Glycolytic inhibitor, 2-deoxy-D-glucose, does not enhance radiation-induced apoptosis in mouse thymocytes and splenocytes in vitro

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Earlier studies have shown that 2-deoxy-D-glucose (2-DG), a glucose analogue and inhibitor of glycolytic ATP production selectively enhances radiation-induced damage in cancer cells by inhibiting the energy (ATP) dependent post-irradiation DNA and cellular repair processes. A reduction in radiation induced cytogenetic damage has been reported in normal cells viz., peripheral blood lymphocytes and bone marrow cells. Since induction of apoptosis plays a major role in determining the radiosensitivity of some most sensitive normal cells including splenocytes and thymocytes, we investigated the effects of 2-DG on radiation induced apoptosis in these cells in vitro. Thymocytes and splenocytes isolated from normal Swiss albino mouse were irradiated with Co\(^{60}\) gamma-rays and analyzed for apoptosis at various post-irradiation times. 2-DG added at the time of irradiation was present till the termination of cultures. A time dependent, spontaneous apoptosis was evident in both the cell systems, with nearly 40% of the cells undergoing apoptosis at 12 hr of incubation. The dose response of radiation-induced apoptosis was essentially similar in both the cell systems and was dependent on the incubation time. More than 70% of the splenocytes and 60% of the thymocytes were apoptotic by 12 hr following an absorbed dose of 2 Gy. Presence of 2-DG marginally reduced the fraction of splenocytes undergoing apoptosis at all absorbed doses, while no change was observed in thymocytes. Presence of 2-DG did not significantly alter either the level or the rate of induction of spontaneous apoptosis in both these cell systems. These results are consistent with the earlier findings on radiation-induced cytogenetic damage in human PBL in vitro and mouse bone marrow cells and lend further support to the proposition that 2-DG does not enhance radiation damage in normal cells, while radiosensitizing the tumors and hence is an ideal adjuvant in the radiotherapy of tumors.

Keywords: Apoptosis, 2-Deoxy-D-glucose, Flow cytometry, Splenocytes, Thymocytes

The success of conventional radiotherapy of tumors is limited by two main factors: (a) The adverse effects of radiation in normal tissues at higher therapeutic doses; and (b) the presence of hypoxic, intrinsically radioresistant and repair proficient sub-population of tumor cells\(^{1,2}\). Therefore, approaches directed towards selectively enhancing the manifestation of radiation damage in tumors while minimizing the damage to normal cells could improve radiotherapy of tumors\(^3\).

Ionizing radiation induces a number of DNA (and non-DNA) lesions\(^4\), causing alterations in the gene expression, cell proliferation and apoptosis\(^5,6\). The nature and extent of these lesions, the enzymatic processes involved in their repair or fixation, and the consequences of altered gene expression lead to cell death, which contributes to the loss of colonogenicity\(^7\). It has been demonstrated that the cellular processes leading to repair or fixation of radiation induced damage require continuous supply of metabolic energy which is provided mainly by the elevated levels of glycolysis in cancer cells, while respiratory metabolism accounts for most of the energy production in normal cells\(^8,9\). Based on the metabolic differences between normal and tumor cells and differences in the quantitative relationship between energy flow and repair/fixation processes, it has been suggested that inhibitors of glycolysis can selectively enhance radiation damage to cancer cells and therefore improve radiotherapy\(^10,11\). Subsequent studies have indeed shown that the glycolytic inhibitor 2-DG selectively enhances radiation-induced damage in cancer cells by inhibiting the energy (ATP) dependent post-irradiation DNA and cellular repair processes\(^12-18\). Most importantly a reduction in the radiation induced cytogenetic damage has been observed in normal cells (polymorpho-nuclear lymphocytes, bone marrow cells) possibly due to reduction in the fixation processes associated with cell death\(^18,20\). The constituents of hematopoietic system...
Materials and Methods

Isolation of thymocytes and splenocytes—Thymocytes and splenocytes were isolated from normal, 6-8 weeks old, female Swiss albino mice. The animals were dissected following cervical dislocation and spleen and thymus were removed under aseptic conditions in a laminar flow. The isolated tissues were washed in cold Hank’s balanced salt solution (HBSS) and minced into small pieces. These tissue pieces were gently crushed between two sterile, clean, frosted microscopic slides and single cell suspension was obtained. The suspension was washed twice with HBSS at 600 g; subsequently RBCs were lysed with RBC lysis solution and cells were resuspended in a known volume of medium, mixed thoroughly, and counted using haemocytometer. Approximately $2 \times 10^6$ cells ($\sim$100 µl) were resuspended in phosphate buffered saline (PBS) or RPMI 1640 containing 10% fetal calf serum (FCS).

Treatment procedure—Approximately $2 \times 10^6$ cells were seeded in a 60-mm Petri dish containing 4 ml of PBS or RPMI-1650 with 10% FCS. 2-Deoxy-D-glucose (Sigma Chemicals, USA) was added at a final concentration of 5 mM (equimolar with glucose) was present throughout the incubation period. The cells were irradiated at room temperature as a dose rate 1.82 Gy/min using a Co$^{60}$ gamma ray source (Eldorado 78, Teletherapy unit, AECL, Canada). Immediately after irradiation cells in PBS were transferred into RPMI-1650 with 10% FCS. Cells were incubated in a humidified 5% CO$_2$ incubator at 37°C and harvested at various time intervals. Harvested cells were fixed with either 80% chilled ethanol for flow cytometry or acetic acid:methanol (1:3) for fluorescence microscopy. Part of the cells was used for DNA electrophoresis.

Studies on apoptosis—Viability was examined flow cytometrically by staining unfixed live cells with propidium iodide (PI) based on the principle that dead cells retain the PI stain. Apoptosis was studied by analyzing the morphological features, DNA ladder formation and appearance of hypo-diploid (sub G1) population in flow cytometric measurements of DNA content.

Morphological studies—Morphologically, marked condensation and margination of chromatin, fragmentation of nuclei and cell shrinkage characterize apoptotic cells and a good correlation between these morphological changes and DNA ladder (one of the hallmarks of cells undergoing apoptosis) has been demonstrated$^{17}$. Slides containing acetic acid-methanol (1:3 v/v) fixed and 2-aminophenylindolehydrochloride (DAPI) stained cells were examined under fluorescence microscope using UV excitation filter and the fluorescing nuclei were visualized using a blue emission filter as described earlier$^{21}$. The percentage of apoptotic cells was calculated as—Apoptosis (%) = $N_A/N_T \times 100$; where $N_A$ is the number of apoptotic cells and $N_T$ is total number of cells analyzed.

DNA electrophoresis—Inter-nucleosomal cleavage of DNA, caused by the caspase activated DNase (CAD) is considered one of the hallmarks of apoptosis. Formation of DNA ladder was studied by electrophoresis carried out with 1.2% agarose gel as described earlier$^{25}$. Gels were stained with ethidium bromide (1 µg/ml) and examined using UV trans-illuminator.

DNA analysis by flowcytometry—In flow cytometric DNA analysis, the presence of hypodiploid (sub G0/G1) population is indicative of the apoptotic cell population. Flow cytometric measurements of cellular DNA content were performed with ethanol fixed cells using the intercalating DNA fluorochrome, propidium iodide (PI) as described earlier$^{25}$. Measurements were made with a laser based (488-nm) flow cyrometer (Facs Calibur; Beckton and Dickenson, USA) and data were acquired using the Cell Quest software (Beckton and Dickenson, USA).

Statistical analysis—Chi-square test was used to compare the percentages of cell death in different groups. $P$ value at <0.05 was considered to be significant.

Results

In the present study, we have investigated the effects of 2-DG on gamma ray induced apoptosis in mouse
spleenocytes and thymocytes in vitro by analyzing the morphological features, reduced DNA content and DNA ladder. Further, the viability of the cells was also examined by flow cytometry. After 6 hr of incubation, 70% of untreated thymocytes were viable, while 55% of the irradiated thymocytes were viable (absorbed dose: 2 Gy). No significant change in the viability was observed in cells treated with 2-DG alone or in combination with radiation (2 Gy; Fig. 1). Similar results were observed in spleenocytes (not shown).

Morphological analysis—Morphological examination of cells stained with DNA specific fluorochrome DAPI revealed nuclear features of thymocytes and spleenocytes undergoing apoptosis viz., marked condensation and margination of chromatin, fragmentation of nuclei and cell shrinkage. The induction of apoptosis was dependent, both on the absorbed dose and post-irradiation time in spleenocytes as well as thymocytes (Fig. 2a, b). The maximum apoptotic fraction was observed at 12 hr post-irradiation, with more than 70% of the spleenocytes and 60% of the thymocytes undergoing apoptosis by this time at an absorbed dose of 2 Gy. Presence of 2-DG marginally reduced the fraction of spleenocytes undergoing apoptosis at all absorbed doses (Fig., while no effect could be observed in thymocytes (Fig. 2a,b). Under these conditions, 2-DG did not enhance the level or the rate of induction of spontaneous apoptosis in both these cell systems (Fig. 2a, b) complementing the observations of flow cytometric DNA content measurement (Figs 4, 5).

![Diagram](image1)

**Fig. 1**—Viability assay thymocytes by flowcytometry. Propidium iodide stained live (unfixed) cells were analyzed by flow cytometry following PI staining after treatment with 2-DG alone or in combination with radiation (2 Gy).

![Diagram](image2)

**Fig. 2**—Effects of 2-DG on spontaneous and radiation induced apoptosis, studied by morphological examination in mouse (a) thymocytes; and (b) spleenocytes in vitro.

![Diagram](image3)

**Fig. 3**—DNA ladder showing inter-nucleosomal degradation of nuclear DNA in mouse thymocytes (1-4) and spleenocytes (5-8) observed after 2 hr of irradiation. (Lane 1 and 5 control; lane 2 and 6 2-DG; lane 3 and 7 Radiation (2Gy) and lane 4 and 8 2-DG+Radiation).
DNA gel electrophoresis—Electrophoresis of DNA from both thymocytes and splenocytes showed the characteristic DNA ladder, indicating the internucleosomal cleavage associated with spontaneous as well as radiation-induced apoptosis at all incubation times studied, viz., 2, 6 and 12 hr. A typical gel showing the DNA ladder observed in thymocytes and splenocytes at 6 hr following irradiation (absorbed dose: 2 Gy) has been shown in Figure 3. An increase in the intensity of bands at the lower end of the gel (representing mono-, di-, tri- and tetra-nucleosomes etc.) was evident with increasing time of incubation (not shown). No significant change in the pattern of the bands or band intensities could be observed in DNA isolated from unirradiated or irradiated cells treated with 2-DG.

Flow cytometric analysis of DNA content—Sample preparation used in the present studies clearly permitted the visualization of hypo-diploid peak (sub G0/G1 population) in DNA histogram obtained from unirradiated as well as irradiated thymocytes and splenocytes (Fig. 4a, b). The hypo-diploid fraction increased with the incubation time as well as the absorbed dose in both the cell systems (Fig. 4a, b). The apoptotic frequency observed in the present study was in agreement with the fraction of non-viable cells reported earlier for thymocytes under comparable incubation condition. The dose response of

![Relative DNA content](image)

Fig. 4—Flow cytometric DNA analysis showing the effects of 2-DG on radiation induced apoptosis in mouse (a) thymocytes; and (b) splenocytes in vitro.
radiation-induced apoptosis analyzed by flow cytometric DNA content was essentially similar in both the cell systems (Fig. 5a, b) and correlated well with the observations from morphological analysis (Fig. 2a, b). Presence of 2-DG had no significant effect on either the time course or extent of radiation induced apoptosis in thymocytes (Fig. 4a), whilst a small (=12%) decrease was observed in case of splenocytes irradiated with a dose of 2Gy at 6 hr (Fig. 4b). Since certain constituents of the complete media containing 10% FCS used for preparing the cell suspension can influence the radiomodifying effects of 2-DG, we also examined the effects of suspending the cells in PBS during irradiation followed by incubation in complete medium. Effects of 2-DG on the radiation induced apoptosis at 2 and 6 hr post-irradiation were similar to the cells irradiated in complete medium (Table 1).

Table 1—Effects of 2-DG on the radiation induced apoptosis of splenocytes irradiated in PBS and complete media.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell death (%)</th>
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<tbody>
<tr>
<td></td>
<td>Complete medium</td>
</tr>
<tr>
<td>None</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>2-DG</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>2Gy</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>2-DG+2Gy</td>
<td>52 ± 6</td>
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![Graph showing effects of 2-DG on cell death](image)

Fig. 5—Effects of 2-DG on spontaneous and radiation induced apoptosis analyzed from flow cytometric measurements of mouse (a) thymocytes; and (b) splenocytes in vitro.

Discussion

Adjuvant, which selectively enhance cytotoxicity in tumors and/or reduce normal tissue toxicity, can significantly enhance the efficacy of radiotherapy. Most of the currently used radio-sensitizers and radioprotectors are either toxic or do not show a differential effect between tumor and normal tissue at therapeutically effective doses. Therefore, potential radiomodifiers suggested as adjuvant for improving therapeutic efficacy need to be critically evaluated for their protective affects against radiation damage in normal tissues, besides the radiosensitizing effects on tumors.

Our earlier studies have shown that glucose analogue and glycolytic inhibitor 2-DG enhances radiation damage in tumor cells by inhibiting energy dependent repair processes. On the other hand, protection of normal cells such as peripheral blood lymphocytes (PBL) and bone marrow cells were observed under these conditions, presumably due to a reduction in the lesion fixation processes. Therefore, 2-DG offers a unique opportunity to enhance the therapeutic efficacy. The present results in two most radiosensitive normal tissues (belonging to hematopoietic system) complement our earlier observations on peripheral blood lymphocytes and bone marrow cells where a reduction in cytogenetic damage was observed. Mitotic death related to cytogenetic damage and apoptosis are the two important mechanisms of cell death induced by radiation, which determines the cell survival. Radiation induced DNA damage and ceramide release due to membrane damage are two important candidates for radiation-induced apoptosis. Absence of any significant increase in induction of apoptosis in presence of 2-DG suggests that metabolic changes induced under these conditions do not lead to the up-regulation of processes, particularly the phosphorylation linked events, that facilitate apoptosis.

2-DG has been shown to enhance radiation induced delayed apoptosis observed in human glioma cell lines carrying wild type p53 gene. Although the mechanisms involved in the alterations of radiation induced apoptosis by 2-DG are not clear at present, it is likely that many of the signal transduction processes downstream to DNA as well as membrane damage involving various kinases such as DNAPK, SAPK and JNK could be modified under conditions of energy limitation (ATP) in presence of 2-DG,
However, inhibition in GTP-dependent signal transduction processes cannot be ruled out, as significant fall in GTP levels induced by 2-DG has been reported in cells with high rates of glycolysis like the cancerous cells18,19.

It has been shown that proliferation of partially purified splenic lymphocytes following concanavalin-A stimulus was inhibited by 2-DG25. Further, in vivo administration of 2-DG has also been shown to induce dose-dependent changes in thymus and spleen cell distribution and function33,34. Mature T cells account for almost 50% of splenocytes, while 85% of thymocytes are immature T cells. Although, the mechanisms are not very clear, but a small but significant decrease in rate as well as the level of apoptosis induced by radiation observed in splenocytes in the presence of 2-DG (Figs 2, 4, 5) could be partly due to the differences in the mechanisms of regulation of apoptosis in mature (CD4+ or CD8+) and immature (CD4+CD8+) T cells35. Ionizing radiation has been shown to enhance CD95-mediated apoptosis; animals expressing wild-type p53 have been found to substantially up-regulate CD95 mRNA in the spleen and the modest up-regulation in the thymus, while no significant change has been noted in other tissues such as heart and liver31. Further, wild type thymocytes readily undergo apoptosis after treatment with ionizing radiation, while homozygous null p53 thymocytes are resistant to radiation-induced apoptosis31. From the results of the present study, it seems that 2-DG does not interfere with p53 dependent or CD95-mediated signalling pathways to enhance radiation or spontaneously induced cell death. Moreover, our recent studies on gene expression changes using microarray analysis show that there is no significant change in the expression of these genes following 2-DG treatment37. The absence of enhanced apoptosis in irradiated thymocytes and splenocytes also imply that immunocompetence may not be severely compromised following the administration of combined (radiation + 2-DG) therapy, which is an important determinant of systemic response. It is pertinent to note that in phase-I/II clinical studies no significant late radiation damage to the brain has been observed in glioblastoma patients following the administration of the combined therapy 2-DG+ radiation) involving whole brain as well as local irradiation36,38.

In summary, the present results clearly demonstrate that presence of 2-DG does not enhance radiosensitivity of thymocytes and splenocytes, the two important radiosensitive tissues that also contributes to the systemic response of radiation. Since radiosensitization of a number of tumor cells in vitro and in vivo by 2-DG has been well-established13,14,15, these results lend further support to the proposition that 2-DG may be an ideal adjuvant for improving the efficacy of radiotherapy.

References
11 Jain V, Pohlit W & Purohit S C, Influence of energy metabolism on the repair of X-ray damage in living cells. II. Effects of 2-deoxy-D-glucose in liquid holding reactivation in yeast, Biophysik, 10 (1973) 137.