Alterations in radiation induced cell cycle perturbations by 2-deoxy-D-glucose in human tumor cell lines

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In the present studies, effects of glucose analogue, 2-deoxy-D-glucose (2-DG) on radiation-induced cell cycle perturbations were investigated in human tumor cell lines. In unirradiated cells, the levels of cyclin B1 in G2 phase were significantly higher in both the glioma cell lines as compared to squamous carcinoma cells. Upon irradiation with Co60 gamma-rays (2 Gy), the cyclin B1 levels were reduced in U87 cells, while no significant changes could be observed in other cell lines, which correlated well with the transient G2 delay observed under these conditions by the BrdU pulse chase measurements. 2-DG (5 mM, 2 hr) induced accumulation of cells in the G2 phase and a time-dependent increase in the levels of cyclin B1 in both the glioma cell lines, while significant changes could not be observed in any of the squamous carcinoma cell lines. 2-DG enhanced the cyclin B1 level further in all the cell lines following irradiation, albeit to different extents. Interestingly, an increase in the unscheduled expression of B1 levels in G1 phase 48 hr after irradiation was observed in all the cell lines investigated. 2-DG also increased the levels of cyclin D1 at 24 hr in BMG-I cell line. These observations imply that 2-DG-induced alterations in the cell cycle progression are partly responsible for its radiomodifying effects.

Irradiation of eukaryotic cell results in a dose-related delayed progression through G1, S, and G2 phases of cell cycle1-2. These delays arise on account of the presence of complex cell surveillance mechanisms, referred to as checkpoints, that operate mainly in response to a variety of DNA damages, although non-DNA damages have also been implicated3. At low and moderately absorbed doses, G1 phase arrest occurs predominantly in cells with wild type p53 function while G2 phase delay occurs regardless of the p53 status4,5. The delay in G2 phase of the cell cycle presumably allows the repair and recovery processes leading to cell survival6-7, while lack or abolition of the delay has been correlated with enhanced cell death8,9. Studies have shown that yeast cells with a mutation in the rad 9 gene are highly sensitive to radiation and do not undergo a G2 delay, thus suggesting that duration of G2 delay plays a significant role in determining the radiosensitivity of cells in a dose-dependent manner9,10. The position of cells in cell cycle phases at the time of irradiation also markedly influences the extent of division delay with irradiation in G1 phase showing minimal delay, while irradiation in S and G2 is associated with progressively greater delays11. Studies on transformed cell lines predominantly show a G2 delay, even at low radiation doses, while delay in S phase has been observed at relatively high doses12,13. Although the mechanisms of these delays are not completely understood, the extent of the delay appears to be under genetic control13.

Mammalian cell cycle is regulated by a set of complex mechanisms involving several cyclins and cyclin-dependent kinases resulting in a cascade of phosphorylation-dephosphorylation of target proteins directly involved in DNA synthesis, chromosome segregation and mitotic divisions14. These Cyclin-CDK complexes are influenced by a number of regulatory proteins whose levels are regulated both by growth factor stimulation as well as damage levels through different kinases, p53, p21 and several other gene products15.

Residual DNA damage, the resultant of a competition between processes of repair and fixation of primary DNA lesions appears to be the primary cause of radiation-induced division delay15. Our earlier studies showed that the glucose analog,
Followed by 2-deoxy-D-glucose (2-DG), an inhibitor of glucose transport and glycolysis selectively inhibit the DNA repair and cellular recovery processes, resulting in an enhancement of the radiation damage in cells with high rates of glycolysis (like in cancer cells)\textsuperscript{16,21}.

Under these conditions a good correlation between inhibition of DNA repair, enhanced cytogenetic damage and cell death has been demonstrated in certain cell lines\textsuperscript{16,22}, while a lack of correlation has also been observed in few cases\textsuperscript{23}. Although alterations in cell cycle delay have been indicated in these studies, a clear understanding of the nature of delays as well as mechanisms involved, besides its role in the radiomodification have not emerged so far. Therefore, we have initiated systematic studies to investigate in detail, the cell cycle perturbations caused by 2-DG following irradiation by characterizing the nature of delays as well as alterations in some of the cell cycle regulatory proteins like cyclin B1, D1 etc. Results of these studies carried out in human glioma and squamous carcinoma cell lines are presented here.

Materials and Methods

Cell culture — Four human tumor cell lines, two gliomas (BMG-1, U87 wild type p53) and two squamous carcinomas (4197 wild type p53, 4451 mutated p53) were used in these studies. Cells were maintained in Dulbecco’s modified Eagles medium (DMEM) supplemented with either 5% fetal calf serum (BMG-1) or 10% fetal calf serum (4451 and U87) or 20% fetal calf serum (4197), HEPES and antibiotics as described earlier\textsuperscript{26}. All experiments were carried out in exponentially growing cells.

Treatment procedure — Cells were plated at a density of 6,000 to 8,000 cells/cm\textsuperscript{2} in petridishes. Twenty-four hours later, cells were washed with HBSS and incubated in HBSS with 5 mM 2-DG (equimolar conc. with glucose). Cells were irradiated immediately following 2-DG addition and held in HBSS for 2 hr following irradiation. Appropriate control groups (sham irradiated, 2-DG and radiation alone) were always included in the protocol. Irradiation was carried out at a dose rate of 1.4 to 2 Gy per min (2 Gy) at room temperature using a cobalt\textsuperscript{60} teletherapy unit (El dorado, Canada).

Cell growth and cell cycle distribution — Following different treatments as described above, cells were harvested at every 24 hr time interval. Both floating and attached cells were counted and fixed in 70%-chilled ethanol for the analysis of cell cycle distribution. Cell proliferation was calculated by computing the increase in cell number and an index of proliferation P, was calculated as: \( P = N_t/N_0 \)

where, \( N_t \) = number of cells at time t; \( N_0 \) = number of cells at the time of irradiation.

Flow cytometric measurements of cellular DNA content were performed with ethanol fixed cells using the intercalating DNA fluorochrome, propidium iodide (PI) as described earlier\textsuperscript{24}. Measurements were made with a LASER based (488 nm) flowcytometer (Facs Calibur; Becton Dickinson, (USA)), and data were analysed using Mod-Fit software (Becton Dickinson; USA).

Analysis of S-phase cells labeled with bromodeoxycytidine (BrdU) — Cells pulsed with 10 \( \mu \text{M} \) BrdU for 30 min were trypsinized, fixed in 80% chilled ethanol and stored at 4°C. Immunostaining was performed as described earlier\textsuperscript{26}. Briefly, cells were incubated with pepsin for 10 min to isolate nuclei, and treated with 2N HCl for 30 min to partly denature DNA. Subsequently they were labeled with mouse anti-BrdU antibody (1:40, Becton Dickenson), followed by FITC labeled anti-mouse IgG\textsubscript{2}, secondary antibody (1:100, Sigma Chemicals). The DNA was stained with propidium iodide (PI, 50 \( \mu \text{g/ml} \)). Green (FITC) and red (PI) fluorescence were recorded for 10,000 events using cell quest software (Becton Dickinson, USA). Data were analyzed by applying appropriate gates for labeled and unlabeled cells.

Measurement of cyclins — Immunostaining was performed as described earlier\textsuperscript{26}. Briefly, ethanol fixed cells was first permeabilized with 0.25% Triton X-100 in PBS for 5 min on ice. Cells were then washed with PBS and incubated with respective primary antibodies diluted (1:400) in PBS containing 1% BSA, (Phar megen, a Becton Dickinson Co.) overnight at 4°C. Cells were then washed with PBS containing 1% BSA and incubated with FITC labeled goat anti-mouse IgG\textsubscript{2} secondary antibody (Sigma chemicals) diluted 1:100 in PBS containing 1% BSA for 30 min at 4°C. After washing, cells were treated with 0.1% RNase and propidium iodide (PI, 10 \( \mu \text{g/ml} \)) for 30 min. Green (FITC) and red (PI) fluorescence were recorded for atleaset 10,000 events as described earlier. Cell cycle was also analyzed from the measurement of DNA content from PI fluorescence.

Results

Cell growth and cell cycle progression — A significant reduction in cell number was observed upon treatment with 2-DG in squamous cell
carcinomas (4197 and 4451). A further reduction was observed when cells were treated with 2-DG (5 mM, 2 hr) and gamma radiation (2 Gy). However, no significant effects were observed in the growth of BMG-1 and U87 cell lines (Fig. 1). Cell cycle analysis showed that both the duration and extent of G2 delay differ among these cell lines investigated (Fig. 3b). A predominant G2 delay observed at 4 hr was nearly equal in both the glioma cell lines (BMG-1 and U87) but higher in 4197 and 4451 cells (Fig. 3b).

**Pulse labeling and follow up studies**—Incorporation of BrdU by the DNA synthesizing cells serves as a good marker for studying the progression of cells through cell cycle. Pulse labeling (20 min) of BMG-1 cells with BrdU at different post irradiation time intervals showed a transient delay in G2 phase of the cell cycle (data not shown). When cells were chased by monitoring the label S phase cells at various time intervals, a similar distribution of cells was observed in unirradiated cells as in pulse labeling experiment with nearly 35% of the cells in S-phase. A significant delay in the cell cycle progression induced by 2-DG was evident particularly in irradiated cells (Fig. 2).

At 12 hr post-irradiation, nearly 41% of the labeled cells treated with 2-DG were still in G2 phase, while this value was 34% in untreated and 36% in gamma-ray irradiated cells. However, by 24 hr, the unirradiated cells appeared to have overcome the 2-DG-induced block, with only 20% of the labeled cells in G2 phase with a simultaneous increase in the fraction of labeled cells in G1 phase from 11% at 12 hr to 27% at 24 hr. Similar observations were also made in the irradiated cells treated with 2-DG, where a release of cells from G2 block was evident with nearly 10% of the labeled cells in G2 phase and a concomitant increase in the fraction of labeled cells in G1 phase from 7% at 12 hr to 35% at 24 hr.

**Cyclins**—Constitutive as well as treatment-induced changes in cyclin B1 levels were measured in all cell lines by immunoflowcytometry. The constitutive levels of cyclin B1 were higher in both the glioma cell lines compared to squamous carcinoma cell lines (Fig. 3a). However, treatment-induced alterations in the cyclin B1 levels varied considerably among the different cell lines. Upon irradiation (2 Gy), the cyclin B1 levels were reduced in U87 cells at 24 hr post-irradiation, while no significant change could be observed in other cell lines. However, in unirradiated cells, presence of 2-DG for 2 hr enhanced the level of cyclin B1 in all the cell lines investigated, which was elevated further in irradiated cells at 24 hr (Fig. 3a).

Since events responsible for disturbances in cell cycle progression are linked to the radiation-induced damage and competes with DNA repair processes immediately following the induction of damage, the effects of 2-DG on cell cycle kinetics were studied at short time intervals up to 2 hr following irradiation. Cyclin B1 levels were significantly higher at 4 hr but reduced subsequently by 12 hr following the treatment in BMG-1 cells (Fig. 4a), indicating a transient disturbance by this short exposure to 2-DG. The extent of this increase in cyclin B1 levels were more in both the glioma cells as compared to carcinoma cells to 2-DG treatment (data not shown). Although, the cyclin B1 levels were observed higher in G2 phase, where it plays a role in facilitating G2-M transition, interestingly a significant unscheduled expression was observed in the G1 phase in all the cell lines after 48 hr of treatment (Fig. 4a).
Since 2-DG was also found to induce disturbances in the progression of cells from G₁ to S phase, we investigated the levels of cyclin D₁ in BMG-1 cells under these conditions. 2-DG significantly increased cyclin D₁ levels in unirradiated and irradiated cells at 24 hr post treatment, while no significant change could be observed further at later time intervals (Fig. 5).

**Discussion**

Primary lesions (both DNA as well as non DNA) caused by radiation initiate a set of competitive cellular responses such as DNA repair, cell cycle perturbations, disturbances in signal transduction pathways and apoptosis, involving complex molecular processes driven by various kinases, phosphatases and polymerases. Some of which are highly regulated by flow of metabolic energy. Therefore, modification of cellular radiation response by metabolic inhibitors such as 2-DG (and others like 6AN, hematoporphyrin derivative) needs to be understood from their integrated effects on these processes. Residual DNA damage, resultant of a competition between DNA repair and damage fixation processes is one of the main determinants of cellular radiation response. Present studies indicate that 2-DG-induced alterations in the progression of cells through different phases of cell cycle occurs through DNA damage mediated checkpoint arrest, which vary among different cell types.

![Bivariate plots of DNA and anti-BrdU antibody levels showing the effects of 2-DG (5 mM, 2 hr) on radiation-induced cell cycle perturbations analyzed by chasing BrdU labeled exponentially growing BMG-1 cells.](image-url)
types. DNA damage has been implicated as the primary signal responsible for the operation of cell cycle checkpoints at G1/S and G2/M transition although, recent evidences indicate that non DNA damage could also play a role. Heterogeneity in the alterations in cell cycle progression observed here (Fig. 4 b) may be partly responsible for the observed differences in the survival by 2-DG and could arise on account of variations in the effects of 2-DG on processes of DNA repair, induction of p21-mediated apoptosis or DNA damage-dependent p53-mediated progression in cell cycle.

2-DG-induced energy linked differences in strand break rejoicing among human tumor cell lines have been reported. While no significant differences in the residual DNA damage were observed upon treatment with 2-DG in a squamous carcinoma cell line (4197), the residual DNA damage under similar conditions was significantly higher in both the glioma cell lines (BMG-1 and U87) and in another squamous carcinoma cell line (4451). Presence of 2-DG for 2 hr following irradiation resulted in a higher growth inhibition in 4197 cells than 4451 cells (Fig. 1) while,

Fig. 3 — Immuno-flow cytometric measurement showing effects of gamma rays (2 Gy) and 2-DG (5 mM, 2 hr) on cyclin B1 levels in exponentially growing human tumor cell lines, observed 24 hr after treatment. (a) Bivariate plots of cyclin B1 and relative DNA content; and (b) Content showing the treatment induced disturbances in the cell cycle progression.

Fig. 4 — Bivariate plots of cyclin B1 and DNA content showing kinetics of cyclin B1 levels observed upon treatment with gamma rays (2 Gy) and 2-DG (5 mM, 2 hr) in (a) exponentially growing BMG-1 cells. (b) Histogram plots of relative DNA contents showing treatment induced disturbances in cell cycle progression.
no significant inhibition of growth was observed in both the glioma cell lines (BMG-1 and U87). 2-DG (5 mM) induced delay in cell cycle progression was more pronounced in the G2 to M phase transition. While delay in the transition of G1 phase cells into the S-phase was evident, no correlation was observed in residual DNA damage and cell survival, therefore the role of DNA damage-dependent p53-mediated cell cycle progression was speculated. However, we observed that 4197, BMG-1 and U87 carrying wt p53 differ in their sensitivity to 2-DG treatment (Fig. 1). Thus, it appears that neither residual damage nor p53 status is sole determinant of 2-DG-induced cell cycle perturbations as reported earlier.4,29.

A direct effect of transient alterations in the metabolic disturbances caused by 2-DG leading to alterations in some of the cell cycle regulatory processes particularly involving the maintenance of appropriate cyclins is indicated by the present studies. Small, yet significant changes in the cyclin B1 and D1 levels observed in unirradiated BMG-1 cells, 24–72 hr after a short exposure (2 hr) to 2-DG is an indication of such a possibility (Fig. 4a). The transient increase in the levels of cyclin B1 at 4 hr in both unirradiated and irradiated cells treated with 2-DG correlates well with the delayed progression from G2 to M phase of cell cycle (Fig. 4b). Earlier studies have indeed shown a correlation between fall in ATP levels and accumulation of cells in G2 and G1 phases of the cell cycle and concentration-dependent growth inhibition.22,23. BrdU pulse chase experiments have clearly shown that the treatment of cells with 2-DG enhanced the radiation-induced G2 delay later at 4 hr (Fig. 2). It is interesting to note that in both squamous carcinoma cell lines where radiosensitization by 2-DG was not observed, the constitutive cyclin B1 levels were significantly lower as compared to the glioma cells (Fig. 3a). Therefore, 2-DG-induced radiosensitization in U87 and BMG-1 cells (both wt p53) appears to be partly related to the constitutive as well as treatment-induced alterations in cyclin B1 levels. One of the reasons could be the damage-dependent cyclin B1-mediated enhanced cell death through apoptosis, as has been reported in cells with higher level of cyclin B1. Indeed, the radiation-induced delayed apoptosis was higher in both glioma cell lines as compared to the squamous carcinoma cell lines. Since the role of cyclin D1 in radiosensitization has been indicated earlier, we investigated the changes in cyclin D1 levels in altered cell cycle progression upon treatment with 2-DG. Further, enhanced levels of cyclin D1 observed 24 hr after treatment with 2-DG alone or in combination with radiation (Fig. 5) indicates another pathway possibly modulated by 2-DG in the regulation of cell cycle progression. These responses could, however, vary among cell lines as shown here between the two glioma and squamous carcinoma cell lines, which could partly contribute to heterogeneity in the radiosensitization by, 2-DG.

Taken together, the results of the present studies suggest that presence of 2-DG for few hours following irradiation significantly alters the progression of cells through the cell cycle, which may be partly responsible for 2-DG-induced alterations in cellular response observed earlier.32,33. Quantitatively, these alterations were different in different cell lines, in line with the heterogeneity in the overall radiomodification by 2-DG observed in these cells. Although changes in cyclin B1 level are implicated to be responsible for alterations in cell cycle progression, role of transient changes in energy levels, changes in levels of cyclin D1 which manifest at earlier time

Fig. 5 — Immunoflow cytometric measurements of cyclin D1 levels in exponentially growing BMG-1 cells, 24 hr after treatment with gamma rays (2 Gy) and 2-DG (5 mM, 2 hr). Lower panel indicates DNA histograms of respective groups.
intervals upon short duration treatment of 2-DG could also play important roles. Further studies are required to establish a causal relationship between energy status and cyclin levels as well as their roles in radiomodification by 2-DG.

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References


32 Manickam J, Dwarakanath B S, Adhikari J S & Mathew T Lazar, XXIV All India cell biology conference held at Jawaharlal Nehru University, New Delhi (24-26 Nov 2000).
