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Enhancement of radiation induced cell death in chicken B lymphocytes by withaferin A

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Withaferin A (WA), a plant withanolide, has shown significant radiosensitizing effect *in vitro* and *in vivo*. Inhibition of DNA repair has been suggested as a mechanism of radiosensitization by WA. To test this, the effect of withaferin A on survival of DT40 chicken B-lymphocyte cell line and its repair deficient single gene mutants Rad54$^{-/-}$, Ku70$^{-/-}$ and double mutant Ku70$^{-/-}$ /Rad54$^{-/-}$ after irradiation was studied . Exponentially growing cells were treated for 1 hr with 5 μM WA and then exposed to different doses of X-rays. Cell survival was studied by clonogenic assay. WA significantly reduced survival of DT40, Ku70$^{-/-}$ and Ku70$^{-/-}$ /Rad54$^{-/-}$, but not Rad54$^{-/-}$ cells, suggesting that WA enhances radiosensitivity by interfering with homologous repair, the major pathway of DSB repair in these cells. Inhibition of DNA repair is further indicated in a significant decrease in surviving fraction of DT40 cells by post-irradiation incubation with WA. This could have relevance to cancer radiotherapy.

Keywords: Chicken B-lymphocytes, DNA repair, Surviving fraction, X-radiation, Withaferin A

Withaferin A is a natural withanolide found in the roots and leaves of the Indian medicinal plant *Withania somnifera.* This compound has shown significant radiosensitizing effect on experimental mouse tumors *in vivo*[1-4] and on mammalian cells *in vitro*[5,6]. Based on *in vitro* study, inhibition of repair of radiation damage has been suggested[6] as a possible mechanism of its radiosensitzing action. This was tested by cell survival assay in cultured DT40 chicken B-lymphocytes and its mutants deficient in DNA repair genes.

Materials and Methods

Medium and chemicals—Modified Eagle's minimum essential medium (DMEM/F12) with L-glutamine and pyridoxine hydrochloride and without $NAHCO_3$ and Hepes buffer (GIBCO BRL, Japan), MEM alpha, with L-glutamine, $NAHCO_3$ and 25 mM Hepes, without RNA and DNA (Nikken Seibutsu Kenkyujo, Japan), bovine calf serum (BCS, Hyclone Laboratories, USA), chicken serum (Tissue Culture Biologicals, USA), methyl cellulose (Aldrich Chemicals, USA), phosphate buffer (PBS), L-glutamine (GIBCO BRL, Japan), 2-mercaptoethanol (Nakalai Tesque, Japan), were used.

Medium for clonogenic assay was prepared as follows: methylcellulose (7.5 g) and 216 ml double distilled water were autoclaved and mixed well by constant stirring. To this was added 216 ml DMEM/F12, 75 ml BCS, 7.5 ml chicken serum, 5 ml 200 mM L-glutamine and 500 μl of 50 mM 2-mercaptoethanol and mixed and incubated overnight at 4°C.

Withaferin A—Dried root powder of *Withania somnifera* was obtained from Vaipa Pharmaceuticals, Surat, India. Methanolic extract was prepared in a Soxhlet apparatus as described by Suffness and Douros[7] and withaferin A was isolated by chromatography[8]. The compound was identified by UV and NMR spectra. Withaferin A is not soluble in water. Stock solution was prepared by dissolving it in a few drops of absolute ethanol and then diluting with 10% dimethylsulfoxide (DMSO). Stock solutions of 1 and 10 mM concentrations were prepared and stored in the freezer. Working solutions were freshly prepared by diluting with MEM alpha to the appropriate micro molar concentrations before each experiment.

Cells—DT40: Wild type chicken B lymphocyte clones; Ku70$^{-/-}$:Deficient in non-homologous end

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joining (NHEJ) repair; Rad54$^{-/-}$:Deficient in homologous recombination (HR) repair; and Ku70$^{-/-}$/Rad54$^{-/-}$:Deficient in both NHEJ and HR repair.

The repair deficient clones were generated[9] and cells were grown in methylcellulose medium. Exponentially growing cells (24 hr old cultures) were used for the experiments.

Experimental design—The following experiments were performed:

Experiment 1: DT40 cells were incubated with 1.0, 2.5, 5.0 7.5 or 10 μM of WA for 1 hr at 39.5°C, then washed in PBS, resuspended and diluted to the required cell concentration in MEM alpha and plated in methyl cellulose medium in 60 mm culture dishes (Nunc) for clonogenic assay.

Experiment 2: DT40 cells were treated with 1 μM or 5 μM of WA for 1-5 hr and then plated in methylcellulose for clonogenic assay.

Experiment 3: DT40, Ku70$^{-/-}$, Rad54$^{-/-}$ and Ku70$^{-/-}$/Rad54$^{-/-}$ cells were incubated for 1 hr in 5 μM WA or vehicle, and then exposed to X-rays, as follows:

DT40: 0, 2, 4, 6, 8 and 10 Gy;

Rad54$^{-/-}$: 0, 1, 2, 3 and 5 Gy;

Ku70$^{-/-}$: 0, 0.5, 1, 3, 5, 10 and 14 Gy;

Ku70$^{-/-}$/Rad54$^{-/-}$: 0, 0.4, 0.8, 1.2 and 1.6 Gy.

The dose rate for Ku 70$^{-/-}$/Rad54$^{-/-}$ was 1.8 cGy/sec, while for all other cell lines the dose rate was 6.62 cGy/sec. The cells were then washed and plated for clonogenic assay.

Experiment 4: If WA is inhibiting the DNA repair, then it should be able to increase the cell killing effect of radiation even when treated after irradiation. To test this, DT40 cells were exposed to 8Gy of X-rays, immediately incubated for 1hr with different concentrations of WA, then washed and plated for clonogenic assay.

Survival curves—After incubation for sufficient time to develop visible colonies of 50 or more cells (5-6 days for DT40, Rad54$^{-/-}$ and Ku70$^{-/-}$, 9-10 days for Ku70$^{-/-}$/Rad54$^{-/-}$), the colonies were counted under a stereomicroscope. In all experiments the plating efficiency (PE) was calculated as the ratio of the number of colonies to the number of cells plated and surviving fraction was calculated as the ratio of the PE in the treated to the PE in the sham-irradiated control, using Microsoft Excel software. The survival curves were plotted with log of surviving fraction against radiation dose on linear scale, using Origin 6 software. Statistical analysis was done by the Student's *t* test and P value of ≤0.05 was taken as significant. WA treated groups were compared with the respective radiation alone treated groups.

Statistical analysis—The surviving fractions were expressed as mean±SD of 3 experiments. Statistical comparison between the different treatments for each cell line was done by the Student's *t* test.

Results

Experiment 1—Incubation for 1 hr at concentrations below 10 μM did not produce any significant decrease in DT40 survival. Increasing WA concentration to 10 μM resulted in a decrease in the surviving fraction (Fig. 1)

Experiment 2—Incubation at 1 μM for 5 hr did not have any toxic effect on DT 40 cells. Incubation at 5 μM for 1-2 hr did not have any effect on survival, while incubation for 3 hr produced a non-significant decrease in the surviving fraction. Increasing the incubation period to 5 hr in 5 μM WA significantly reduced the survival (Fig. 2).

Based on the above results, 5 μM for 1 hr was selected for further studies.

Experiment 3—The different cell lines showed different levels of radiosensitivity (Figs 3-6). The doses needed to reduce the surviving fraction to approximately 0.01 of control were as follows:

DT40 = 8 Gy; Rad54$^{-/-}$ = 4.5 Gy; Ku70$^{-/-}$ =14 Gy; Ku70$^{-/-}$/Rad54$^{-/-}$=1.5 Gy. Incubation with 5 μM of WA for 1 hr before irradiation resulted in different responses in these cell lines. While DT 40 (Fig. 3), Ku70$^{-/-}$ (Fig. 5) and the double mutant Ku70$^{-/-}$/Rad54$^{-/-}$ (Fig. 6) showed a significant decrease in surviving fraction compared to the respective radiation alone

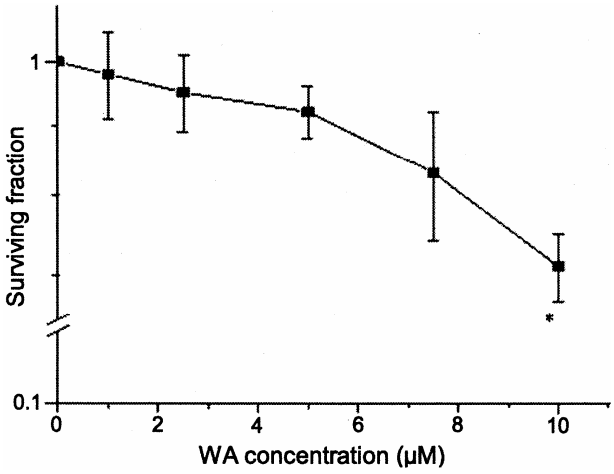

Fig. 1—Effect of increasing concentrations of withaferin A on survival of DT40 cells [*P<0.01, compared to untreated cells].

treated groups, survival of $Rad^{-/-}$ was not affected by WA treatment (Fig. 4).

$Ku70^{-/-}$ cells showed maximum sensitization by WA pretreatment. Radiation produced a steep fall in surviving fraction at the low doses up to 1 Gy, followed by a gradual decrease; the survival curve showed a shallow slope between 1 and 14 Gy. WA treatment produced a significant increase in cell death, the difference between radiation alone and radiation + WA groups increased at higher radiation doses (Fig. 5).

DT40 cells showed a linear dose-dependent decrease in surviving fraction after exposure to X-rays. WA treatment with 2 Gy produced a small effect on the radiation response. The enhancement in cell killing became significant at higher radiation doses (Fig. 3), but was less pronounced than that of $Ku70^{-/-}$ cells. The $Ku70^{-/-}/Rad54^{-/-}$ cells showed a similar response to radiation and WA + radiation as that of the wild type DT40, with a linear dose-dependent decrease in the surviving fraction, but the enhancement in radiation effect by WA was more pronounced than in the latter (Fig. 6).

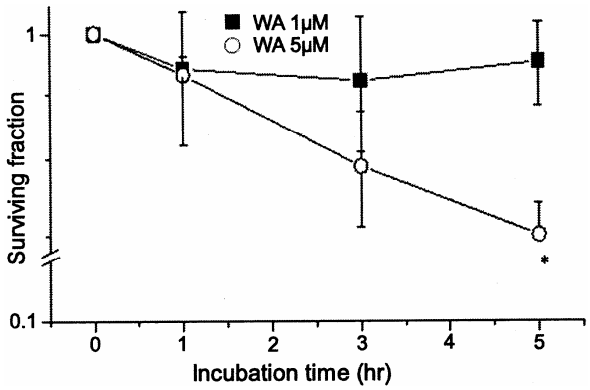

Fig. 2—Effect of incubation time in withaferin A on the survival of DT40 cells [* P<0.001, compared to 1 μM]

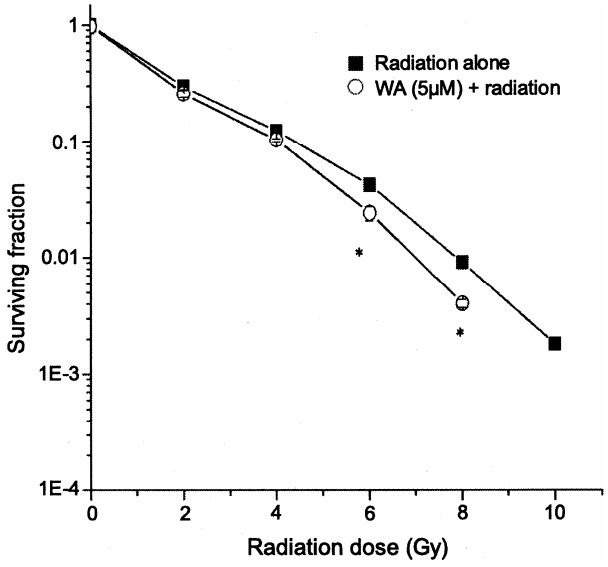

Fig. 3—Effect of withaferin A (5 μM) on the survival of DT40 cells exposed to X-rays [*P<0.001, compared to radiation alone].

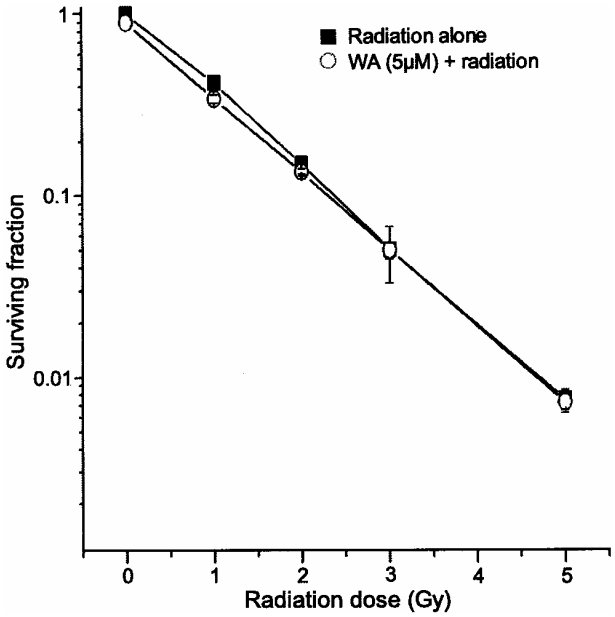

Fig. 4—Effect of withaferin A (5 μM) on the survival of HR deficient ($Rad^{-/-}$) cells exposed to X-rays. There was no significant difference in survival between radiation alone and WA + X-ray treated cells.

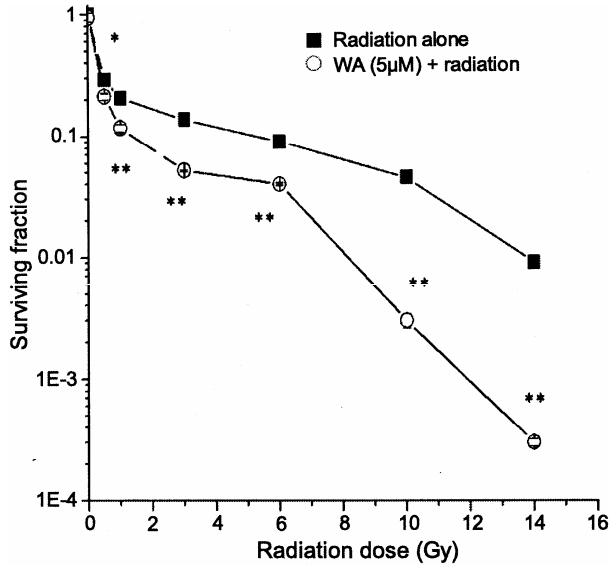

Fig. 5—Effect of withaferin A (5 μM) on the survival of NHEJ deficient ($Ku70^{-/-}$) cells exposed to X-rays [P values: * <0.01, ** <0.001, compared to radiation alone].

Experiment 4—Post-irradiation treatment for 1 hr with 2.5 μM of WA did not have any effect on the radiation lethality. But higher concentrations of WA produced a significant increase in the radiation effect, the surviving fraction decreasing to 0.008 at 5 μM to 0.0013 at 10 μM of WA (Fig. 7).

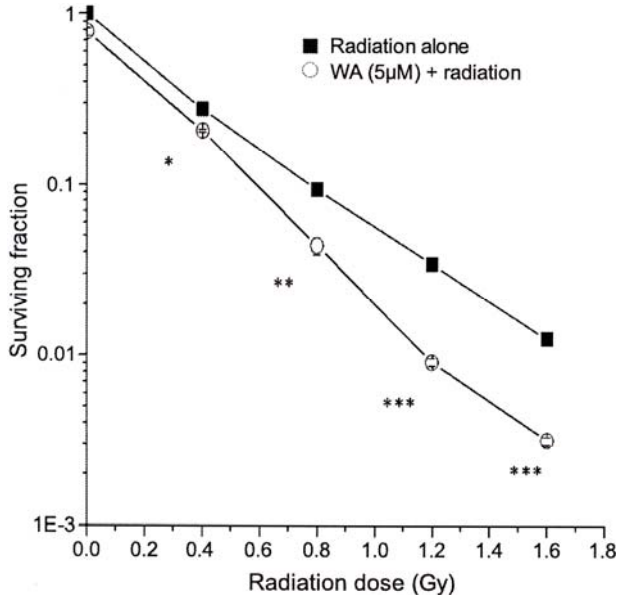

Fig. 6—Effect of withaferin A (5 μM) on the survival of $Ku70^{-/-}$ / $Rad^{-/-}$ double mutant cells exposed to X-rays [*P* values: *<0.05, **<0.01, ***<0.001, compared to radiation alone].

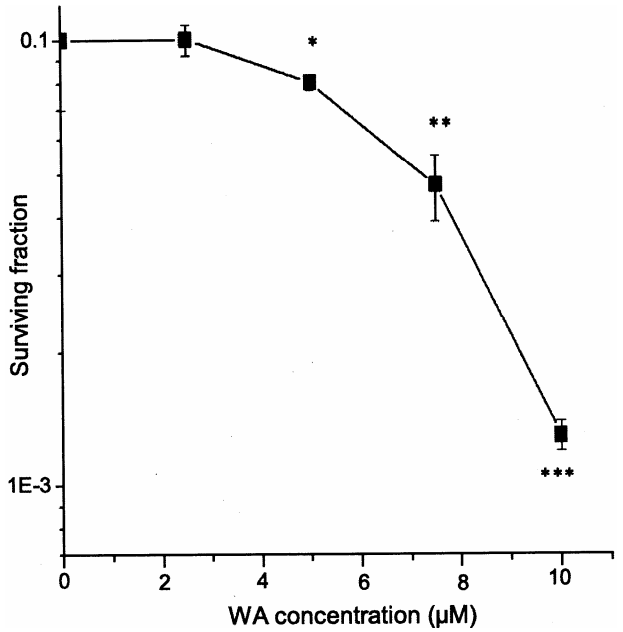

Fig. 7—Effect of post-irradiation treatment with withaferin A on survival of DT40 cells [*P* values: *<0.05, **<0.01, ***<0.001, compared to 8 Gy alone].

Discussion

The chicken B lymphocyte cell line DT40 has been proposed as a model to investigate the genetics of homologous recombination in vertebrates[10]. At least two repair pathways, the homologous recombination (HR) and non-homologous end-joining (NHEJ) are involved in the repair of DNA double strand breaks (DSB) in the eukaryotic cells. NHEJ using Ku70 plays a dominant role in the repair of X-ray induced DSB during G1/early S phase, while HR, using Rad54 is preferentially used in the late S/G2 phase[9]. HR is a more precise pathway controlled by the Rad52 epistatic group of genes, Rad51, Rad52 and Rad54 and requires the presence of homologous sequences in a homologous chromosome or in a sister chromatid[11]. The chicken lymphocyte cell lines used in the present investigation are suitable to study the effect of radiosensitizers on the DSB repair. Through split dose experiments using these cell lines, Utsumi *et al.*[12] have shown that sublethal damage (SLD) repair occurs through DSB repair by a Rad54-dependent HR pathway, and that SLD is primarily DSBs in homologous or sister DNA. In the present study, the treatment of these cells with withaferin A before and during irradiation increased the lethal effect of X-rays. There was significant decrease in the surviving fraction even when the wild type DT40 cells were first exposed to X-rays (8Gy) and then incubated with a non-cytotoxic concentration (5 μM) of WA, indicating that this compound possibly inhibits the repair of radiation damage in DNA. WA has been shown to induce apoptosis *in vivo* (unpublished) and *in vitro*[13]. Withaferin A is a potent inhibitor of protein kinase C[14]. Malik *et al.*[13] demonstrated that withaferin A primarily induces oxidative stress in human leukemia cells and in several other cancer cell lines by inhibiting protein kinase.

The finding that WA did not show any radiosensitizing effect on the cells deficient in HR ($Rad54^{-/-}$), while significant sensitization was seen in cells lacking NHEJ ($Ku70^{-/-}$), suggests that this compound may inhibit DNA repair by interfering with the HR pathway. Takata *et al.*[9] have shown that in the DT40 cells NHEJ plays a dominant role in repairing γ-radiation induced DSB during G1-early S-phase, while HR pathway is preferentially used in late S-G2 phase. They also showed that the two repair pathways are complementary. Adachi *et al.*[15] reported that topoisomerase I-mediated DNA damage is repaired primarily by HR. HR pathway has been suggested to

be more important for DSB repair in DT40 cells than in mammalian cells[9,16,17]. This could explain the lack of sensitization in the HR deficient ($RAD54^{-/-}$) cells observed in the present study. The Ku70-dependent NHEJ pathway or the Ku protein itself may act as a backup for SLD repair[12]. Takata *et al*.[9] reported that while disruption of Rad54 increased radiosensitivity of the chicken B lymphocytes, inactivation of Ku70, which encodes a component of the NHEJ pathway, had no detectable effect on survival after gamma irradiation. In the present study also the lethal effect of X-rays on $Ku70^{-/-}$ mutant cells was less pronounced than that on the Wild type cells. The double mutant cell line showed the maximum radiosensitivity to X-rays, as was also reported earlier[9].

In the repair efficient cells like the wild type DT40, where both the pathways are intact, the NHEJ pathway may be able to repair SLD and hence the sensitization by WA is not marked at the lower doses. At the higher doses, where HR pathway will have a more prominent role, the sensitizing effect of WA is also found to increase. This may account for the high increase in radiation lethality by WA of the $Ku70^{-/-}$ cells, which have only the HR repair pathway. Kaileh *et al.*[18] have reported that withaferin A potently inhibits NFκB activation by preventing TNF induced activation of 1 κB kinase beta via a thioalkylation sensitive redox mechanism. WA also enhanced the cytotoxic effect of radiation on the double mutant $Ku70^{-/-}/Rad54^{-/-}$, even though the sensitizing effect was less pronounced than on the $Ku70^{-/-}$ cells. This is difficult to explain as the double mutant lacks both repair pathways and needs to be investigated.

Conclusions

The plant withanolide, withaferin A is a good radiosensitizer of exponentially growing chicken B lymphocytes. Inhibition of repair of radiation induced DNA double strand breaks, possibly by interfering with the HR pathway, seems to have an important role in the radiosensitization by WA. If confirmed by further studies, it would have significant implication for tumor sensitization in cancer radiotherapy.

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