2-Deoxyglucose Induces Noxa-Dependent Apoptosis in Alveolar Rhabdomyosarcoma

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Abstract

Alveolar and embryonal rhabdomyosarcomas are childhood tumors that do not respond well to current chemotherapies. Here, we report that the glycolytic inhibitor 2-deoxyglucose (2-DG) can efficiently promote cell death in alveolar, but not embryonal, rhabdomyosarcoma cell lines. Notably, 2-DG also induced cell differentiation accompanied by downregulation of PAX3/FOXO1a, the chromosome translocation-encoded fusion protein that is a central oncogenic driver in this disease. Cell death triggered by 2-DG was associated with its ability to activate Bax and Bak. Overexpression of the antiapoptotic Bcl-2 homologues Bcl-2 and Mcl-1 prevented apoptosis, indicating that cell death proceeds through the mitochondrial pathway. Mechanistic investigations indicated that Mcl-1 downregulation and Noxa upregulation were critical for 2-DG-induced apoptosis. In addition, 2-DG promoted eIF2α phosphorylation and inactivation of the mTOR pathway. Mcl-1 loss and cell death were prevented by downregulation of the endoplasmic reticulum (ER) stress–induced protein ATF4 and by incubating cells in the presence of mannose, which reverted 2-DG–induced ER stress but not ATP depletion. Thus, energetic stresses created by 2-DG were not the primary cause of cell death. Together, our findings suggest that glycolysis inhibitors such as 2-DG may be highly effective in treating alveolar rhabdomyosarcoma and that Noxa could offer a prognostic marker to monitor the efficacy of such agents. Cancer Res; 71(21): 6796–806. ©2011 AACR.

Introduction

Rhabdomyosarcoma is the most common soft tissue tumor in children and adolescence, accounting for 4% to 5% of pediatric tumors. The 2 common histiotypes are a favorable group comprising embryonal rhabdomyosarcoma and an unfavorable group comprising alveolar rhabdomyosarcoma (1). Standard therapeutic regimens are a combination of vincristine, actinomycin-D, and cyclophosphamide, with other drugs being tested in clinical trials (2). Although the introduction of chemotherapy has greatly improved survival, overall survival rate is 70%, which indicates that new chemotherapeutic approaches need to be developed (3).

Tumor metabolism is receiving an ever-increasing attention as an antitumor target. Several metabolic pathways function differently in tumor and nontransformed cells (4). In particular, glycolysis is frequently upregulated in tumor cells and respiration is inhibited. This makes tumor cells particularly sensitive to glycolytic inhibitors such as 2-deoxyglucose (2-DG) or 3-bromopyruvate (5, 6). Some pieces of evidence suggest that targeting glycolysis could be a good strategy against rhabdomyosarcoma. These cell display mitochondrial respiratory defects (7) and an energy-producing metabolic phenotype compared with a more catabolic metabolism of primary myocytes (8). This could be due to hyperactivation of the PI3K/Akt/mTOR pathway, which is frequently observed in rhabdomyosarcoma (9). Second, p53 is frequently inactivated in rhabdomyosarcoma (10). p53 sustains the production of ATP through respiration, and its loss promotes glycolysis. Therefore, loss of p53 has been shown to promote susceptibility of tumor cells to glucose deprivation (11). In addition, rhabdomyosarcoma can be detected in patients by the positron emission tomographic technique, which is based on uptake of a glucose analogue (12).

Sensitivity to antglycolytics is known to be regulated by a number of proteins involved in response to metabolic stress. However, little is known about the cell death proteins that respond to these drugs (13). Cell death in animals occurs mainly through apoptosis or necrosis; these forms of cell death promote different responses in the tissue, with necrosis being a proinflammatory form of cell death, as opposed to apoptosis (14). About apoptosis, 2 major pathways have been described: the extrinsic, death ligand–mediated pathway, and the intrinsic or mitochondrial pathway (15). The first pathway is initiated by death ligands such as TNF, Fas ligand, or TRAIL and is mediated by the protease caspase-8. The mitochondrial pathway is initiated by "BH3-only" proteins such as Bim, Noxa, or Puma, which act as stress sensors and promote activation of...
Bax and Bak on the mitochondrial membrane. This allows the release of cytochrome c, which promotes formation of the apoptosome and caspase activation.

We aimed to determine whether rhabdomyosarcoma cells are sensitive to the glycolytic inhibitor 2-DG. We describe that alveolar rhabdomyosarcoma cells are sensitive to this drug. Moreover, we provide evidence that the BH3 protein Noxa mediates apoptosis.

Materials and Methods

Cell culture and treatments

Alveolar rhabdomyosarcoma cell lines [Rh4, Rh30 (obtained from the original repository, Peter Houghton’s laboratory in 2008), and Rh41 purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen 2008] and embryonal rhabdomyosarcoma cells (RD from European HPACC 2009 and A-204 from Deutsche Sammlung von Mikroorganismen und Zellkulturen 2008) were expanded and frozen within 2 weeks of purchase and used for a maximum of 2 months after resuscitation of frozen aliquots. They were authenticated by the provider on the basis of viability, recovery, growth, morphology, as well as by cytogenetic analysis, antigen expression, DNA profile, and isoenzymology. Cells were maintained in high-glucose [25 mmol/L], pyruvate-free Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 2 mmol/L L-glutamine, 200 mg/mL penicillin, 100 mg/mL streptomycin sulfate, and 10% FBS (Invitrogen).

For treatments, cells were plated at a concentration of 200,000/mL and treated in fresh medium 24 hours later at 70% confluence (600,000/mL). Q-VD-OPH (SM Biochemicals LLC) was used at 20 μmol/L and added simultaneously with 2-DG. An equal amount of DMSO was added to the controls.

Western blotting

Cells were trypsinized, washed with PBS, lysed by resuspending them in lysis buffer [5 mmol/L Tris-HCl/2% SDS, Complete Antiprotease Cocktail (Roche)], and frozen. For analysis of phosphoproteins and hypoxia-inducible factor 1-alpha (HIF-1α), cells were lysed in radioimmunoprecipitation assay buffer plus orthovanadate and 2-glycerol-phosphate. After sonication, protein concentration was measured with BCA Protein Assay Reagent (bicinchoninic acid; Pierce). Equal amounts of protein were mixed with Laemmli loading buffer. After electrophoresis, protein was transferred to a polyvinylidene fluoride membrane (Millipore). Membrane was blocked with 5% nonfat dry milk in Tris-buffered saline–Tween (0.1%). Antibodies are detailed in Supplementary Methods.

Immunocytochemistry

Attached and detached cells were collected by trypsinization, fixed in suspension with 4% paraformaldehyde in PBS for 20 minutes at room temperature, washed, and blocked with 0.1% bovine serum albumin + 0.1% Triton X-100 in PBS for 1 hour. They were then incubated overnight with anti-active Bax (6A7, catalog no. 556467; BD Pharmingen; 1:100) or anti-active Bak (Ab-1/TC100; Calbiochem/Merck) diluted in blocking buffer. See more details in Supplementary Methods.

Immunoprecipitations

Attached cells were collected by trypsinization and washed in PBS together with detached cells. They were lysed in 500 μL of CHAPS immunoprecipitation buffer [2% CHAPS, 20 mmol/L Tris-HCl (pH 7.4), 137 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol] plus Complete Protein Inhibitor Cocktail Tablets (Roche) for 30 minutes. Thirty microliters of Protein G Magnetic Beads (Millipore) was washed 3 × in immunoprecipitation buffer without CHAPS and then incubated in 1 mL CHAPS buffer with 2 μg of antibody for 4 hours at 4°C under rotation. Five hundred micrograms of total cell extract was incubated overnight in 1 mL of beads coupled with 2 μg of antibody. The next day, beads were washed 3 times with immunoprecipitation buffer and incubated with 45 μL of immunoprecipitation buffer containing 2% SDS and 15 μL of SDS loading buffer (containing 5% β-mercaptoethanol) for 10 minutes at 95°C. Eluted proteins were subjected to SDS-PAGE.

Measurement of cell death

For sub-G1 analysis, detached and attached cells were trypsinized, washed in PBS, fixed in 70% cold ethanol while vortexing, and incubated for 1 to 10 days at −20°C. For experiments of transient transfection, fixation was done in 4% paraformaldehyde dissolved in PBS at room temperature for 15 minutes before washing. Cells were further washed, resuspended in PBS with 40 μg/mL PI and 100 μg/mL RNase A, and incubated for 30 minutes at 37°C before fluorescence-activated cell-sorting analysis.

DNA and RNA transfections, plasmids, and generation of cell lines

For DNA transfection, cells were incubated in 10-cm dishes in antibiotic-free DMEM and incubated for 6 hours with 25 μL Lipofectamine 2000 (Invitrogen) and 10 μg of DNA. For generation of Rh4 cells stably expressing Bcl-xL, they were transfected as described earlier and selected with 0.5 μg/mL puromycin. pBABE-Bcl-xL was provided by Dr. J. Goldstein, and the pcDNA plasmids encoding Mcl-1 and Bcl-xL were provided by Dr. J-E. Ricci (INSERM, Nice) and Prof. Seamus Martin (TCD, Ireland), respectively. For transfections of siRNA, cells were incubated in antibiotic-free DMEM for 8 hours (6 hours for Mcl-1) with siRNA 100 nmol/mL premixed with DharmaFECT 1 (Dharmacon). Sequences are detailed in Supplementary Methods.

Reverse transcription PCR

Total RNA (3 μg), extracted with the Nucleospin RNA II Kit (MACHERY-NAGEL), was used for cDNA synthesis with SuperScript II Reverse Transcriptase (Invitrogen). Amplifications were carried out with specific primers (Noxa: forward 5’-CTCGACAAGGGCTTGCCTC, reverse 5’-CAACTGGACACCTGGG; Mcl-1: forward 5’-GAGGAGAGATGTGGTACC-GG, reverse 5’-CAGACGCTCATTGGTGC-β-actin: forward 5’-GGGAGCTAGCTAGACTCACT, reverse 5’-CTTCATTGTGCTGGTGTC). For each set of primers, the number of cycles was adjusted so that the reaction endpoints fell within the exponential phase of product amplification, thus providing a semiquantitative estimate of relative mRNA abundance.
Results

Alveolar rhabdomyosarcoma cell lines are sensitive to 2-DG

2-DG induces different effects in different tumor cell lines: It reduces proliferation, induces cell-cycle arrest, or promotes apoptosis (16). We analyzed the effects of 2-DG in a panel of alveolar rhabdomyosarcoma cell lines. 2-DG inhibited the growth of Rh4 alveolar rhabdomyosarcoma cells incubated with doses of 2 mmol/L or higher, even though glucose is present at 25 mmol/L in the culture medium (Supplementary Fig. S1). We observed cell death at doses over 5 mmol/L (Fig. 1A). Cell death was likely due to apoptosis because DNA was degraded and cells displayed sub-G1 DNA content. Furthermore, DNA cleavage was prevented by incubating cells in the presence of the caspase inhibitor Q-VD. The same results were observed in Rh30 or Rh41 alveolar rhabdomyosarcoma cell lines (Fig. 1B and C).

Embryonal rhabdomyosarcoma generally have a better prognosis than alveolar rhabdomyosarcoma. We observed that RD and A-204 embryonal rhabdomyosarcoma cells were much more resistant than alveolar rhabdomyosarcoma to 2-DG. Only RD cells showed cell death at high doses, almost equimolar with glucose concentration in the medium (Fig. 1D). A-204 cells were completely resistant at all doses tested (Fig. 1E). We did, however, observe inhibition of cell growth at higher doses (Fig. 1F). To determine whether this cell growth arrest was irreversible, after 72 hours, cells were washed and were left to grow in the absence of 2-DG. Cell growth arrest was reversible because cells started to grow back immediately after removal of the drug (Fig. 1F). Because HIF-1α frequently regulates tumor glycolytic phenotype, one possibility was that HIF-1α was differentially expressed in embryonal versus alveolar rhabdomyosarcoma and it could be regulating uptake and toxicity of 2-DG in rhabdomyosarcomas. Thus, we analyzed the levels of this protein in the 5 cell lines. HIF-1α was virtually

![Figure 1. Alveolar rhabdomyosarcoma, but not embryonal rhabdomyosarcoma, cell lines are sensitive to 2-DG. Rh4 (A), Rh30 (B), Rh41 (C), RD (D), or A-204 (E) cells were treated with 2-DG at indicated concentrations in the presence of Q-VD or dimethyl sulfoxide as indicated, collected after 72 hours, and subjected to sub-G1 analysis. For control samples, cells were plated at half concentration to avoid death due to overgrowth. Mean ± SEM of at least 3 experiments is shown. F, 24 hours after plating A-204 cells, one well was stained with crystal violet (C, control), and the rest were grown in the absence (C72) or the presence of 20 mmol/L 2-DG for 72 hours. Cells were washed and further incubated with fresh medium for indicated times. Adhered cells were stained with crystal violet 0.2% in 2% ethanol for 20 minutes and solubilized in 10% SDS. Absorbance was measured at 595 nm.](image-url)
undetectable in all untreated cells. Upon treatment, the levels of this protein did not increase, and its downregulation using siRNA did not provide protection from 2-DG. Rather, a slight sensitization was observed (Supplementary Fig. S2).

Although alveolar rhabdomyosarcoma cells express markers of muscular differentiation, they lack the possibility of terminal differentiation, what is believed to be caused by PAX/FOXO1a chimeras (17, 18). Because differentiated cells stop proliferating, one of the aims of the therapy is to promote differentiation of the tumor cells. We observed fusion of alveolar rhabdomyosarcoma cells characteristic of myotube formation and terminal differentiation after treatment with 2-DG (Fig. 2A and Supplementary Fig. S1). This was likely due to the fact that 2-DG induced the downregulation of the fusion protein PAX3/FOXO1a (Fig. 2B), which promotes differentiation of these cells (17). This effect was more pronounced when cells were cultured in differentiation medium (without serum) and was not inhibited by the caspase inhibitor Q-VD, indicating that differentiation was not a consequence of caspase activity.

2-DG induces apoptotic cell death

Inhibition of glucose metabolism induces cell death by necrosis or by apoptosis, probably depending on the cell type (13). Results shown in Fig. 1 suggest apoptosis as the form of cell death, because death is inhibited by the caspase inhibitor Q-VD. However, we were unable to detect cleavage of caspase-3 by Western blotting (not shown). To verify that cell death is due to apoptosis, we analyzed cleavage of the caspase substrate PARP. Treatment with 2-DG promoted PARP cleavage, which was inhibited by Q-VD (Fig. 3A). Moreover, 2-DG induced caspase activity as measured by cleavage of a peptidic caspase substrate (Fig. 3B). In addition, we analyzed the nuclear morphology of Rh4 cells after treatment with 2-DG and observed classical apoptotic chromatin condensation (Fig. 3C).

Apoptosis induced by 2-DG proceeds through the mitochondrial pathway

In hematopoietic cells, apoptosis induced by glucose deprivation proceeds through the mitochondrial pathway (13). However, we have described that in other cell types, apoptosis induced by lack of glucose is mediated by caspase-8 and it does not require a functional mitochondrial apoptotic pathway (19). We observed that the most sensitive cell lines did not express detectable caspase-8 (Supplementary Fig. S3). We thus analyzed the mitochondrial pathway by immunostaining with antibodies that detect the active forms of Bax and Bak, the proteins that mediate mitochondrial permeabilization during apoptosis. As shown in Fig. 3C, 2-DG promoted Bax and Bak activation even when cells were treated in the presence of Q-VD, indicating that these events are not a consequence of caspase activity.

To further show the involvement of the mitochondrial pathway in death by 2-DG, we overexpressed the antiapoptotic Bcl-2 homologues Bcl-xL and Mcl-1 in Rh4 cells (Fig. 4A and B and Supplementary Figs. S4 and S5). Both proteins prevented death induced by 2-DG. Conversely, downregulation of these proteins sensitized cells to 2-DG (Fig. 4C and D and Supplementary Fig. S5).

Inhibition of glucose metabolism by limitation of glucose (20) or by 2-DG (21) promotes downregulation of the
antiapoptotic Bcl-2 family member Mcl-1. In Rh4 cells, we observed downregulation of Mcl-1, which could not be prevented by inhibition of caspases (Fig. 4E). Levels of other antiapoptotic Bcl-2 family members (Bcl-2 and Bcl-xL) increased during treatment, whereas Bax and Bak did not change significantly. We analyzed the levels of putative BH3-only proteins that could be responsible for cell death induced by 2-DG (Fig. 5A). We observed that Puma was not induced by the treatment. However, Bim and Noxa showed moderate increases, which prompted us to analyze their role by employing siRNA (Fig. 5B and Supplementary Fig. S5). As shown in Fig. 5C, siRNA-mediated ablation of Noxa, but not Bim, conferred significant resistance to 2-DG. The combined ablation of the 2 proteins did not confer further resistance to cell death, indicating that Noxa is the major cell death mediator.

Noxa is constitutively bound to Mcl-1 in many cell lines, and it has also been described to be bound to Bcl-xL (22). These antiapoptotic proteins can also sequester Bak and Bax. Proapoptotic proteins such as Noxa can free Bax/Bak, allowing them to promote cytochrome c release. We studied interactions between these proteins and observed that Mcl-1 is constitutively bound to Bim and Noxa (Fig. 5D and E). In addition, we could detect very weak interactions between Bak and Bcl-xL or Mcl-1 (Fig. 5F and Supplementary Fig. S6). Bcl-2 and Bax could not be coimmunoprecipitated with Bak or Bcl-xL/Mcl-1, respectively (data not shown). We could not observe changes in the binding pattern of these proteins when treated with 2-DG.

The Noxa/Mcl-1 axis has been described to be involved in cell death induced by inhibition of cell metabolism, and its levels and activity have been shown to be regulated by multiple transcriptional and posttranscriptional mechanisms (21, 23–25). We checked for transcriptional regulation and observed that mRNA levels of these proteins barely changed (Fig. 6A). By blocking protein synthesis with cycloheximide, we observed that stability of the short-lived Mcl-1 and Noxa was dramatically enhanced when cells were treated with 2-DG (Fig. 6B). Because Mcl-1 is very sensitive to inhibition of translation, we analyzed the status of 2 pathways that lead to translation.
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inhibition and Mcl-1 downregulation: Ser\textsuperscript{51} phosphorylation of the eukaryotic initiation factor 2-\alpha (eIF2\textsubscript{\alpha}) and mTOR inactivation (21, 26). We observed both phosphorylation of eIF2\textsubscript{\alpha} and inactivation (dephosphorylation) of the marker of mTOR status S6 (Fig. 6C). In addition, we observed that Noxa contributed modestly to downregulation of Mcl-1 (Fig. 6D).

Endoplasmic reticulum stress, but not ATP loss, correlates with apoptosis and regulation of Noxa and Mcl-1

Glucose deprivation and 2-DG impair generation of ATP, synthesis of macromolecules, and protein modifications such as acetylation and glycosylation. Although 2-DG is frequently used as a caloric restriction mimic, it has been shown to kill some tumor cell lines by interference with protein N-glycosylation rather than by causing energetic stress (27). We aimed to identify the major cause of death induced by 2-DG by incubating cells in the presence of the sugar mannose, which rescues some cell lines from the toxicity of 2-DG by restoring glycosylation. We observed that coinubation with mannose completely prevented cell death induced by 2-DG (Fig. 7A). This suggested that death was due to impairment of glycosylation in the endoplasmic reticulum (ER) and the subsequent ER stress. To further analyze this, we treated the cells with the ER stressor tunicamycin at a dose that promoted cell death with the same kinetics as 2-DG (Fig. 7D). We observed that tunicamycin also promoted a late increase in Noxa levels and a rapid decrease in Mcl-1. Moreover, we downregulated the transcription factor ATF4, which is induced by the PKR-like endoplasmic reticulum kinase.

Figure 4. Apoptosis induced by 2-DG proceeds through the mitochondrial pathway. A and B, RH4 cells were transfected with 2.5 \mu g of membrane-targeted GFP vector and none (–), 2.5 \mu g (low), or 7.5 \mu g (high) of vector encoding Mcl-1 (A; Supplementary Fig. S5) or Bcl-x\textsubscript{L} (B). Empty vector was added up to 10 \mu g of total DNA. Cells were rinsed and, 2 hours later, they were trypsinized and replated. Fifteen hours later, medium was replaced, and cells were either left untreated or treated with 10 mmol/L 2-DG for 48 hours. Sub-G\textsubscript{1} analysis of GFP (–) cells (–10%–15%) is shown. C and D, cells were transfected with siRNA against Bcl-x\textsubscript{L} or Mcl-1 and subjected to further treatment with 2-DG for the indicated times and sub-G\textsubscript{1} analysis. * significant differences versus the controls (P < 0.05). Western blots are shown in Supplementary Fig. S5. E, RH4 cells were cultured for the indicated times with 10 mmol/L 2-DG in the presence or absence of Q-VD. Indicated proteins were resolved by immunoblotting.
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2-DG when HIF-1α was downregulated, in accordance with the studies of Maher and colleagues, which indicate that HIF-1 protects cells under hypoxia from 2-DG (31).

In this work, we have used the most commonly used glycolytic inhibitor 2-DG that has been tested in clinical trials and has been proven to be well tolerated by patients (32). Other drugs that target glycolysis are being tested in preclinical models, and the findings indicate that different cell lines die in different manners when subjected to low glucose availability. In general, glucose deprivation kills hematopoietic cells by mitochondrial apoptosis, whereas mesenchymal or epithelial cells die by necrosis or by caspase-8–dependent apoptosis (19, 33). We observed that Rh4 and Rh30 cells died by necrosis when incubated in the absence of glucose (not shown) but by apoptosis when cultured with 2-DG, indicating that 2-DG and acute glucose deprivation do not promote death in the same manner. 2-DG is widely used to mimic glucose starvation. However, recent studies indicate that toxicity of 2-DG may be due to effects that are different from those in the absence of glucose. 2-DG inhibits glycolysis and usage of glucose to
produce ATP or fatty acids. However, 2-DG can be metabolized through the pentose phosphate pathway in some conditions (34), and a catalytic block does not sufficiently explain the toxicity of 2-DG (35). Moreover, 2-DG alters protein glycosylation in a manner that is different from that of glucose deprivation: While it inhibits N-glycosylation, it enhances O-GlcNAcylation. 2-DG has been shown to kill some cells in normoxia by inhibition of N-glycosylation, and the subsequent ER stress, rather than by inhibition of glycolysis (27). Our experiments indicate that ER stress mediates rhabdomyosarcoma cell death, because a sugar that reverts the effects of 2-DG on N-glycosylation, mannose (36), completely protected from cell death, and inhibition of the ATF4 ER stress pathway partially prevented apoptosis. Inhibition of glycolysis does not seem to be critical for the toxicity of 2-DG over rhabdomyosarcoma. The fact that mannose did not prevent ATP loss induced by 2-DG rules out the possibility that mannose prevents death because it is being metabolized and used as a glycolytic intermediate. Indeed, Kurtoglu and colleagues showed that mannose cannot revert 2-DG toxicity in anaerobic conditions, in which cells are more dependent on glucose, and toxicity of 2-DG would be primarily due to inhibition of anaerobic glycolysis (27).

The apoptotic mechanism by which 2-DG induces cell death has remained underexplored, even though this compound has been used in clinical trials. Apoptosis induced by glucose deprivation in hematopoietic cells is inhibited by overexpression of Bcl-2 or Bcl-xL, and it has been shown to be mediated by Noxa, Puma, or Bim (20, 25). We could not detect induction of Puma, possibly because this protein is usually induced in a p53-dependent manner, but the alveolar rhabdomyosarcoma cells used in this study are deficient in p53 (29, 30). Consistent with data that suggest that death induced by 2-DG is due to ER stress, we observed induction of Bim, which mediates death induced by ER stress in some systems (37). However, when Bim accumulation was prevented by RNA interference, no effect on cell death induced by 2-DG was observed (Fig. 6). Moreover, Bim induction was only partially prevented by mannose, although this sugar completely prevented cell death.

We observed that Noxa was critical for cell death. Noxa is a well-studied BH3-only protein that has recently been described to play a role in glucose metabolism by promoting glucose uptake but directing glucose flux away from the glycolytic pathway by an yet uncharacterized mechanism (23). Noxa/PMAIP1 mRNA had been observed to be induced in response to 2-DG (38), but its role in cell death had not been tested. In

Figure 6. Noxa and Mcl-1 levels are regulated posttranscriptionally. A, cells were treated with 10 mmol/L 2-DG for indicated times and collected for RT-PCR analysis. Results are representative of 3 independent experiments. B, RH4 cells were incubated for 24 hours in regular medium (control) or treated for 72 hours with 10 mmol/L 2-DG in the presence of Q-VD to prevent caspase-mediated protein degradation. Cells were further treated with 100 μg/mL cycloheximide (CHX) for indicated times and collected for Western blot analysis. Results are representative of 2 experiments, plus an experiment in which cells were treated for 48 hours, and identical results were obtained. C, cells were treated for indicated times with 10 mmol/L 2-DG and collected for the analysis of phosphorylation of eIF2α (P-eIF2) and S6 (P-S6) by Western blotting. D, cells were transfected with siRNA against Noxa, treated as in Fig. 5C, and subjected to Western blotting. Levels of Mcl-1 versus tubulin were analyzed by densitometry. Values shown are relative to levels of Mcl-1 in untreated cells after transfection of control siRNA.
It has been described that glucose deprivation activates AMP-activated protein kinase and inactivates mTOR, which leads to a decrease in Mcl-1 levels. In response to 2-DG, Mcl-1 decrease was also associated with inactivation of translation (21, 39).

To our knowledge, this article shows the first evidence of the implication of a BH3-only protein in death induced by 2-DG. Another glycolytic inhibitor, 3-bromopyruvate, promotes dephosphorylation of the BH3-only protein Bad (6), which has also been involved in cell death induced by glucose deprivation in hepatocytes (40). Our results indicate that Noxa is critical for sensitivity to cell death induced by 2-DG. A likely scenario would be that downregulation of Mcl-1 and its inactivation by Noxa would release Bak, which could then be activated. Our immunoprecipitation experiments suggest that Mcl-1 blocks apoptosis, in part, by sequestering Noxa and Bak. In addition, Bcl-xL prevented cell death when overexpressed, and we could detect (weakly) interaction of Bak with Bcl-xL. However, we were unable to detect release of Bak from Mcl-1 or Bcl-xL upon treatment with 2-DG. It is possible that only a very minor fraction is released, and the use of other antibodies would perhaps improve detection of interactions. In addition, other...
BH3-only proteins may also participate in the induction of apoptosis. Altogether, our results suggest that expression of Noxa could predict sensitivity to antilygcolytic drugs, that inhibition of glycolysis could be an effective novel strategy to treat alveolar rhabdomyosarcoma, and that antilygcolytic drugs should be further tested in clinical trials against this type of tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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