouse cells (SR-1, SX9) were irradiated in single cell suspension. Irradiations were carried out with an X-ray machine (Softex Co., Tokyo, Japan) at a dose rate of 7.5 cGy/s, with a 2.0-mm Al filter, operating at 150 kVp and 20 mA. For radiosensitization studies, the X-ray doses that kill approximately 10^{-1} cells were selected from the dose response curve of various cells and given as a pretreatment to wortmannin. All irradiations were carried out at room temperature.

Wortmannin treatment and cell survival assay

Wortmannin (Sigma, USA) was dissolved in DMSO to get a stock concentration of 1mM and stored in aliquotes at −20°C that were protected from light. Working drug solutions were further prepared by dissolving the stock in 1% FCS in PBS. Appropriate controls were used with the experimental groups. Wortmannin treatment for attached cells was performed at various times immediately after irradiation. After different time intervals, the drug was removed by aspiration and washed twice with 1% FCS in PBS. After washing, about 7 ml of α-MEM fresh medium was added and incubated at 37°C inside a CO_2 incubator. The number of cells per dish was chosen to ensure that 50–150 colonies would survive a given treatment. The colonies were stained and counted 8–10 days after plating as described earlier, and the percentage survival was calculated relative to the number of colonies from the control[24,25]. The cell survival following irradiation was normalized to unirradiated controls exposed to the same concentration of DMSO or wortmannin.

In regard to nonattached cells, they were diluted to get appropriate cell counts (2×10^5 cells/ml), divided into various groups, and exposed to either X rays alone or underwent wortmannin posttreatment. Immediately after treatment, the drug was removed by centrifugation and washed twice with 1% FCS in

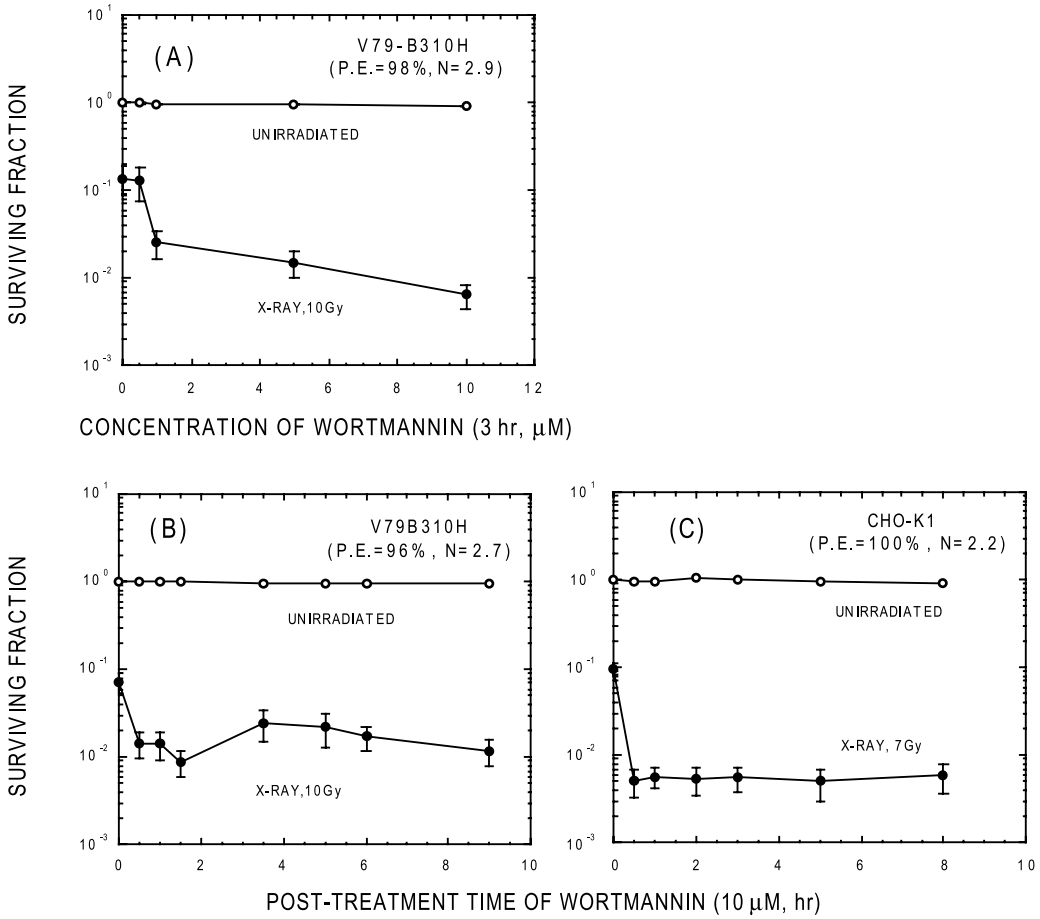

Fig. 1. The effects of various wortmannin concentrations (3 h) (A: V79-B310H) or the treatment time of wortmannin (10 μM) posttreatment (B: V79-B310H, C: CHO-K1) following an irradiation on Chinese hamster cells. The radiation dose for about 10^{-1}% survival was selected from the various dose response curves for the cells used (given inside respective graphs) and were used for combination with wortmannin. The X-ray dose was 10 Gy in A and B, but 7 Gy in C. The error bars indicate uncertainties. Symbols: unirradiated (open cirlces) and radiation + wortmannin (solid circles) treatments. Cell multiplicity at the time of exposure (N) ranged from 2.2–2.9.

PBS. Following centrifugation, a fresh medium was added and serially diluted, and the cells were plated in triplicate culture dishes with about 7 ml of 1.5% (w/v) methylcellulose (Aldrich, Milwaukee, WI) containing D-MEM/F-12 (Gibco-BRL), 5% chicken serum and 10 μM β-mercaptoethanol. The colonies were counted and analyzed as described earlier[24,25]. The plating efficiencies of the cells used in this study ranged from 20–100%. All the experiments were repeated 2 or 3 times to obtain consistent results.

RESULTS

The radiation dose response curves for all cells used in these studies were carried out, and the radiation doses for 10^{-1} survival were used for wortmannin radiosensitization. Wortmannin alone produced no cytotoxicity against the various cells used, as described in the materials and methods, even at the maximum concentration of 10 μM. At first we evaluated the cytotoxicity of increasing doses of wortmannin treatment per se in conjunction with the fixed dose of X ray (10 Gy) and fixed 3-h drug exposure. The radiosensitizing effect of wortmannin was clearly noticed in V79-B310H cells in drug-dose-dependent a manner (Fig. 1A). A wortmannin dose, 10 μM/ml, with maximum radiosensitization was used to study the effect of the duration of drug treatment on the radiosensitizing potential on those cells (Fig. 1B). The wortmannin posttreatment for 8 hours did not result in a time-dependent increase in the radiosensitizing effect in either the V79-B31OH or the CHO-K1 cells (Fig. 1, B and C), confirming a similar observation by Boulton *et al.*[31] of CHO-K1 cells. Therefore a 3-h posttreatment of wortmannin (10 μM) following radiation was used in further studies.

In the next experiment, we studied the wortmannin radiosensitization in repair-deficient cells. Figure 2 demonstrates a significant radiosensitization by wortmannin posttreatment in a transformed human fibroblast cell line, ATM-deficient AT2KYSV and its normal counterpart, N2KYSV (Fig. 2, A and

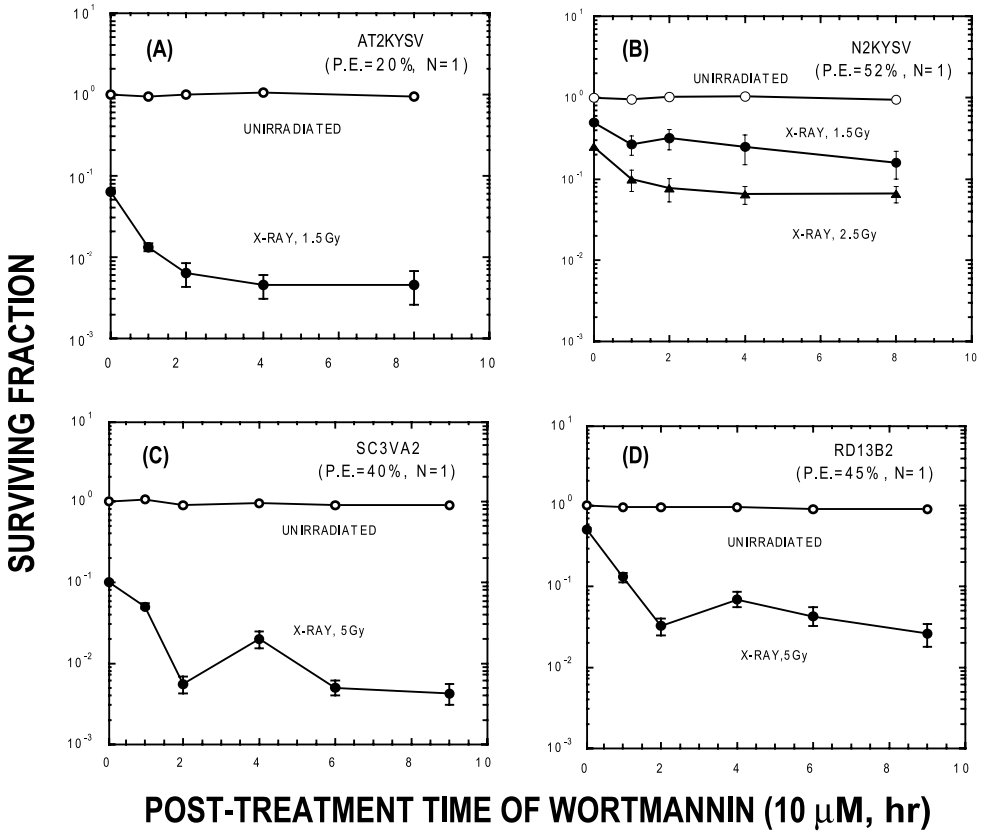

Fig. 2. The effect of wortmannin (10 μM/ml) posttreatment for various time intervals following irradiation. AT2KYSV (2A) and N2KYSV (2B) are transformed human fibroblast cell line; SC3VA2 (2C) is an SV40 transformed SCID mouse fibroblasts cell line; and RD13B2 (2D) is an SCID hybrid cell line complemented with human chromosome No. 8. The X-ray dose was 1.5 Gy (solid circles) or 2.5 Gy (solid triangles) in A and B, but 5 Gy in C and D. Symbols: unirradiated (open circles) and radiation + wortmannin (solid circles and solid triangles) treatments. The cell multiplicity at the time of exposure (N) was near one. Other details are as in Fig. 1.

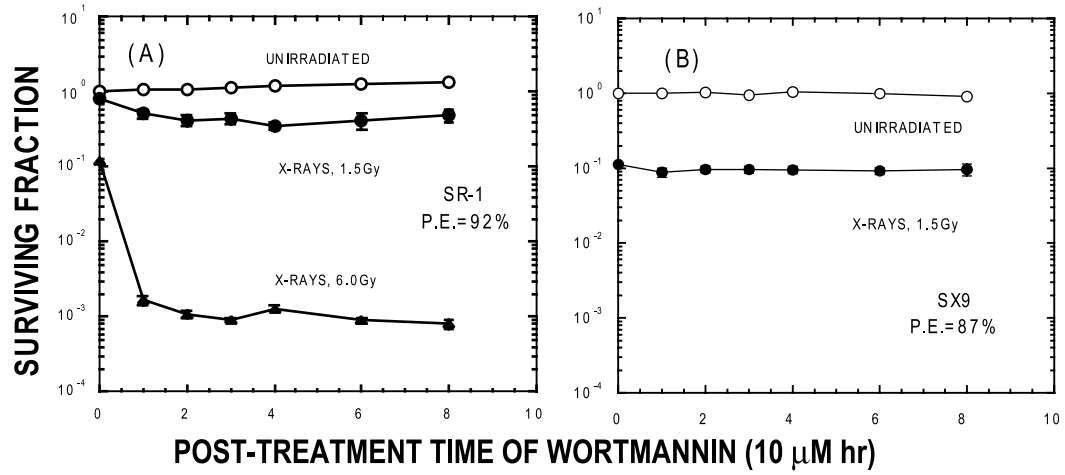

Fig. 3. The effect of wortmannin (10 μM /ml) posttreatment for various time intervals following an irradiation on cells derived from the mouse mammary carcinoma cell line. SR-1 (A) is control cells, and SX9 (B) is DNA-PK mutant cells derived from SR-1 cells. The X-ray dose was 1.5 Gy (soid circles) in A and B and 6 Gy (solid triangles) in A. Symbols: unirradiated (open circles) and radiation + wortmannin (solid circles and solid triangles) treatments. Other details are as in Fig. 1.

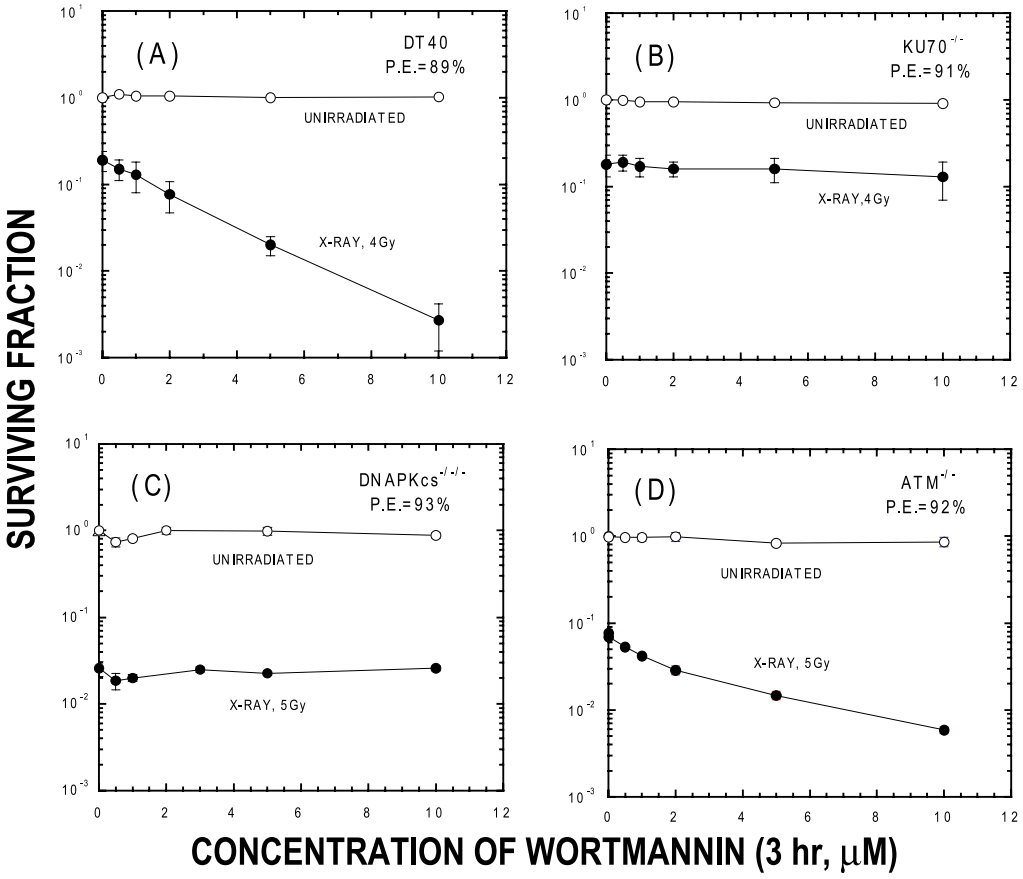

Fig. 4. The effect of various concentrations of wortmannin posttreatment to irradiation on parental DT40 chicken B-lymphocyte cells and its knockout cells. The X-ray dose was 4 Gy in A (DT40) and B ($KU70^{-/-}$) and 5 Gy in C ($DNAPKcs^{-/-/-}$) and D ($ATM^{-/-}$). Symbols: unirradiated (open circles) and radiation + wortmannin (solid circles) treatments. Other details are as in Fig. 1.

B). AT2KYSV cells showed a higher radiosensitivity and also a more pronounced radiosenstization in comparison to its normal counterpart (N2KYSV). Both SCID cell lines, RD13B2 (with a normal DNA-PK activity), which regained the ability to repair DSB because of the presence of fragments of human chromosome 8, and SV40 transformed SCID mouse lung fibroblast cell line, SC3VA2 (representing a cell line with a defective DNA-PKcs gene)[27], exhibited a pronounced wortmannin radiosensitization (Fig. 2, C and D). It may be noted that there was a significant increase in wortmannin sensitization in SC3VA2 and RD13B2 cells because of the length of the drug treatment (Fig. 2, C and D).

In another experiment (Fig. 3), we used SX9 cells, derived from a mouse mammary carcinoma cell line (FM3A), which has shown to be hyperradiosensitive with a defect in DNA-PKcs[28]. SR-1 cells served as a control for these cells. SX9 cells exhibited higher radiosensitivity, as indicated by a 10^{-1} radiation survival dose of 1.5 Gy in comparison with its normal counterpart, SR-1 (5 Gy). Although wortmannin posttreatment could result in a significant radiosensitization in the control SR-1 cells with a normal DNA-PK activity (Fig. 3A), a similar effect was not observed in SX9 cells (Fig. 3B)[28].

Recently, DT40 chicken B lymphocytes and their repair gene knockout cells are successfully used as a good model to study the role of different regulatory proteins in DSB repair by the HR/NHEJ pathway[24,25]. We used parental DT40 cells and mutant $RAD54^{-/-}$, $KU70^{-/-}$, $KU70^{-/-}RAD54^{-/-}$, $DNA\text{-}PKcs^{-/-}$[29], and $ATM^{-/-}$[30] cells to study the role of wortmannin radiosensitization mediated through DSB repair. Figures 4 and 5 summarize the radiation effect of chicken cells in combination with wortmannin. The concentration-dependent-increase in the radiosensitizing effect of wortmannin was seen in mutant $ATM^{-/-}$ cells (Fig. 4D) and in parental DT40 cells (Fig. 4A). The results of chicken mutant $ATM^{-/-}$ cells (Fig. 4D) essentially were similar to those of human ATM-deficient AT2KYSV cells (Fig. 2A). However, radiosensitization was not seen in chicken mutant $Ku70^{-/-}$ (Fig. 4B). As expected, chicken mutant $DNA\text{-}PKcs^{-/-}$ cells showed no radiosensitization by wortmannin posttreatment (Fig. 4C). Similar results in parental (Fig. 5A) and mutant $Ku70^{-/-}$ (Fig. 5B) were also reflected when wortmannin treatment at 10 μM was given immediately after irradiation at various times. Chicken mutant $RAD54^{-/-}$ cells deficient in the HR repair of DSB and hypersensitive to radiation also exhibited wortmannin radiosensitization (Fig. 5C). However, wortmannin gave no sensitizing

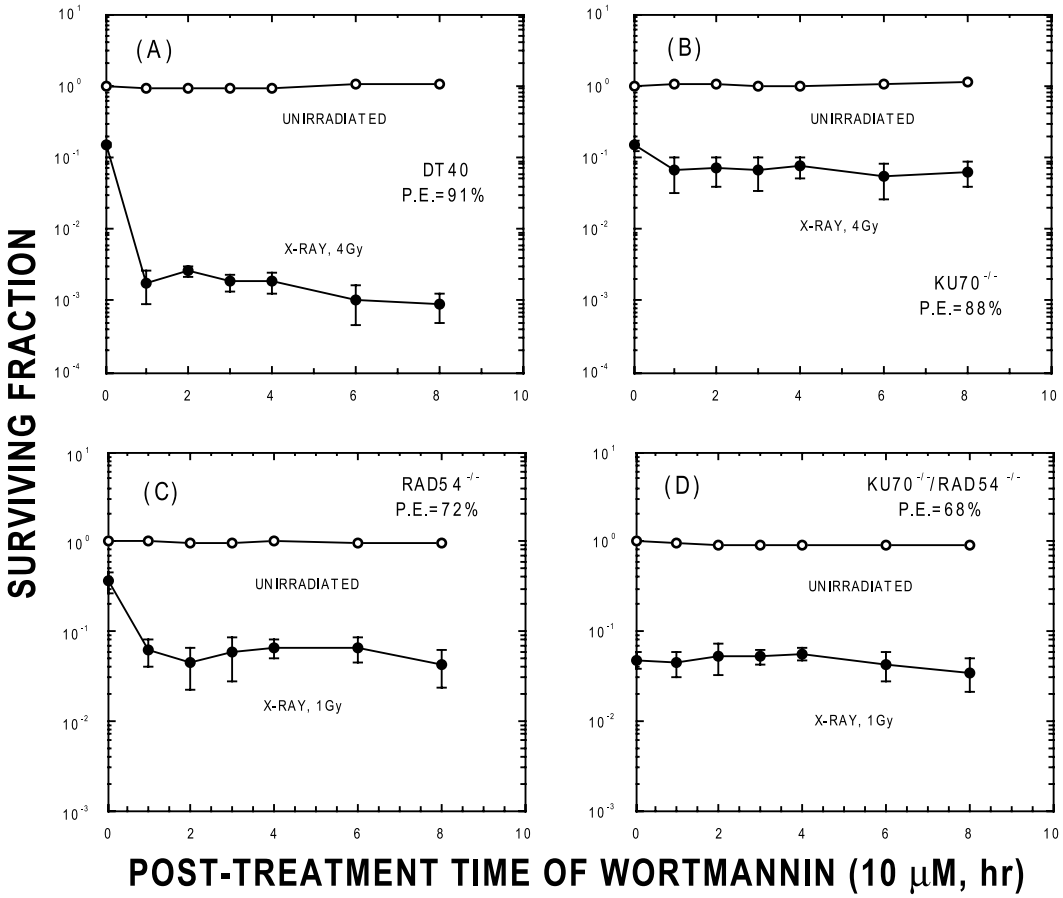

Fig. 5. The effects of wortmannin (10 μM/ml) posttreatment for various time intervals following an irradiation of parental DT40 chicken B-lymphocyte cells and its knockout cells. The X-ray dose was 4 Gy in A (DT40) and B (KU70$^{-/-}$) and 1 Gy in C (RAD54$^{-/-}$) and D (KU70$^{-/-}$/RAD54$^{-/-}$). Symbols: unirradiated (open circles) and radiation + wortmannin (solid circles) treatments. Other details are as in Fig. 1.

effect on double-mutant KU70$^{-/-}$/RAD54$^{-/-}$ cells (Fig. 5D), the most radiosensitive cell line among DT40 mutant cells used in this study. In mutant RAD54$^{-/-}$ and parental DT40 cells, radiosensitizing effects by wortmannin were not increased with a treatment time similar to those of different species [Chinese hamster (Fig. 1, B and C), mouse (Fig. 2, C and D, and Fig. 3A) and human (Figs. 2, A and B)].

DISCUSSION

Our present experimental observations of Chinese hamster (Fig. 1), mouse (Figs. 2, C and D, and 3A), human (Fig. 2, A and B), and chicken (Figs. 4, A and D, and 5, A and C) cells corroborates several earlier studies demonstrating wortmannin as an efficient radiosensitizer against Chinese hamster, mouse, human normal and cancerous cells[7,8,12,31]. It is clear from the earlier reports that wortmannin radiosensitization depends on various factors, such as growth medium supplements[8], phases of the cell cycle, cell types, and drug concentrations[7]. In most of these studies, wortmannin was given as a pretreatment to irradiation. However, a drug generally disturbs the cell cycle and cell metabolism. To undo this side effect and to examine a DSB repair inhibitory effect of wortmannin, our studies looked into the efficacy of wortmannin radiosensitization by adding it immediately after irradiation.

The effects of wortmannin on the kinetics of sensitization were evaluated over an 8-h time course. However, wortmannin (10 μM/ml) sensitized only within a 3-hour period (Figs. 1, B and C, 2, 3A, and 5, A and C), suggesting that wortmannin mainly inhibits a fast component of DSB repair[32].

In SCID cells, it is already known that the DNA-PKcs is not functional[33]. Therefore the loss of DNA-PK activity may account for the increased sensitivity to radiation and the inability to repair DSBs in SCID cells[18,33,34]. Cell lines defective in DNA-PK mediated wortmannin-related radiosensitization had little or no effect on SCID, irs20, xrs-6 or XR-1 cells, as reported recently[8]. Contrary to the later findings, however, the SV40-transformed SCID (SC3VA2) cell line showed the radiation sensitization by wortmannin (Fig. 2C). The discrepancy in the sensitizing effect of wortmannin may have resulted from differences in treatment, since Chernikova et al. treated it before irradiation but we applied treatment after irradiation. Furthermore, in con-

trast to SC3VA2, the SX9 cells were not further radiosensitized by wortmannin (Fig. 3B). In murine SCID cells, only the V(D)J coding joint formation is defective[34], but murine SX9 cells have been found to be defective in coding and in signal joint formation[28]. Although the role of DNA-PKcs in V(D)J signal joint formation is still in controversy, the difference in the radiosensitizing effect of wortmannin might provide compelling evidence that DNA-PKcs is required not only for its well-documented in role V(D)J coding joint formation, but it is also necessary for V(D)J signal joint formation. It might be presumed that the DNA-PK defect in murine SCID was only partial, though this remains to be proved experimentally.

It is presumed that mammalian cells, unlike yeast cells, repair DSBs mainly *via* the NHEJ pathway. In the cycling of mammalian cells, however, a significant fraction of chromosomal DSBs are repaired via the conservative HR pathway[35]. It is also observed that both these HR and NHEJ pathways are competitive and complementary to each other[24,25]. Since DNA-PK mediate NHEJ is the major DSB repair pathway in G_1/early S phase cells of the cell cycle, HR is preferentially used in the late S/G_2 cells[6,19]. Boulten and co-workers[31] demonstrated a direct cause–effect relationship of the wortmannin inhibition of DSB repair with a subsequent enhancement of radiation-induced cytotoxicity. In our studies, the parental DT40 chicken cells exhibited a significant radiosensitization (Figs. 4A and 5A). Earlier studies[25,29] in these cells demonstrated that Ku can interfere with HR-mediated DSB repair, perhaps competing with HR for DSB recognition. With the normal DNA-PK activity, it may be reasoned that the observed radiosensitization in parental DT40 cells is mainly because of the inhibition of DNA-PK. A similar explanation could also be applied to mutant RAD54$^{-/-}$ cells (Fig. 5C) because they also have normal DNA-PK activity, though they are deficient in DSB repair via the HR pathway. These findings, like earlier reports using various other cell lines, further confirm that the radiosensitizing effect of wortmannin is mainly by the inhibition of the DSB via the NHEJ pathway. Moreover, this view is further substantiated with the observation that KU70$^{-/-}$ mutant cells (Figs. 4B and 5B) and RAD54$^{-/-}$/KU70$^{-/-}$ double-mutant cells (Fig. 5D) did not exhibit radiosensitization by wortmannin. These cells are deficient in Ku70 protein, which is one component of the DNA-PK complex, the binding of which is essential for the activation of DNA-PKcs-mediated NHEJ-type DSB repair. It is possible that even though there is wortmannin-mediated inhibition of DNA-PK in these cells, the lack of Ku protein itself might have also contributed to the observed effect. The crude extracts of either Ku70- or Ku80-deficient cells[36] and SCID cells[17] showed no biochemically detectable levels of DNA-PK activity. Therefore it may be argued that the lack of DNA-PK activity itself in Ku deficient cells might have contributed to the absence of wortmannin-mediated radiosensitization. Further studies will be needed to confirm this possibility.

There was a good correlation between the inhibition of DNA-PK activity and the increased radiosensitivity in various cells[7,10,37]. To elucidate the importance of DNA-PK and ATM in wortmannin radiosensitization, we used chicken parental DT40 and mutant cells (DNA-PKcs$^{-/-}$ and ATM$^{-/-}$) defective in each of these proteins. Although a significant wortmannin radiosensitization was observed in the parental DT40 (Figs. 4A and 5A), the chicken DNA-PKcs$^{-/-}$ cells failed to show a similar response (Fig. 4C). However, chicken ATM$^{-/-}$ cells (Fig. 4D) exhibited wortmannin sensitization as in parental cells. Therefore it is clear that ATM protein has no role in wortmannin radiosensitization. The same conclusions were drawn in a study using human DNA-PK deficient cells (M059J) and other AT cells(AT3BISV and ATBIVA)[8,32,38].

It is well known that the p53 gene in V79 cells is mutated, and therefore it has a nonfunctional p53 protein[39]. Furthermore, it may be noted that AT cells are extremely sensitive to ionizing radiation and are also known to be deficient in the p53 DNA damage-response pathway[40,41]. The lack of p53 expression in DT40 cells[42] rules out the role of p53 in wortmannin radiosensitization because the parental and mutant ATM$^{-/-}$ cells exhibited a remarkable radiosensitization by wortmannin.

To conclude, the present findings with the earlier reports clearly demonstrate wortmannin as a good radiosensitizer, which by itself has shown little or no cytotoxicity against murine or human normal and cancerous cells. Therefore it may be an ideal candidate for use in the combined treatment approach against human cancers in combination with radiotherapy. Even if it is argued that its application in clinics is of slight possibility because it does not show its effect throughout the cell cycle and also because the use is restricted mainly to cells undertaking DSB repair mediated via DNA-PK, the elucidation of molecular mechanisms underlying wortmannin radiosensitization may definitely be beneficial to the design of better radiosensitizers.

ACKNOWLEDGEMENTS

We thank Dr. K. Komatsu (Hiroshima University), Dr. Y. Ejima (Kyoto University), and Drs. K. Tatsumi and M. Abe (National Institute of Radiological Sciences) for providing cells. This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. The results reported are the responsibility of the authors and do not represent the official views of the Ministry.

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Received on February 2, 2003
1st Revision on April 1, 2003
2nd Revision on April 19, 2003
Accepted on April 21, 2003