

2-Deoxy-D-Glucose-induced Cytotoxicity and Radiosensitization in Tumor Cells Is Mediated via Disruptions in Thiol Metabolism¹

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ABSTRACT

Exposure to ionizing radiation is believed to cause cell injury via the production of free radicals that are thought to induce oxidative damage. It has been proposed that exposure to agents that enhance oxidative stress-induced injury by disrupting thiol metabolism may sensitize cells to the cytotoxic effects of ionizing radiation. Recently, it has been shown that glucose deprivation selectively induces cell injury in transformed human cells via metabolic oxidative stress (J. Biol. Chem., 273: 5294–5299; Ann. N.Y. Acad. Sci., 899: 349–362), resulting in profound disruptions in thiol metabolism. Because 2-deoxy-D-glucose (2DG) is a potent inhibitor of glucose metabolism thought to mimic glucose deprivation *in vivo*, the hypothesis that exposure to 2DG might be capable of inducing radiosensitization in transformed cells via perturbations in thiol metabolism was tested. When HeLa cells were exposed to 2DG (4–10 mM) for 4–72 h, cell survival decreased (20–90%) in a dose- and time-dependent fashion. When HeLa cells were treated with 6 mM 2DG for 16 h before ionizing radiation exposure, radiosensitization was observed with a sensitizer enhancement ratio of 1.4 at 10% isosurvival. Treatment with 2DG was also found to cause decreases in intracellular total glutathione content (50%). Simultaneous treatment with the thiol antioxidant N-acetylcysteine (NAC; 30 mM) protected HeLa cells against the cytotoxicity and radiosensitizing effects of 2DG, without altering radiosensitivity in the absence of 2DG. Furthermore, treatment with NAC partially reversed the 2DG-induced decreases in total glutathione content, as well as augmented intracellular cysteine content. Finally, the cytotoxicity and radiosensitizing effects of 2DG were more pronounced in *v-Fos*-transformed *versus* nontransformed immortalized rat cells, and this radiosensitization was also inhibited by treatment with NAC. These results support the hypothesis that exposure to 2DG causes cytotoxicity and radiosensitization via a mechanism involving perturbations in thiol metabolism and allows for the speculation that these effects may be more pronounced in transformed *versus* normal cells.

INTRODUCTION

It is well established that cancer cells demonstrate altered metabolism when compared with normal, nontransformed cells (1–4). The most common alterations in cellular metabolism appear to involve intracellular glucose utilization and the loss of regulation between glycolytic metabolism and respiration (1–4). In general, cancer cells exhibit increased rates of glycolysis, as well as pentose phosphate cycle activity, and slightly reduced rates of respiration (1–4). These metabolic differences have been hypothesized to arise as a result of “damage” to the respiratory mechanism in cancer cells, and cancer cells were hypothesized to compensate for this defect by increasing

glycolysis (1). However, the nature of the damage to the cancer cell’s respiratory mechanism was never clearly delineated, and the precise mechanism responsible for the obvious differences in metabolism between normal and cancer cells remains unknown.

Recently, glucose deprivation of human cancer cells has been shown to cause cytotoxicity, as well as activation of multiple signal transduction and gene expression pathways involved in maintenance of phenotypic characteristics associated with malignancy, including angiogenesis and expression of cellular homologues of oncogenes (5–10). These results indicated that some biochemical process provided a mechanistic link between glucose metabolism and expression of phenotypic characteristics associated with malignancy (5–10). Interestingly, increased pro-oxidant production and profound disruptions in thiol metabolism were also noted during glucose deprivation, suggesting that metabolic oxidative stress was induced (7, 9–11). In addition, simultaneous treatment with a thiol antioxidant, NAC,³ was capable of suppressing glucose deprivation-induced cytotoxicity, parameters indicative of oxidative stress, as well as activation of signal transduction and gene expression pathways associated with the malignant phenotype (7, 11). These results led to the speculation that glucose deprivation-induced metabolic oxidative stress provided the mechanistic link between glucose metabolism, signal transduction, and gene expression in these human cancer cells (7, 10). Finally, transformed human cell lines were shown to be more susceptible to glucose deprivation-induced cytotoxicity and oxidative stress, relative to nontransformed human cell types (10).

Exposure to IR for the treatment of cancer is believed to cause cell injury via the production of free radicals that are thought to induce oxidative damage. It has been proposed that exposure to agents that enhance oxidative stress-induced injury by disrupting thiol metabolism may sensitize cells to the cytotoxic effects of IR (12, 13). Because glucose deprivation causes oxidative stress, as well as profound disruptions in thiol metabolism, and 2DG is an analogue of glucose believed to mimic the effects of glucose deprivation (14, 15), the current experiments were designed to determine whether 2DG-induced cytotoxicity and/or radiosensitization was dependent on 2DG-induced disruptions in thiol metabolism. The results indicate that treatment of human cervical cancer cells (HeLa) with 2DG causes decreases in total glutathione content, as well as cytotoxicity and radiosensitization. Both the disruptions in thiol metabolism and the cytotoxicity and radiosensitization induced by exposure to 2DG were reversed by treatment with the thiol antioxidant NAC. Finally, the cytotoxicity and radiosensitization induced by exposure to 2DG were found to be more pronounced in *v-Fos*-transformed rat cells, relative to their nontransformed counterparts, and this radiosensitization was also inhibited by NAC. These results support the hypothesis that 2DG-induced cytotoxicity and radiosensitization result from disruptions in thiol metabolism and support the speculation that these effects may be more pronounced in transformed *versus* normal cells.

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³ The abbreviations used are: NAC, N-acetylcysteine; 2DG, 2-deoxy-D-glucose; IR, ionizing radiation; GSSG, oxidized glutathione.

MATERIALS AND METHODS

Cell Culture and IR Conditions. HeLa (human cervical carcinoma) cells were grown in minimum essential medium (α modification), supplemented with 10% heat-inactivated (56°C, 30 min) calf serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml), and rat fibroblast cell lines 208F and FBj/R were cultured in DMEM supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). Cells were grown in a humidified 5% CO₂ incubator at 37°C. For clonogenic cell survival assays, cells were seeded into 100-mm tissue culture dishes at a density of 2×10^5 and grown to 75% confluence before experimental treatment. HeLa cells were exposed to IR (2–10 Gy) in a Pantak high-frequency 220 kV and 10 mA X-ray generator. The exposure chamber of the X-ray machine supports a 5% CO₂ atmosphere at 37°C, and control cells were placed into a similar environmentally controlled chamber adjacent to the X-ray machine.

Clonogenic Cell Survival Assays. Cells were assayed for clonogenic cell survival as described previously (16). Briefly, HeLa cells were seeded at densities of 2×10^5 cells/100-mm tissue culture dish and allowed to grow in a 37°C incubator until they reached 75% confluence. 2DG was dissolved in water and added directly to the growth media for the indicated times, after which the plates were exposed to IR. Immediately after IR exposure, cells were trypsinized, diluted, counted, and seeded into 60-mm cloning dishes at densities of 200–20,000 cells/dish. Colonies were allowed to grow in a humidified 5% CO₂, 37°C environment for 7–10 days, after which they were fixed, stained, and counted. Individual assays were performed with multiple dilutions with a total of six cloning dishes per data point, repeated at least twice. Dose response survival curves were plotted on a log-linear scale. Statistical significance of survival differences between vehicle and treated cells was determined by two-sample, two-sided *t* tests performed at the 0.05 level of significance.

Thiol Analysis. Cells were washed with ice-cold PBS, scraped into cold PBS, and centrifuged at 4°C for 5 min at $400 \times g$ to obtain cell pellets that were then frozen at -80°C . Pellets were thawed and homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1.34 mM diethylenetriaminepentaacetic acid. Total glutathione content was determined by the method of Anderson (17). Reduced and GSSG were distinguished by the addition of 2 μ l of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 μ l of sample followed by incubation for 1.5 h and assay as described previously by Griffith (18). Cysteine and NAC were determined using the high-performance liquid chromatography methodology described by Ridnour *et al.* (19). All biochemical determinations were normalized to the protein content of whole homogenates using the method of Lowry *et al.* (20). The statistical significance of thiol differences between vehicle and treated cells was determined by two-sample, two-sided *t* tests performed at the 0.05 level of significance.

RESULTS

2DG-induced Cytotoxicity and Radiosensitization. The cell survival curves shown in Fig. 1A demonstrated both the dose and time dependence of the clonogenic inactivation induced in HeLa human cervical carcinoma cells exposed to 4, 6, and 10 mM 2DG for 24, 48, and 72 h. Fig. 1B shows the dose and time dependence of the radiosensitization caused in HeLa cells by pretreatment with either 4 or 6 mM 2DG for 4 or 16 h before radiation exposure. Sensitizer enhancement ratios at 10% isosurvival ($\text{SER}_{10\%} = \text{dose to reach 10\% survival}/\text{dose to reach 10\% survival in the presence of 2DG}$) of 1.3–1.5 were obtained for the higher dose and longer time intervals of exposure to 2DG. The results demonstrate that these exposures to 2DG caused cytotoxicity and radiosensitization in human cervical carcinoma cells in culture.

2DG-induced Cytotoxicity and Radiosensitization Are Inhibited by NAC. Because glucose deprivation of human tumor cells had been shown previously to induce cytotoxicity and oxidative stress that was inhibited by exposure to the thiol antioxidant NAC (7, 11), the ability of NAC to alter 2DG-induced cytotoxicity and radiosensitization was determined. HeLa cells were treated with 30 mM NAC for 1 h before and during exposure to 4, 6, and 8 mM 2DG for 4 and 8 h (Fig. 2, A and B), respectively. These results demonstrate that exposure to NAC significantly inhibits the cytotoxic effects of 2DG. The survival data shown in

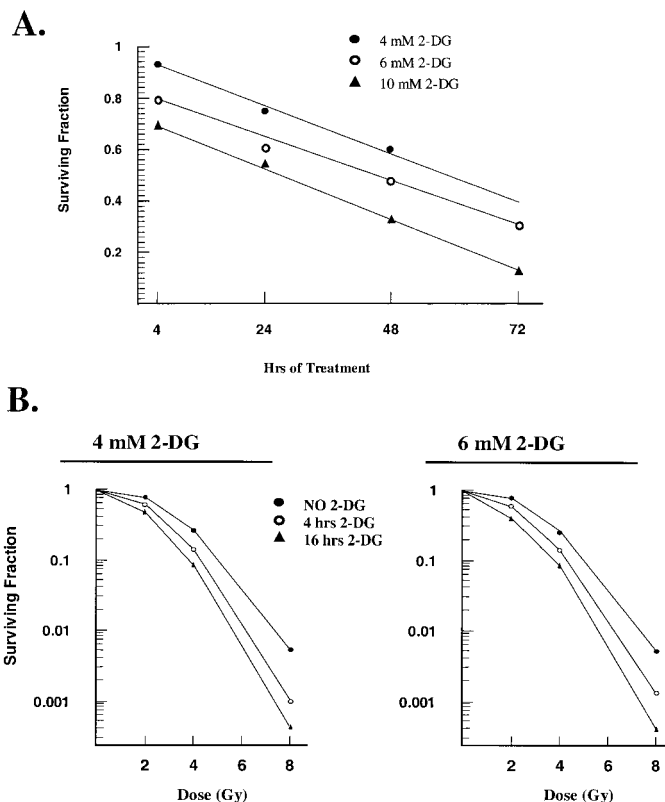


Fig. 1. 2DG-induced cytotoxicity and radiosensitization in HeLa cells. A, clonogenic survival curves using HeLa cells treated with 4, 6, or 10 mM 2DG for 4, 24, 48, or 72 h. Surviving fractions were normalized to untreated controls. A linear regression model was used to assess the correlations between surviving fraction and 2DG time/dose, and these parameters were found to correlate linearly ($R^2 = 0.98$). B, clonogenic cell survival curves using HeLa cells treated with 4 or 6 mM 2DG for 4 or 16 h before exposure to IR at 2, 4, or 8 Gy. Survival data were normalized to unirradiated controls from each respective treatment group.

Fig. 3 demonstrate that treatment with 30 mM NAC is also capable of statistically and significantly inhibiting the radiosensitizing effects of a 16-h exposure to 6 mM 2DG, while having no effect on the toxicity of IR in the absence of 2DG. These results indicate that treatment with a thiol antioxidant is capable of significantly inhibiting the cytotoxicity and radiosensitization induced by exposure to 2DG.

2DG-induced Alterations in Thiol Pools Are Partially Inhibited by NAC. Because glucose deprivation-induced cytotoxicity in human tumor cells had been shown previously to be accompanied by profound disruptions in intracellular thiol pools (7, 10, 11), thiol analysis was done on HeLa cells exposed to 2DG and NAC to determine: (a) if disruptions in thiol metabolism were occurring after treatment with 2DG and/or IR; and (b) if NAC caused any effects on the major pools of intracellular thiols in cells treated with 2DG and/or IR. Fig. 4, top panel shows that treatment for 8 h with 6 mM 2DG caused a 50% decrease in total glutathione content that was largely reversed by coincubation with 30 mM NAC. Fig. 5, top panel shows in the same experiment that NAC was only detectable in the cell homogenates from NAC-treated cells, and NAC treatment also resulted in a 2-fold increase in cellular cysteine content (Fig. 5, bottom panel). In contrast, 2DG treatment alone did not appear to alter cysteine levels (Fig. 5, bottom panel), and radiation exposure did not appear to alter any parameter of thiol metabolism under the various conditions (Figs. 4 and 5). Interestingly, 2DG exposure caused percentage of GSSG to decrease from 10 to 7% (Fig. 4, bottom panel), and when the cells were treated with 2DG in the presence of NAC, percentage of GSSG was decreased from 10 to 3%, relative to untreated controls. The results of these experiments provide support for the hypothesis that the cytotoxic and radiosensitizing effects of 2DG could be mediated by

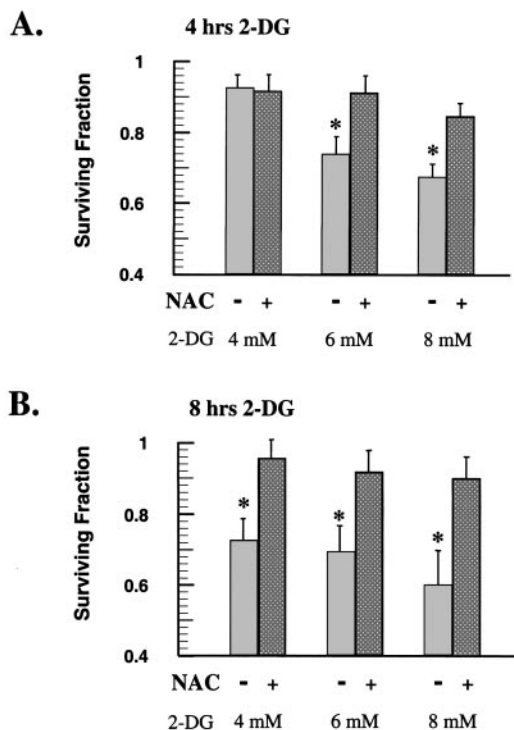


Fig. 2. 2DG-induced cytotoxicity is inhibited by NAC. Clonogenic cell survival of HeLa cells treated with 4, 6, or 8 mM 2DG for 4 (A) or 8 (B) h with (+) or without (-) treatment with 30 mM NAC for 1 h before and during 2DG exposure. Clonogenic cell survival data were normalized to untreated controls, and errors represent ± 1 SD. Statistical relationships between treated and untreated cells were determined by two-sample, two-sided *t* tests comparing the means obtained \pm NAC at each dose of 2DG. *, statistical significance at the 0.05 level.

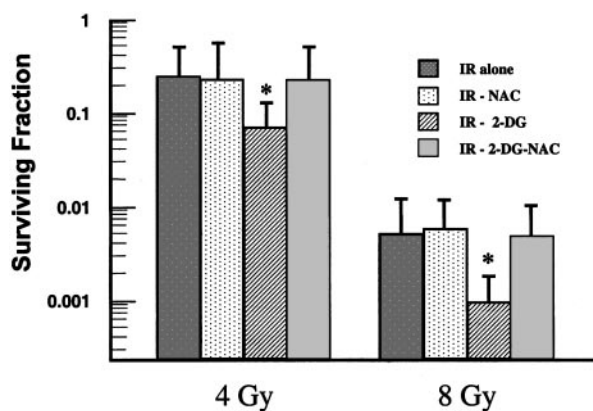


Fig. 3. 2DG-induced radiosensitization is inhibited by NAC. Clonogenic survival of HeLa cells treated with 6 mM 2DG for 16 h before exposure to 4 or 8 Gy of IR with (+) or without (-) treatment with 30 mM NAC for 1 h before and during 2DG exposure. Clonogenic cell survival data were normalized to unirradiated controls from each respective treatment group, and errors represent ± 1 SD. *, statistically significant comparisons of 2DG-treated cells with 2DG + NAC-treated cells.

disruptions in thiol metabolism that were partially reversed by exposure to the thiol antioxidant NAC.

Increased 2DG-induced Cytotoxicity and Radiosensitization in Transformed Fibroblasts. Because transformed human cells had been shown previously to be more susceptible to glucose deprivation-induced cytotoxicity than untransformed human cells (7, 10), 2DG-induced cytotoxicity and radiosensitization were determined in a matched pair of nontumorigenic (208F) *versus* *v-Fos*-transformed (FBJ/R) fibroblast cell lines. These cell lines have been characterized previously and were a generous gift from Dr. Tom Curran (21, 22). Cell survival after exposure

of 208F and FBJ/R cells to 6 or 10 mM 2DG for 4, 8, 16, or 24 h was accomplished (Fig. 6). The results showed that the FBJ/R oncogene-transformed fibroblasts were statistically and significantly more susceptible to the cytotoxic effects of 2DG than the 208F parental cell line (Fig. 6). The intrinsic radiosensitivities of 208F and FBJ/R cells were found to be similar as demonstrated by the clonogenic cell survival curves shown in Fig. 7A. In contrast, treatment with 6 mM 2DG for 4 or 8 h before and during exposure to 2 Gy of IR resulted in statistically and significantly more radiosensitization in the FBJ/R oncogene-transformed cells, relative to the parental 208F cells (Fig. 7B). The results of these experiments indicate that *v-Fos*-transformed cells (FBJ/R) are more susceptible to 2DG-induced cytotoxicity and radiosensitization than their nontransformed counterparts (208F). The survival data in Fig. 8 show that, similar to what was seen with HeLa cells, 2DG-induced radiosensitization in the *v-Fos*-transformed cells was inhibited by exposure to NAC. The only statistically significant difference is noted between FBJ/R cells treated with 2Gy IR and 2DG *versus* FBJ/R cells treated with 2Gy IR, 2DG, and NAC at 8 h. These results suggest that 2DG-induced radiosensitization in *v-Fos*-transformed rat fibroblasts may also be caused by disruptions in thiol metabolism.

DISCUSSION

It has been known for many years that 2DG is cytotoxic in yeast, mammalian tumor cells *in vitro*, and animal tumors *in vivo* (14, 15, 23, 24). It has also been suggested that the effects of 2DG are more pronounced in cancer cells exhibiting high rates of glycolysis *versus* normal

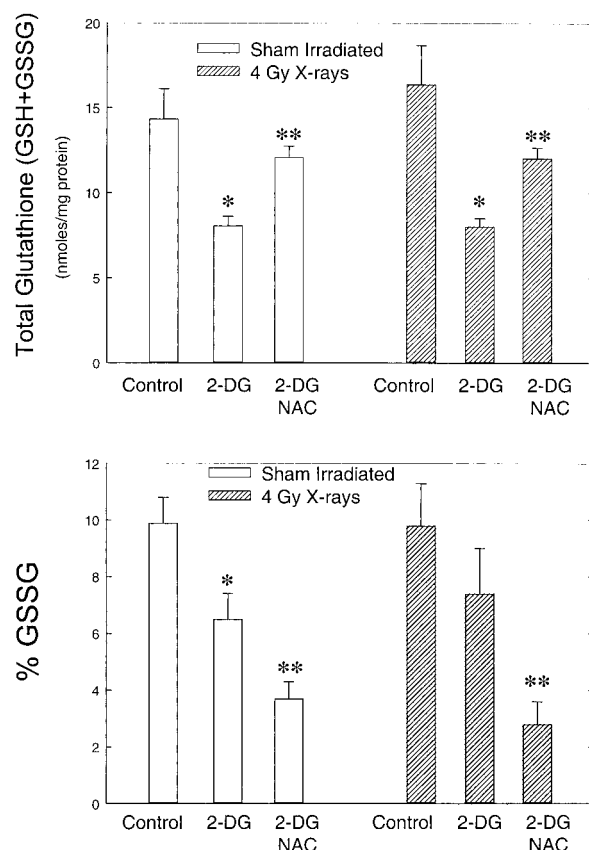
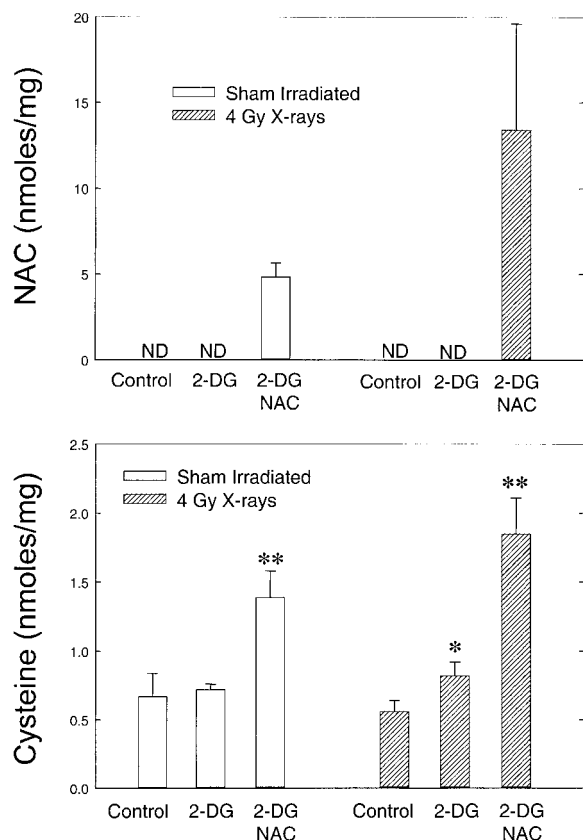


Fig. 4. The effect of 2DG and NAC on glutathione levels in control and irradiated HeLa cells. HeLa cells were treated for 8 h with 6 mM 2DG in the presence and absence of 30 mM NAC, irradiated, and harvested for glutathione analysis using the spectrophotometric recycling assay. Errors represent ± 1 SD of $n = 3$ samples. Statistical relationships between groups were determined by two-sample, two-sided *t* tests. *, statistically significant comparisons of 2DG alone-treated cells to control. **, statistically significant comparisons of 2DG + NAC-treated cells to 2DG alone-treated cells.



ND = not detectable
errors represent ± 1 SD of 3 separately measured samples

Fig. 5. The effect of 2DG and NAC on thiol levels in control and irradiated HeLa cells. HeLa cells were treated for 8 h with 6 mM 2DG in the presence and absence of 30 mM NAC, irradiated, and harvested for high-performance liquid chromatography thiol analysis. Errors represent ± 1 SD of $n = 3$ samples. Statistical relationships between groups were determined by two-sample, two-sided t tests. *, statistically significant comparisons of 2DG alone-treated cells to control. **, statistically significant comparisons of 2DG + NAC-treated cells to 2DG alone-treated cells.

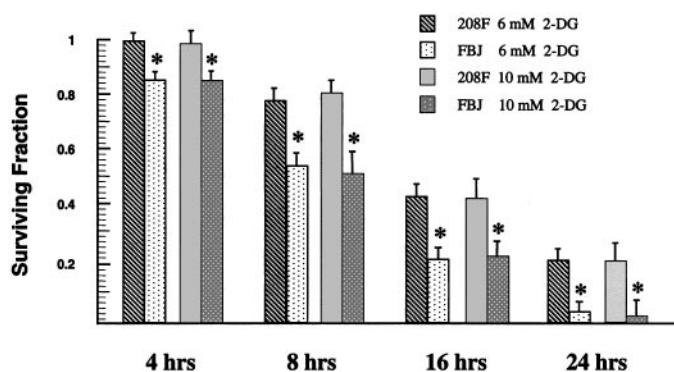


Fig. 6. Enhanced susceptibility of v-Fos-transformed fibroblasts to 2DG-induced cytotoxicity. Clonogenic cell survival of 208F (immortalized rat fibroblasts) and FBJ/R (v-Fos-transformed 208F cells) treated with 6 or 10 mM 2DG for 4, 8, 16, or 24 h. Clonogenic cell survival data were normalized to untreated controls, and errors represent ± 1 SD. *, statistically significant comparisons of FBJ/R cells to the identically treated 208F cells.

cells (24). However, the precise mechanism(s) responsible for the cytotoxic and radiosensitization effects of 2DG has been elusive. There is some data to support the hypothesis that 2DG leads to an inhibition of DNA repair pathways after exposure to radiation (24). However, the obvious mechanism proposed to explain this inhibition of DNA repair (impairment of ATP production and energy metabolism) has not been supported by the data gathered to date (24).

Recently, a significant amount of data has accumulated showing that glucose deprivation induces cytotoxicity via metabolic oxidative stress (7, 10, 11, 25). Glucose deprivation-induced oxidative stress is characterized by profound disruptions in thiol metabolism in human cancer cells, and treatment with NAC has been shown to inhibit the disruption of thiol metabolism, as well as the cytotoxicity induced by glucose deprivation (7, 10, 11, 25). In addition, glucose deprivation-induced oxidative stress was more pronounced in transformed *versus* normal human cells (10). Glucose deprivation-induced oxidative stress was hypothesized to involve impairment of hydroperoxide metabolism, because the products of glycolysis (pyruvate) and the pentose cycle (NADPH) have been shown to be integrally related to peroxide detoxification (reviewed in Ref. 10). Pyruvate can directly react with peroxides to detoxify them (26), and NADPH serves as the source of reducing equivalents for the glutathione-dependent decomposition of hydroperoxides via the glutathione peroxidase/glutathione reductase system, as well as the thioredoxin-dependent decomposition of hydroperoxides via the thioredoxin peroxidase/thioredoxin reductase system (27, 28).

Using this theoretical construct, cancer cells were hypothesized to increase glucose metabolism to enhance the metabolic decomposition of hydroperoxides formed as byproducts of one-electron reductions of O_2 to form superoxide from mitochondrial electron transport chains (10). In this regard, cancer cells were hypothesized to have a funda-

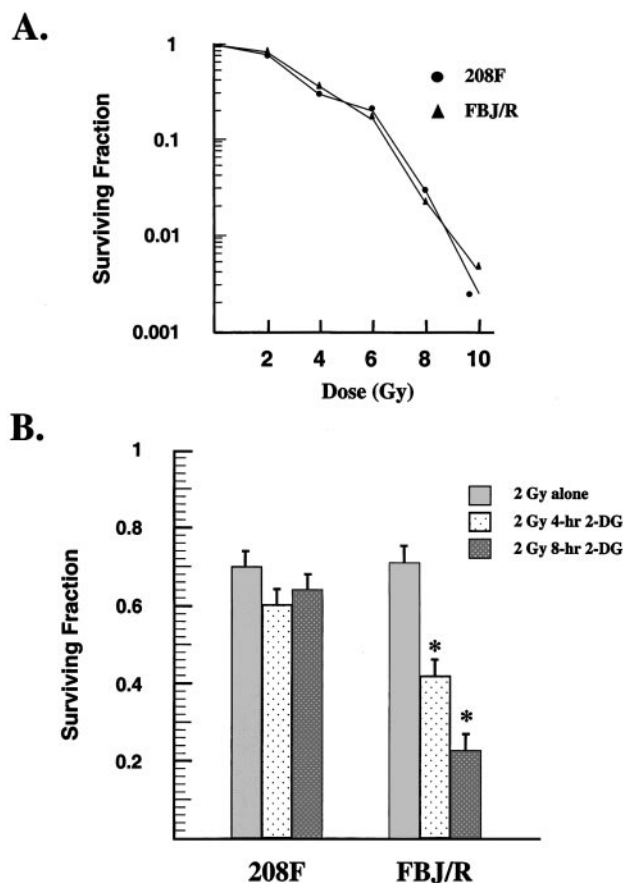


Fig. 7. 2DG-induced radiosensitization in oncogene-transformed fibroblasts. A, clonogenic cell survival of 208F (immortalized rat fibroblasts) and FBJ/R (v-Fos-transformed 208F cells) treated with 2, 4, 6, 8, or 10 Gy of IR. Surviving fractions are normalized to sham-treated controls from each cell line. B, clonogenic cell survival of 208F and FBJ/R treated with 6 mM 2DG for 4 or 8 h before and during treatment with 2 Gy IR. Survival data were normalized to unirradiated controls from each respective group, and errors represent ± 1 SD. Statistical relationships between identically treated samples of 208F and FBJ/R cells were determined by two-sample, two-sided t tests. *, statistically significant comparisons.

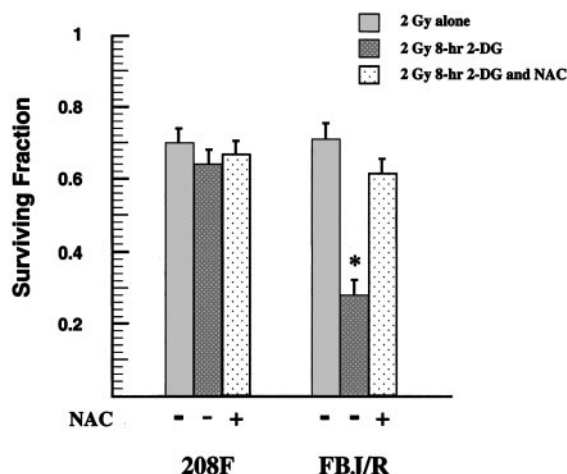


Fig. 8. 2DG-induced radiosensitization in oncogene-transformed fibroblasts is inhibited by treatment with NAC. Clonogenic cell survival of 208F (immortalized rat fibroblasts) and FBJ/R (*v-Fos*-transformed 208F cells) treated with 2 Gy of IR in the presence and absence of 6 mM 2DG and 30 mM NAC. Surviving fractions are normalized to unirradiated controls from each respective drug-treated group, and errors represent ± 1 SD. Statistical relationships between identically treated samples of 208F and FBJ/R cells were determined by two-sample, two-sided *t* tests. *, statistically significant comparisons.

mental defect in their electron transport chains, leading to increased leakage of electrons to form O_2^- and H_2O_2 , relative to normal cells (10). Glucose deprivation-induced oxidative stress was therefore hypothesized to be more severe in cancer cells because of the excess formation of prooxidants during mitochondrial respiration. Furthermore, a recent report has also shown that 2DG-induced apoptosis in leukemia cells is selectively inhibited by overexpression of the mitochondrial phospholipid hydroperoxide glutathione peroxidase enzyme (23). This previous report provided the first direct evidence that glutathione peroxidase-sensitive decomposition of hydroperoxides originating from mitochondrial metabolism might be an important contributor to 2DG-induced apoptosis.

The results in the current study using HeLa cells confirm previous reports showing that the competitive inhibitor of glucose metabolism, 2DG, is both cytotoxic and capable of inducing radiosensitization in human cancer cells *in vitro* (15, 24). In addition, the current report shows for the first time that simultaneous treatment with the thiol antioxidant, NAC, suppressed both the cytotoxicity and radiosensitization induced by 2DG in a human cancer cell line. Furthermore, NAC has now been observed to partially inhibit 2DG-induced cytotoxicity in PC-3 human prostate cancer cells.⁴ In addition, 2DG exposure was shown to induce disruptions in thiol metabolism that were partially reversed by exposure to NAC. Finally, the cytotoxicity and radiosensitization induced by exposure to 2DG appeared to be more pronounced in an oncogene-transformed cell line, when compared with the parental cell line, and this radiosensitization was also inhibited by treatment with a thiol antioxidant. The results of these studies are consistent with the previous observations obtained with glucose deprivation (7, 10, 11) and support the hypothesis that 2DG-induced cytotoxicity and radiosensitization are mediated by disruptions in thiol metabolism resulting from metabolic oxidative stress. Finally, the results also support the speculation that transformed cells may be more susceptible to 2DG-induced cytotoxicity and radiosensitization than nontransformed cells.

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