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2-Deoxyglucose combined with wild type p53 over expression enhances cytotoxicity in human prostate cancer cells via oxidative stress

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Abstract

Over expression of the tumor suppressor gene, wild type p53 (wtp53), using adenoviral vectors (Adp53) has been suggested to kill cancer cells by hydroperoxide-mediated oxidative stress [1,2] and nutrient distress induced by the glucose analog, 2-deoxyglucose (2DG), has been suggested to enhance tumor cell killing by agents that induce oxidative stress via disrupting hydroperoxide metabolism [3,4]. In the current study clonogenic cell killing of PC-3 and DU-145 human prostate cancer cells (lacking functional p53) mediated by 4 h exposure to 50 plaque forming units (pfus)/cell of Adp53 (that caused the enforced over expression of wtp53) was significantly enhanced by treatment with 2DG. Accumulation of glutathione disulfide was found to be significantly greater in both cell lines treated with 2DG+Adp53 and both cell lines treated with 2DG+Adp53 showed a ~2-fold increases in dihydroethidine (DHE) and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CDCFH₂) oxidation, indicative of increased steady-state levels of O₂^{•-} and hydroperoxides, respectively. Finally, over expression of catalase or glutathione peroxidase using adenoviral vectors partially, but significantly, protected DU-145 cells from the toxicity induced by 2DG+Adp53 treatment. These results show that treatment of human prostate cancer cells with the combination of 2DG (a nutrient stress) and over expression of the tumor suppressor gene, wtp53, enhances clonogenic cell killing by a mechanism that involves oxidative stress as well as allowing for the speculation that inhibitors of glucose and hydroperoxide metabolism can be used in combination with Adp53 gene therapy to enhance therapeutic responses.

Keywords

2DG; p53; Cancer; Glucose; oxidative stress; reactive oxygen species; superoxide and hydrogen peroxide

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INTRODUCTION

Adenocarcinoma of the prostate is a commonly diagnosed cancer that poses a significant health problem by virtue of being the second leading cause of cancer death among males in the United States [5]. Androgen ablation therapy for advanced prostate cancer is rarely curative because it is targeted to the androgen-dependent prostate cancer cell population leaving the androgen independent cells relatively unaffected and resistant to further treatment [6]. Therefore new therapies effective against androgen independent prostate cancer cells are needed.

Replication-deficient adenovirus gene therapy vectors, which induce over expression of human recombinant wild-type p53 in human cancer cells, have demonstrated cytotoxic effects that are suggested to involve hydroperoxide-mediated oxidative stress both *in vitro* and *in vivo* as well as having been associated with increased mitochondrial respiration [1,2,7,8,9,10]. Moreover, strategies combining adenovirus-mediated wild-type p53 gene transfer with DNA-damaging treatments, such as irradiation and chemotherapeutic agents, have also been explored in glioblastoma, lung, colorectal, ovarian, and head and neck cancers. These combination therapies involving p53 over expression have consistently demonstrated enhanced tumor suppressing activity, relative to single agents [11,12]. Finally, in androgen-independent prostate cancer cells, significant cytotoxicity has been previously reported with over expression of recombinant wild-type p53 using an adenovirus vector (Ad5CMV-P53) [13,14].

2-Deoxy-d-glucose competitively inhibits metabolism of glucose and has been suggested to be selectively cytotoxic to fully transformed cells [3], via a mechanism that involves hydroperoxide-mediated oxidative stress [4]. Since glucose is a major source of electrons for hydroperoxide metabolism [15–18] and tumor cells are believed to produce relatively high steady-state levels of hydroperoxides [19], the mechanism by which 2DG enhances oxidative stress in cancer cells was suggested to involve limitation of hydroperoxide detoxification [4]. Recently p53-induced apoptosis in cancer cells has been shown to occur through induction of p53-induced genes (PIGs) and the tumor suppressive effects of some PIGs are thought to be the result of increased formation of reactive oxygen species and mitochondrial respiration (ROS) (*i.e.* $O_2^{\bullet-}$ and H_2O_2), which in turn lead to oxidative stress [1,2,9,10,20]. Since 2DG would be expected to inhibit the formation of pyruvate and NADPH, which function in the detoxification pathways of H_2O_2 [3,4,15], we hypothesized that the combination of 2DG and wt p53 over expression would lead to increased steady-state levels of ROS and enhanced cell killing by oxidative stress. In the current report, the interaction between 2DG and p53 gene therapy in two androgen-independent prostate cancer cell lines (DU-145 and PC-3) was evaluated *in vitro*. Exposure of DU-145 and PC-3 cells to 2DG combined with Adp53 resulted in over expression of wtp53, enhanced cytotoxicity, as well as increased steady-state levels of intracellular $O_2^{\bullet-}$, H_2O_2 , and glutathione disulfide (GSSG). In addition, over expression of catalase or glutathione peroxidase using adenoviral vectors partially, but significantly, protected DU-145 cells from the toxicity induced by 2DG+Adp53 treatment. These results show that treatment of androgen independent human prostate cancer cells with the combination of 2DG and over expression of wtp53 enhances cancer cell clonogenic inactivation by a mechanism that appears to involve oxidative stress mediated by hydroperoxides. These results support the hypothesis that inhibitors of glucose and hydroperoxide metabolism can be used in combination with Adp53 gene therapy to enhance cell killing in human prostate cancer cells.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions

PC-3 human prostate cancer cells were obtained from ATCC, and maintained in RPMI media supplemented with 10% fetal bovine serum. DU-145 human prostate cancer cells were a gift from Dr. Mary Hendrix, University of Iowa, and maintained in RPMI media supplemented

with 10% fetal bovine serum [21]. All stock cultures were maintained in 5% CO₂ and air in a humidified 37°C incubator in the absence of antibiotics. All cells were routinely tested for mycoplasma and found to be negative.

Drug Treatment

The competitive inhibitor of glucose metabolism, 2DG, was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Since RPMI media contains 11 mM glucose, 2DG was added to cell culture media at a final concentration of 20 mM to ensure inhibition of glucose metabolism. Stock solutions of 1 M 2DG were dissolved in PBS and the required volume added directly to achieve the desired final concentration.

Measurement of Glutathione Levels

Following treatment, cells were scrape-harvested in PBS at 4°C, centrifuged, the PBS discarded and the cell pellets frozen at -80°C. Samples were thawed and whole homogenates were prepared as described [3,4,15,22,23]. Total glutathione (GSH+GSSG), and glutathione disulfide (GSSG) were determined using a spectrophotometric recycling assay [3,4,15,22, 23]. All biochemical determinations were normalized to the protein content using the method of Lowry *et al.* [24].

Viral Transduction

Replication incompetent adenoviral vectors, AdCMV Bgl II (AdBglII), AdCMV mitochondrial catalase (AdMitCat) and AdCMV glutathione peroxidase (AdGPx) were manufactured at The University of Iowa Vector Core Facility by inserting the gene of interest into the E1 region of an Ad5 E1/particle E3 deleted replication deficient adenoviral vector [25,26]. The cDNAs were all under the control of a CMV promotor. Except for AdMitCat, the adenovirus constructs were originally prepared by John Engelhardt, University of Iowa [26]. The full-length catalase cDNA with the MnSOD mitochondrial leader sequence added to the construct was originally prepared by Dr. Andre Melendez [27]. Cells were seeded until attached (overnight), and then the desired amount of viral particles was added with 1.8 mL of complete media per 60 mm dish for 24 h, then the media was changed and replaced by 4 mL complete media and left for another 24 h prior to each experiment.

Replication incompetent adenoviral vectors, Ad5CMVwtp53/eGFP (Adp53), and Ad5CMV/eGFP (AdGFP) were manufactured at the University of Iowa's Vector Core Facility by inserting the gene of interest into the E1 region of an Ad5 E1/partial E3 deleted replication deficient adenoviral vector. The cDNAs were under the control of a CMV promoter. The Adp53 and AdGFP constructs were originally prepared by Dr. Vogelstein [28,29]. Cells were seeded until attached (overnight), and then 20 mM 2DG was added for 24 h; then 50 MOI of p53 virus was added to the same media for up to 4 h and then removed and the cells trypsinized and plated for clonogenic cell survival assay. After the incubation with virus particles, cultures for western blot assay; transduction efficiency assay (GFP assay); DHE and CDCFH₂ oxidation assay; and thiol assay were placed in fresh media for another 12 h prior to harvest.

Transduction Efficiency

Adp53/GFP-transduced cells were harvested at 37°C using trypsin, resuspended in medium, and then centrifuged to obtain the pellet. The pellet was resuspended with 0.5 mL PBS, placed on ice and analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems; excitation 488 nm, emission 530 nm band-pass filter). Transduction efficiency was calculated by dividing the number of GFP positive cells by total number of at least 10,000 cells counted.

Western Blot Analysis

To assay for p53 immunoreactive protein levels, whole homogenates were sonicated at a duty cycle of 50% and an output control of 4 for 20 s and the protein concentration was measured as described in Bradford *et al* [30]. Denatured protein (5–10 μ g) was resolved on 12% SDS-PAGE and electroblotted into nitrocellulose membranes (Bio Rad). The membrane was incubated with anti-human p53 monoclonal antibody diluted at 1:1000 in TBS-T for 1 h at room temperature. After washing, the membrane was incubated with a horseradish peroxidase-labeled anti-mouse second antibody at a 1:2000 dilution for 1 h at room temperature. After washing signals were detected on an X-ray film using an enhanced ECL detection system (Amersham).

Catalase Activity Assay

Catalase activity was determined on whole homogenates by measuring the disappearance of 10 mM hydrogen peroxide ($\Delta\epsilon_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$) in 50 mM potassium phosphate, pH 7.0, monitored at 240 nm and the units were expressed as milli-k units/mg of protein as described [31].

Intracellular $\text{O}_2^{\bullet-}$ Determination using Flow Cytometry

Superoxide levels were estimated using the fluorescent dye, dihydroethidium (DHE), obtained from Molecular Probes (Eugene, Oregon). DU-145 cells were washed with PBS and labeled on culture plates at 37°C for 40 minutes in PBS (containing 5 mmole/L pyruvate) with DHE (10 μ mole/L; in 0.1% DMSO). Culture plates were placed on ice, trypsinized and resuspended in ice-cold PBS. Samples were analyzed using a FACScan flowcytometer (Becton Dickinson Immunocytometry Systems, Inc., Mountain View, CA; excitation 488 nm, emission 585 nm band-pass filter). The mean fluorescence intensity (MFI) of at least 10,000 cells was analyzed and corrected for autofluorescence from unlabeled cells [32]. SOD inhibitable DHE fluorescence was calculated by subtracting the MFI of cells treated with 100 U/mL polyethylene glycol-conjugated CuZn containing superoxide dismutase (PEG-SOD) from the MFI from co-cultures labeled in the absence of PEG-SOD [32].

Measurement of intracellular prooxidant levels

Steady-state levels of prooxidants (presumably hydroperoxides) were determined using the oxidation-sensitive [5- (and-6)-carboxy-2,7 -dichlorodihydrofluorescein diacetate; CDCFH₂, 10 μ g/mL] fluorescent dye (dissolved in 0.1% DMSO) obtained from Molecular Probes. The oxidation insensitive analog of the dye [5-(and-6)-carboxy-2, 7 -dichlorofluorescein diacetate] was used to control for changes in uptake, ester cleavage, and efflux of the dye [25]. The cells were washed once with PBS and labeled with the fluorescent dyes for 15 minutes at 37°C in PBS. At the end of the incubation time, the plates were placed on ice. Cells were then trypsinized on ice, resuspended in PBS, and analyzed using a FACScan flow cytometer (Bacton Dickinson Immunocytometry Systems; excitation 488 nm, emission 530 nm band-pass filter). The MFI of 10,000 cells was analyzed in each sample and corrected for auto-fluorescence from unlabeled cells [25]. To confirm that any observed changes in MFI represented changes in steady-state levels of hydroperoxides, cells were pretreated with 50 MOI AdGPx or AdMitCat to over express peroxide metabolizing enzymes and the ability of these manipulations to suppress MFI was determined.

Statistical Analysis

All results are expressed as means $\pm$ 1 standard deviation (SD). For analysis limited to two groups, student's t-test was employed ($p < 0.05$). Statistical comparisons among treatment groups were accomplished using analysis of variance and the least significant difference test ($p < 0.05$) to determine differences between individual means.

RESULTS

Expression of p53 and transduction efficiency in PC-3 and DU-145 human prostate carcinoma cells exposed to 2DG and infected with Ad5CMVwtp53/eGFP (Adp53)

In Figure 1A exponentially growing PC-3 and DU-145 cells were exposed to 20 mM 2DG (lanes 1 and 3) or sham treatment (lanes 2 and 4) for 24 hours, then 50 MOI AdGFP or Adp53 was added to the media for 4 hours, followed by washing the cell monolayers, and addition of fresh complete media for another 12 hours. The cells were then harvested and subjected to Western blot analysis which showed that p53 immunoreactive protein was clearly over expressed in all cases where Adp53 was present during the exposure interval (Figure 1A). In Figure 1B a similar protocol was used to show that greater than 80% of cells exposed to Adp53 became infected as determined by *GFP* fluorescence using flow cytometry. These results also show that gene transfer efficiencies occurred in a similar fashion in both DU-145 and PC-3 cells.

Clonogenic survival of DU-145 and PC-3 cells exposed to 2DG and Adp53

In order to confirm that treatment with 20 mM 2DG inhibited glucose metabolism in PC3 and DU145 cells, disappearance of glucose from the culture media was measured using a YSI glucose analyzer in 2DG treated vs. non-2DG treated cell cultures at the same cell density. The results confirmed that 24–72 hr treatment with 20 mM 2DG inhibited the apparent uptake of media glucose by both PC3 and DU145 cell lines (data not shown).

To evaluate the toxicity of 2DG combined with Adp53, cells were treated with 20 mM 2DG for 24 h followed by Adp53 treatment for 1, 2, and 4 h. The cells were then trypsinized and plated for clonogenic cell survival assay. Increases in cell death were noted when 2DG was combined with Adp53 in both PC-3 and DU-145 cells and the cytotoxicity was dependent on the time of exposure to Adp53 (Figure 2). The same protocol was repeated using both cell lines at only the 4 h time point of Adp53 exposure, then clonogenic survival assays (Figure 3) and glutathione analysis (Figure 4) were performed. The cytotoxicity of p53 combined with 2DG was found to be significantly enhanced in both PC-3 and DU-145 cells, relative to p53 or 2DG treatment (Figure 3). These results support the hypothesis that the combination of 2DG and Adp53 is more cytotoxic to human prostate cancer cells than either agent alone and appear to be at least additive.

The glutathione analyses in Figure 4 were accomplished to determine if the combination of 2DG with Adp53 caused disruptions in glutathione metabolism in prostate cancer cell lines consistent with the hypothesis that these treatments were inducing oxidative stress. 2DG treatment caused a significant increase in total glutathione as well as GSSG accumulation in both PC-3 cells ($p < 0.05$, Figure 4B) and DU-145 cells ($p < 0.05$, Figure 4D), relative to vector alone groups. This data strongly supports the hypothesis that treatment of human prostate cancer cell lines with 2DG increased the need to synthesize more glutathione and the cells' ability to retain glutathione in the reduced state was compromised in the presence of 2DG. This is consistent with what has been seen before with 2DG treatment in a human breast cancer cell line [4]. In addition Adp53 treatment by itself in DU-145 cells (relative to vector control) caused a significant increase in GSSG accumulation ($p < 0.02$) that was not seen in PC-3 cells (Figure 4D vs. 4B) and this effect of Adp53 was significantly enhanced by 2DG (Figure 4D, $p < 0.0001$). These results are again consistent with the idea that the combination of 2DG and Adp53 induces oxidative stress and this could contribute to the increased cytotoxicity seen with exposure to these agents, relative to either agent alone.

Detection of increases in steady-state levels of $O_2^{\bullet-}$ in DU-145 cells exposed to 2DG and Adp53

In order to determine if exposure to 2DG and p53 led to increases in steady-state levels of $O_2^{\bullet-}$ in DU-145 cells, intracellular oxidation of dihydroethidine (DHE) were monitored [32]. Figure 5A shows using flow cytometry analysis [32], that exposure to Adp53 caused significant increases in DHE oxidation ($p < 0.01$), suggesting exposure to Adp53 could significantly increased steady-state levels of $O_2^{\bullet-}$. Furthermore, when 2DG was combined with Adp53, DHE oxidation increased to levels that were significantly greater than that seen with either single agent alone (Figure 5A, $p < 0.05$). To confirm that increases in DHE oxidation seen in Figure 5A were truly indicative of changes in steady-state levels of $O_2^{\bullet-}$, the experiment was repeated but the cells were pretreated with 100 units/mL PEG-SOD for 2 hours before and during DHE labeling (Figure 5B). Results in Figure 5B show that PEG-SOD-inhibitable DHE oxidation was significantly increased in a pattern nearly identical to that seen in Figure 5A. These results strongly support the hypothesis that 2DG and Adp53 treatment lead to increases in steady state levels of $O_2^{\bullet-}$ that could contribute to the cytotoxicity seen with these agents.

Detection of increases in steady-state levels of hydroperoxides and H_2O_2 in DU-145 cells exposed to 2DG and Adp53

To determine if steady-state levels of hydroperoxides and H_2O_2 were increased in DU-145 cells treated with 2DG and Adp53, cells were labeled with the oxidation-sensitive probe CDCFH₂ that is capable of being oxidized to its fluorescent product (CDCF) by hydroperoxides and other prooxidants [25]. Figure 6A shows significant (~2-fold) increases in CDCFH₂ oxidation in cells treated with either 2DG or Adp53 that was further increased when 2DG was combined with Adp53. To confirm this increase in CDCFH₂ fluorescence was specific for hydroperoxide-mediated oxidation of the probe, the experiment was repeated after pretreating the cells with 50 MOI AdGPx to induce over expression of the hydroperoxide scavenging enzyme, glutathione peroxidase (Figure 6B). The results in Figure 6B show that GPx-inhibitable CDCFH₂ oxidation was increased 2- to 3-fold in cells treated with 2DG + Adp53, relative to cells treated with 2DG alone, supporting the conclusion that hydroperoxides were responsible for the increased oxidation of CDCFH₂ seen in cells exposed to 2DG + Adp53. To determine if increases in CDCFH₂ fluorescence were caused specifically by H_2O_2 -mediated oxidation of the probe, the experiment in Figure 6A was repeated after pretreating the cells with 50 MOI AdMitCat [25] to induce over expression of the H_2O_2 metabolizing enzyme, catalase, in both the cytosol and mitochondria of DU-145 cells (Figure 6C). When cells were transduced with AdMitCat (Figure 6C), MitCat-inhibitable CDCFH₂ oxidation was found to be increased 7-fold relative to cells treated with p53 alone providing further support for the conclusion that steady-state levels of H_2O_2 were contributing to the increases in probe oxidation induced by 2DG + Adp53, relative to 2DG or p53 alone. In addition, this experiment was repeated using CDCF, the oxidation-insensitive analogue of CDCFH₂ to control for possible changes in probe uptake, ester cleavage, or efflux, and the data in (Figure 6D) show no difference in labeling between treatments. Finally, to ensure that a high percentage of the cells treated with the AdGPx and AdMitCat vectors were being infected, β -galactosidase assays were performed with PC-3 cells infected with AdLacZ, which has the same adenoviral backbone as the AdMitCat and AdGPx vectors. When this experiment was accomplished, $\geq 80\%$ of 50 MOI AdLacZ infected cells were positive for β -galactosidase activity (data not shown).

The effect of catalase, and glutathione peroxidase on 2DG/p53-induced cytotoxicity

To determine whether increased steady-state levels of hydroperoxides and H_2O_2 were causally involved with 2DG + Adp53-induced cytotoxicity, human prostate cancer cells (DU-145) were transduced with 50 MOI of AdMitCat or AdGPx and then treated with 2DG and p53 (Figure

7A,B). The treatment with AdMitCat increased catalase activity from 3 mkunits/mg protein to 38 mkunits/mg and the results in Figure 7A demonstrate that this was accompanied by partial but significant protection of DU-145 cells from clonogenic inactivation induced by 2DG + Adp53 ($p < 0.01$). These results combined with the results in Figure 6 provide strong evidence for the hypothesis that mitochondrial H_2O_2 significantly contributes to the cytotoxicity associated with 2DG + Adp53 in this model system. Further confirmation of the involvement of hydroperoxides with 2DG + p53-induced cytotoxicity was obtained when pretreatment with AdGPx was also found to provide partial but significant protection from the cytotoxicity associated with exposure to 2DG + Adp53 in DU-145 cells (Figure 7B, $p < 0.001$). Overall, the results in Figure 7 provide strong support for the hypothesis that the combination of 2DG + Adp53 kills prostate cancer cells by a mechanism that involves hydroperoxides including H_2O_2 .

DISCUSSION

The purpose of the current study was to obtain proof-of-principal cell biology data directed at developing a biochemical rationale for novel combined modality cancer therapy protocols utilizing an inhibitor of glucose metabolism (2DG) combined with over expression of wild type p53 to enhance human prostate cancer cell killing via metabolic oxidative stress. The potential clinical relevance of using 2DG as an adjuvant for combined modality cancer therapy using agents that increased oxidative damage (*i.e.*, ionizing radiation) in cancer cells has been actively pursued for more than 10 years [33,34]. Clinical trials (phase I/II) using the combination of radiotherapy and 2DG on human gliomas have already been conducted and shown to be well-tolerated [33]. Recent reports also documented that the combination of ionizing radiation and adenoviral p53 gene therapy increased radiosensitivity of both p53-mutant and wild type cancer cells [7;8;35]. In addition several reports have suggested that p53 over expression can induce oxidative stress and alterations in respiration in cancer cells [1,2,9,10,20,36] and that 2DG can enhance cell killing in malignant cells by agents that induce metabolic oxidative stress [3,4,37]. Finally, several investigators have reported that androgen-independent prostate cancer cells have a high frequency of p53 mutations [38;39]. Given this background, we chose to focus on human androgen independent prostate cancer cells harboring non-functional p53 (PC-3 and DU-145) as target cancer cells.

In the current study, human prostate carcinoma cells were treated with an inhibitor of glycolysis (20 mM 2DG) and a clinically relevant adenoviral vector (Adp53) capable of enforcing the over expression of wild type p53 in human cancer cells. The biochemical rationale for this combination to enhance cancer cell killing was based on previous results in other human cancer cells suggesting that 2DG would inhibit glucose metabolism leading to a reduction in intracellular pyruvate and NADPH [3,4,37], limiting the capacity of the tumor cells to metabolize hydroperoxides [4,15,25], and p53 would increase metabolic hydroperoxide production [1,2,20,36] leading to enhanced oxidative stress. As shown in our results the combination of 2DG and Adp53 lead to over expression of p53 (Figure 1), increased clonogenic cell killing (Figure 2 and Figure 3), and increased oxidative stress that appeared to be mediated by increased steady-state levels of hydroperoxides including H_2O_2 (Fig 4–Fig 6) in prostate cancer cells.

In order to determine if increases in steady-state levels of specific reactive oxygen species (*i.e.*, superoxide and hydroperoxides) were associated with exposure to 2DG + Adp53, DU-145 cells were pretreated with PEG-SOD (Figure 5B), AdGPx (Figure 6B), or AdMitCat (Figure 6C), before and during labeling with DHE or CDCFH₂, respectively. The significant increases in PEG-SOD inhibitable DHE oxidation (Figure 5B) as well as AdGPx (Figure 6B) or AdMitCat (Figure 6C) inhibitable CDCFH₂ oxidation that were observed, strongly supports the hypothesis that increases in steady state levels of $O_2^{\bullet -}$ and hydroperoxides did occur in

prostate cancer cells treated with 2DG and Adp53. These results also suggested that both $O_2^{\bullet-}$ and hydroperoxides were capable of contributing to the oxidative stress and cytotoxicity seen during 2DG and Adp53 treatment. These results are also consistent with the previous observations that p53-dependent apoptosis was preceded by the induction of the enzyme proline oxidase and increased respiration presumably leading to the increased generation of $O_2^{\bullet-}$ and hydroperoxides [9, 10, 20, 36].

In order to determine if increases in steady-state levels of $O_2^{\bullet-}$ and hydroperoxides were causally related to the clonogenic cell killing associated with exposure to 2DG + Adp53, DU-145 cells were treated with AdMitCat or AdGPx before and during treatment with 2DG + Adp53 (Figure 7). The results showed that AdMitCat (Figure 7A), or AdGPx (Figure 7B), were capable of partially, but significantly, protecting DU-145 cells from the cytotoxicity of 2DG + Adp53. Overall, these results provide strong support for the hypothesis that treatment of human prostate cancer cells with the combination of 2DG and over expression of wild type p53 enhances clonogenic cell killing by a mechanism that involves oxidative stress mediated by hydroperoxides as well as suggesting that inhibitors of glucose and hydroperoxide metabolism can be used in combination with Adp53 gene therapy to enhance therapeutic responses in prostate cancer.

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Abbreviations

2DG, 2-deoxyglucose; GSH, glutathione; GSSG, glutathione disulfide; DHE, dihydroethidium; pfus, plaque forming units; CDCFH₂, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate.

Figure 1. p53 over expression (A) and transduction efficiency (B) in PC-3 and DU-145 human prostate carcinoma cells infected with Ad5CMVwtp53/eGFP (Adp53) and Ad5CMVeGFP (AdGFP)

(A). Western blot analysis of p53 protein in both PC-3 and DU-145 cells transduced with 50 MOI Adp53. Cells were seeded overnight, and then 20 mM 2DG was added for 24 h, then 50 MOI of Adp53 or vector control AdGFP was added to the same media for 4 h, followed by adding fresh media for another 12 h, and harvest for Western blot analysis. Lane 1, cells transduced with AdGFP in combination with 2DG; Lane 2, cells transduced with AdGFP; Lane 3, cells transduced with Adp53 in combination with 2DG; Lane 4, cells transduced with Adp53. **(B).** Cells were seeded overnight, and then 20 mM 2DG was added for 24 h, then 50 MOI of Adp53 was added to the same media for 4 h, followed by adding fresh media for another 12 h prior to harvest. There were no significant differences between cell lines ($p > 0.05$, t-test, $n = 3$).

Figure 2. Clonogenic survival of cells exposed to 2DG and various times of Adp53-infection using DU-145 (A) and PC-3 cells (B)

Cells were plated in complete media overnight, and then 20 mM 2DG was added for 24 h, then 50 MOI of Adp53 was added to the same media for 1 to 4 h. Cells were then trypsinized and subjected to clonogenic cell survival analysis. Survival data were normalized to the respective treatment group at time 0 of the viral exposure. Errors represent ± 1 SD of at least three cloning dishes counted for each point and taken from one treatment dish. Data were normalized to the time 0 h of viral exposure.

Figure 3. Clonogenic survival of cells exposed to 2DG and 4 hr of Adp53-infection using PC-3 (A) and DU-145 cells (B)

Cells were seeded overnight, then 20 mM 2DG was added for 24 h, then 50 MOI of Adp53 was added to the same media for 4 h. Cells were then immediately trypsinized and plated for clonogenic survival. Errors represent ± 1 SD of $n=3$ treatment dishes from each treatment group. The differences between 2DG or p53 treatment groups vs. 2DG+p53 groups was determined using analysis of variance and the least significant difference test and the p values for comparison are specified above the lines pointing to the groups being compared ($n=3$).

Figure 4. Total glutathione and GSSG analysis of PC-3 (A,B) and DU145 cells (C,D) treated with 20 mM 2DG for 24 h followed by 50 MOI Adp53 for 4 h with samples being harvested 12 h following virus exposure

Errors represent ± 1 SD of 3 experiments. The differences between 2DG or p53 treatment groups vs. 2DG+p53 groups was determined using analysis of variance and the least significant difference test and the p values for comparison are specified above the lines pointing to the groups being compared (n=3).

Figure 5. Steady-state levels of $O_2^{\bullet}$ as determined by DHE oxidation (A) and PEG-SOD inhibitable DHE oxidation (B) in DU-145 cells treated with 2DG and Adp53

(A) Cells were treated with 20 mM 2DG for 24 h, then 50 MOI of Adp53 or vector control AdGFP was added to the same media for 4 h followed by adding fresh media for another 12 h. Cells were then incubated with 10 μ M DHE in 2 mL PBS containing 5 mmol/L pyruvate at 37°C for 40 minutes. (B) Cells were treated with 100 units/ml PEG-SOD for 2 hours before and during DHE labeling. Cells were trypsinized on ice and analyzed by flow cytometry. Each sampling measured the MFI of 10,000 cells corrected for auto-fluorescence. The differences between treatment groups was determined using analysis of variance and the least significant

difference test and the p values for comparison are specified above the lines pointing to the groups being compared (n=3).

Figure 6. Steady-state levels of hydroperoxides as determined by CDCFH₂ oxidation (A), GPx-inhibitable CDCFH₂ oxidation (B) and MitCat- inhibitable CDCFH₂ oxidation (C) in DU-145 cells treated with 2DG and Adp53

(A) Cells were treated with 20 mM 2DG for 24 h, then 50 MOI of Adp53 or vector control AdGFP was added to the same media for 4 h followed by adding fresh media for another 12 h. Cells were then incubated with 10 µg/mL CDCFH₂ (panels A, B) in PBS at 37° C for 15 minutes. Cells were trypsinized on ice and analyzed by flow cytometry. Each sampling measured the MFI of 10,000 cells corrected for auto-fluorescence. In cells labeled with the oxidation insensitive probe (CDCF) no increases between treatments that exceeded 40% of control were noted. (B) Cells treated with 2DG + Adp53 showed increased GPx inhibitable

CDCFH₂ fluorescence relative to 2DG treatment alone. Cells were exposed to 50 MOI AdGPx 24 hour after plating. The media was changed 24 hour after AdGPx addition and replaced with fresh media. Cells were then treated with 20 mM 2DG and Adp53 then labeled with CDCFH₂ as described above. **(C)** Cells pretreated with AdMitCat followed by 2DG + Adp53 demonstrated increased MitCAT inhibitable CDCFH₂ fluorescence relative to p53 alone. For these experiments cells were exposed to 50 MOI AdMitCAT 24 hour after plating. The media was changed 24 hour after the virus addition and replaced with fresh media. Cells were then treated with 20 mM 2DG and Adp53 then labeled with CDCFH₂ as described above. **(D)** Cells treated with 2DG/p53 as above were labeled with the oxidation insensitive analog of CDCFH₂ (CDCF) and analyzed as above. The differences between 2DG or p53 treatment groups vs. vector control containing AdGFP groups was determined using analysis of variance and the least significant difference test and the p values for comparison are specified above the lines pointing to the groups being compared (n=3). Asterisks indicate differences between both treatments vs. each treatment alone using analysis of variance and the least significant difference test (*p<0.0001, **p<0.02, ***p<0.004, n = 3).

Figure 7. Transduction of DU-145 cells with AdMitCat, and AdGPx, suppressed the cytotoxicity induced by 2DG + Adp53 treatment

Cells were exposed to vector control or 50 MOI AdMitCat (A) or AdGpx and 30 nM selenium (B, vector controls also got Se) 24 h after plating. The media was changed 24 h after the virus addition and replaced with fresh media. Cells were then treated with 20 mM 2DG for 24 h, then 50 MOI of Adp53 or vector control AdGFP was added to the same media for 4 h and then plated for survival. Errors represent ± 1 SD of 3 experiments. Survival data were normalized to sham treated cultures with appropriate vector controls. The differences between 2DG or Adp53 treatment groups vs. combined treatment groups and the differences between 2DG +

Adp53 group with AdMitCat (or AdGpx) relative to 2DG/p53 were determined using ANOVA as in Fig. 6 (n=3).