2-deoxyglucose induces Noxa-dependent apoptosis in alveolar rhabdomyosarcoma

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Abstract

Alveolar and embryonal rhabdomyosarcoma 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 anti-apoptotic Bcl-2 homologs Bcl-xL 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. Additionally, 2-DG promoted eIF2-alpha 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.

Abbreviations

2-DG, 2-deoxy-D-glucose; aRMS, alveolar rhabdomyosarcoma; eRMS, embryonal rhabdomyosarcoma; Q-VD, Q-VD-OPH.
Introduction

Rhabdomyosarcoma is the most common soft tissue tumor in children and adolescence, accounting for 4-5% of pediatric tumors. The two common histiotype types are a favorable group comprising embryonal rhabdomyosarcoma (eRMS) and an unfavorable group comprising alveolar rhabdomyosarcoma (aRMS) (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 ever-increasing attention as an anti-tumor target. Several metabolic pathways function differently in tumor and non-transformed 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 cells display mitochondrial respiratory defects (7) and an energy producing metabolic phenotype compared to 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). Additionally, rhabdomyosarcoma can be detected in patients using the PET technique, which is based on uptake of a glucose analog (12).
Sensitivity to antiglycolytics 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 pro-inflammatory form of cell death, as opposed to apoptosis (14). Regarding apoptosis, two 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-deoxyglucose. 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, 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 based on viability, recovery, growth, morphology and also cytogenetic analysis, antigen expression, DNA profile and isoenzymology. Cells were maintained in high-glucose (25 mM), pyruvate-free DMEM (Invitrogen) supplemented with 2mM L-glutamine, 200mg/ml penicillin, 100mg/ml streptomycin sulphate and 10% FBS (Invitrogen).

For treatments, cells were plated at a concentration of 200,000/ml and treated in fresh medium 24h later at 70% confluence (600,000/ml). Q-VD-OPH (SM Biochemicals LLC, CA) was used at 20 μM and added at the time of addition of 2-deoxyglucose. An equal amount of DMSO was added to the controls.

Western blots

Cells were trypsinized, washed with PBS, lysed by resuspending them in lysis buffer (5mM Tris-ClH/ 2% SDS, Complete antiprotease cocktail (Roche)) and frozen. For analysis of phospho-proteins and HIF-1α cells were lysed in RIPA buffer plus orthovanadate and 2-glycerol-phosphate. After sonication, protein concentration was measured with BCA (Pierce). Equal amounts of protein were mixed with Laemmli loading buffer. After electrophoresis, protein was transferred to a PVDF membrane (Millipore). Membrane was blocked with 5% nonfat dry milk in TBS-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 min at room temperature, washed and blocked with 0.1% BSA+ 0.1% Triton X-100 in PBS for 1h. Then they were incubated overnight with anti-active Bax (6A7, 556467, BD Pharmingen, 1:100) or anti-active Bak (Calbiochem/Merck Ab-1/TC100) 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, 20mM Tris/HCl (pH 7.4), 137mM NaCl, 2mM EDTA, 10% glycerol) plus Complete protein inhibitors (Roche) for 30 min. 30 µl of Protein G Magnetic Beads (Millipore) were washed 3x in immunoprecipitation buffer without CHAPS and then incubated in 1ml of CHAPS buffer with 2µg of antibody for 4h at 4ºC under rotation. 500µg of total cell extract was incubated overnight in 1ml of beads coupled with 2µg of antibody. The next day, beads were washed three times with immunoprecipitation buffer and incubated with 45 µl of immunoprecipitation buffer containing 2% SDS and 15 µl of SDS loading buffer (containing 5% beta-mercaptoethanol), for 10 min at 95ºC. Eluted proteins were subjected to SDS-polyacrylamide gel electrophoresis.

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 1-10 days at -20ºC. For experiments of transient transfection, fixation was performed in paraformaldehyde 4% dissolved in PBS at room temperature for 15 min before washing. Cells were further
washed, resuspended in PBS with 40µg/ml PI and 100µg/ml RNase A and incubated for 30 min at 37°C before FACS 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 6h with 25µl lipofectamine 2000 (Invitrogen) and 10µg DNA. For generation of Rh4 cells stably expressing Bcl-xL, they were transfected as described earlier, and selected with puromycin 0.5µg/ml. 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 8h (6h for Mcl-1) with siRNA 100nM premixed with Dharmafect1 (Dharmacon). Sequences are detailed in Supplementary Methods.

**RT-PCR**

Total RNA (3 µg), extracted using the Nucleospin RNA II kit (Macherey-Nagel), was used for cDNA synthesis with SuperScript II Reverse Transcriptase (Invitrogen). Amplifications were carried out using specific primers (Noxa forward 5’-CTCGACAAAAGCGTGGTCTC, rev 5’-CAACTGGAGCACCTCGGAC; Mcl-1 fwd 5’-GAGGAGGACGAGTTGTACCGG, rev 5’-CAGACCTGCCCATTGGCTT, β-Actin fwd 5’-CGGGACCTGACTGACTCC, rev 5’-CTTCATTGTGCTGGGTGC). For each set of primers, the number of cycles was adjusted so that the reaction end points fell within the exponential phase of product amplification, thus providing a semi-quantitative estimate of relative mRNA abundance.
RESULTS

Alveolar rhabdomyosarcoma cell lines are sensitive to 2-deoxyglucose

2-deoxyglucose (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 (aRMS) cell lines. 2-DG inhibited the growth of aRMS Rh4 cells incubated with doses of 2mM or higher, even though glucose is present at 25mM in the culture medium (Suppl. Fig. S1). We observed cell death at doses over 5mM (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 aRMS cell lines (Fig.1B and C).

Embryonal rhabdomyosarcoma (eRMS) generally have a better prognosis than aRMS. We observed that eRMS RD and A-204 cells were much more resistant than aRMS to 2-DG. RD cells only 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). In order to determine whether this cell growth arrest was irreversible, after 72h cells were washed and they were left growing in the absence of 2-DG. Cell growth arrest was reversible, since cells started growing back immediately after removing the drug (Fig. 1F).

Since HIF-1α frequently regulates tumor glycolytic phenotype, one possibility was that HIF-1α was differentially expressed in eRMS versus aRMS and it could be regulating uptake and toxicity of 2-DG in rhabdomyosarcomas. Thus, we analyzed the levels of this protein in the five cell lines. HIF-1α was virtually undetectable in all untreated cells. Upon
treatment, the levels of this protein did not increase, and its downregulation using siRNA
did not protect from 2-DG. Rather, a slight sensitization was observed (Suppl. Fig. S2).

Although aRMS express markers of muscular differentiation they lack the
possibility of terminal differentiation, what is believed to be caused by
PAX/FOXO1a chimeras (17, 18). Since differentiated cells stop proliferating, one of the
aims of the therapy is to promote differentiation of the tumor cells. We observed fusion
of aRMS cells characteristic of myotube formation and terminal differentiation after
treatment with 2-DG (Fig.2A and Suppl.Fig.S1). This was likely due to the fact that 2-DG
induced the downregulation of the fusion protein PAX3/FOXO1a (Fig.2B), what 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-deoxyglucose 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 that the form of
cell death is apoptosis, since death is inhibited by the caspase inhibitor Q-VD. However,
we were unable to detect cleavage of caspase-3 by western blot (not shown). In order
to verify that cell death is apoptosis we analyzed cleavage of the caspase substrate poly-
(ADP-ribose)polymerase (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). Additionally, we analyzed the nuclear
morphology of Rh4 cells after treatment with 2-DG and we observed classical apoptotic
chromatin condensation (Fig.3C).

9
Apoptosis induced by 2-deoxyglucose 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 (Suppl. 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.

In order to further demonstrate the involvement of the mitochondrial pathway in death by 2-DG, we overexpressed the anti-apoptotic Bcl-2 homologs Bcl-xL and Mcl-1 in Rh4 cells (Fig. 4A,B and Suppl. Fig. S4,S5). Both proteins prevented death induced by 2-DG. Conversely, downregulation of these proteins sensitized cells to 2-DG (Fig. 4C, 4D and S5).

Inhibition of glucose metabolism by limitation of glucose (20) or by 2-deoxyglucose (21) promotes down-regulation of the anti-apoptotic 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 anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-xL) increased during treatment, while Bax and Bak did not change significantly. We analyzed the levels of putative BH3-only proteins which 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 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 two 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. Pro-apoptotic proteins such as Noxa can free Bax/Bak allowing them to promote cytochrome c release. We studied interactions between these proteins and we observed that Mcl-1 is constitutively bound to Bim and Noxa (Fig. 5D, 5E). Additionally, we could detect very weakly interactions between Bak and Bcl-xL or Mcl-1 (Fig. 5F and Suppl. Fig. S6). Bcl-2 and Bax could not be co-immunoprecipitated with Bak or Bcl-xL/Mcl-1, respectively (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 post-transcriptional mechanisms (21, 23-25). We checked for transcriptional regulation and we 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). Since Mcl-1 is very sensitive to inhibition of translation, we analyzed the status of two pathways that lead to translation inhibition and Mcl-1 downregulation: Ser51 phosphorylation of the Initiation Factor 2-alpha (eIF2α) and mTOR inactivation (21, 26). We observed both phosphorylation of eIF2α and inactivation
(dephosphorylation) of the marker of mTOR status S6 (Fig. 6C). Additionally, we observed that Noxa was contributing 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. While 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 co-incubation 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. We indeed observed induction of several ER stress markers such as ATF4/CREB2, GRP58 and the ER chaperones GRP94/endoplasmin and GRP78/BiP (Fig. 7B). Mannose fully prevented the induction of these proteins. Moreover, mannose prevented accumulation of Noxa and loss of Mcl-1, and it reduced the induction of Bim (Fig. 7B). In order to verify that mannose was not interfering with all effects of 2-DG, for instance by reducing its uptake or providing metabolites for mitochondrial oxidation, we measured the effects of mannose on ATP depletion. As shown in figure 7C, 2-DG promoted a reduction of ATP levels that was not prevented by the addition of mannose. These results suggest that the Noxa/Mcl-1 axis is involved in ER-stress-induced apoptosis. To further analyze this, we treated the cells with the ER stressor tunicamycin at a dose that promoted cell death with the same kinetic 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 PERK ER stress pathway. ATF4 downregulation did not prevent Noxa increase (not shown) but it partially prevented cell death and loss of Mcl-1 (Fig. 7E and F).
DISCUSSION

Rhabdomyosarcoma are aggressive tumors for which more effective chemotherapy needs to be found. We provide evidence here that an inhibitor of the glycolytic metabolism is effective against alveolar rhabdomyosarcoma. This subgroup of rhabdomyosarcoma is characterized by a chromosomal translocation involving PAX3 or PAX7 and the FKHR (Foxo1) genes. PAX-FKHR fusion gene has been shown to alter the expression of some metabolic enzymes (28), which may account for the different sensitivity of these tumor cell lines when compared with the embryonal subtype. Other possible determinants of the sensitivity to 2-deoxyglucose are p53 and HIF-1, which regulate the glycolytic phenotype. p53 is not functional in any of the three –sensitive– aRMS lines tested (29, 30), which would agree with data that indicate that p53 protects cells from metabolic stress. However, RD cells are also deficient in p53 but still insensitive. According to our results, HIF-1α does not seem to be mediating the differential response to 2-DG either (Suppl. Fig. S2). We did however observe a small but reproducible sensitization to 2-DG when HIF-1α was downregulated, in accordance to studies by Lampidis 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-deoxyglucose, that has been tested in clinical trials and has been proven well tolerated by patients (32). Other drugs that target glycolysis are being tested in preclinical models, and may prove more effective in the future (4). Furthermore, it is possible that this drug would be more effective in combination regimes, since 2-DG synergizes with chemo and radio-therapy in vitro and in vivo.
Cell death by glucose deprivation has been studied in several 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, while 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-deoxyglucose is widely used to mimic glucose starvation. However, recent studies indicate that toxicity of 2-DG may be due to different effects than 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 different manner than 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 endoplasmic reticulum (ER) stress, rather than by inhibition of glycolysis (27). Our experiments indicate that ER stress mediates rhabdomyosarcoma cell death, since 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 appear 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, Lampidis 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 aRMS 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 RNAi, 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 which 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 a yet uncharacterized mechanism (23). Noxa/PMAIP1 mRNA had been observed to be induced in response to 2-deoxyglucose (38), but its role in cell death had not been tested. Additionally, Noxa has been shown to mediate death by ER stressors (22). In our studies, both Noxa induction and loss of Mcl-1 seem to be a consequence of ER stress rather than energetic stress, since addition of mannose prevented both events (Fig. 7B). Regulation of both proteins was post-transcriptional. In the case of Noxa, its induction is only observed at long time points (3 days), while we can detect 40% death at 48h. This suggests that Noxa induction and stabilization are not as critical as its activation, which could possibly occur via phosphorylation by CDK5 (23). Mcl-1 is likely to be downregulated due to inhibition of translation. eIF2α phosphorylation had been shown to regulate Mcl-1 levels...
(26). In this line, we show that the ER stressor tunicamycin, which, like 2-DG, induces ER stress by inhibiting N-glycosylation, also promoted loss of Mcl-1 and induction of Noxa. Downregulation of ATF4 partially prevented Mcl-1 downregulation in response to 2-DG, but more experiments are required to determine how ATF4 contributes to maintain its levels. Besides ER-stress, a contribution of the mTOR pathway to downregulation of Mcl-1 is also likely: it has been described that glucose deprivation activates AMPK 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, the present report shows the first evidence of the implication of a BH3-only protein in death induced by 2-deoxyglucose. 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. Additionally, 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. Additionally, other BH3-only proteins may also participate in the induction of apoptosis. Altogether, our results suggest that expression of Noxa could predict sensitivity to antiglycolytic drugs, that inhibition of glycolysis could be an effective novel strategy to treat alveolar rhabdomyosarcoma, and that anti-glycolytic drugs should be further tested in clinical trials against this type of tumors.
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FIGURE LEGENDS

Figure 1. Alveolar rhabdomyosarcoma but not embryonal rhabdomyosarcoma cell lines are sensitive to 2-deoxyglucose

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 DMSO as indicated, collected after 72h and subjected to sub-G1 analysis. For control samples, cells were plated at half concentration to avoid death due to overgrowth. Average and SEM of at least 3 experiments is shown.

F. 24h after plating A204 cells, one well was stained with crystal violet (C=control) and the rest were grown in the absence (C72) or the presence of 2-DG 20mM for 72h. 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 min and solubilized in 10% SDS. Absorbance at 595 nm was measured.

Figure 2. 2-deoxyglucose promotes differentiation of alveolar rhabdomyosarcoma cells

A. 4x10^5 RH4 or 1.8x10^5 RH30 cells were plated in 6-well plates and treated with 10mM 2-DG in the presence of Q-VD in complete media (RPMI + 10%FBS) or differentiation media (DM, serum-free RPMI) for 48h. See microscope settings in Supplementary Methods. Arrows indicate fused cells with myotube morphology. Larger images are shown in Suppl.Fig.S1.

B. Immunoblot showing reduced PAX3/FOXO1a levels in RH4 and RH30 cells in the presence of 2-DG. Lysates of cells treated as indicated were collected at 55h (RH30) or 72h (RH4) and blotted with anti-Foxo1 antibody.
Figure 3. 2-deoxyglucose promotes apoptosis in RH4 cells

A, B. 2-DG induces cleavage of PARP and caspase activity which are inhibited by Q-VD. Rh4 cells were cultured for the indicated times with 2-DG (10mM) in the presence or absence of Q-VD. Cell lysates were prepared as described in Methods and proteins were resolved by immunoblot (A). Lysates were incubated with AC-DEVD-amc (Pharminingen) following manufacturer’s instructions (B). Results show average and SEM of 3 experiments. Fluorescence values (arbitrary units) are divided by the value of fluorescence of untreated cells.

C. Detection of active Bax and Bak and chromatin condensation. Cells were left untreated or treated with 2-DG in the presence or absence of Q-VD for 48h.

Figure 4. Apoptosis induced by 2-deoxyglucose proceeds through the mitochondrial pathway.

A, B. RH4 cells were transfected with 2.5µg of membrane-targeted GFP vector and either none (-), 2.5µg (low) or 7.5µg (high) of vector encoding Mcl-1 (A and Suppl. Fig. S5) or Bcl-xL (B). Empty vector was added up to 10µg total DNA. Cells were rinsed and 2h later they were trypsinized and replated. 15h later medium was replaced and they were either left untreated or treated with 2-DG 10mM for 48h. Sub-G1 analysis of GFP(+) cells (approx. 10-15%) is shown.

C, D. Cells were transfected with siRNA against Bcl-xL or Mcl-1 and subjected to further treatment with 2-DG for the indicated times and sub-G1 analysis. Asterisks denote significant differences versus the controls. Western blots are shown in Fig.S5.

E. Rh4 cells were cultured for the indicated times with 2-DG 10mM, in the presence or absence of Q-VD. Indicated proteins were resolved by immunoblot.
Figure 5. 2-deoxyglucose regulates BH3-only proteins and induces Noxa-dependent apoptosis

A. Rh4 cells were cultured for the indicated times with 2-DG, in the presence or absence of Q-VD. Indicated proteins were resolved by immunoblot. Bands immunoreactive with anti-Puma antibody of approx. 23 and 16 kDa, and of 23 kDa with anti-Bim are shown. Untreated control cells (labeled as “c”) were incubated in regular culture medium for 24h.

B. Cells were transiently transfected with control oligonucleotide (labeled as “C”) or siRNA against Noxa or Bim and subjected to further treatment with 2-DG for indicated times. Western blot against the indicated proteins is shown. N.S. indicates a non-specific band detected by Bim antibody, shown as loading control.

C. Cells were treated as in B with siRNA against Noxa, Bim or both combined, and subjected to sub-G1 analysis. See also Fig. S5 for a second siRNA against Noxa. Asterisks indicate p<0.01 (n=4).

D, E, F. Cells were treated with 2-DG 10mM for 48h. Immunoprecipitation and western blot with indicated antibodies were performed as indicated under Methods. Asterisks denote unspecific bands. Input was 5% of immunoprecipitated protein.

Figure 6. Noxa and Mcl-1 levels are regulated post-transcriptionally.

A. Cells were treated with 2-DG 10mM for indicated times and they were collected for RT-PCR analysis. Results are representative of three independent experiments.

B. RH4 cells were incubated for 24h in regular medium (control) or treated for 72h with 2-DG 10mM in the presence of Q-VD to prevent caspase-mediated protein degradation. Cells were further treated with cycloheximide 100µg/ml for indicated times and collected for western blot analysis. Result is representative of 2 experiments, plus an experiment in which cells were treated for 48h and identical results were obtained.
C. Cells were treated for indicated times with 2-DG 10mM and collected for analysis of phosphorylation of eIF2α and S6 by western blot.

D. Cells were transfected with siRNA against Noxa, treated as in Figure 5C and subjected to western blot. 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.

Figure 7. ER stress response mediates apoptosis induced by 2-deoxyglucose.

A. Rh4 cells were cultured for 72h with or without 2-DG 10mM in the presence or absence of mannose at indicated doses and collected for subG1 analysis. Graph shows average and SEM of 4 experiments.

B. Rh4 cells were incubated with 2-DG 10mM with or without mannose 5mM for indicated times. Control cells were either left untreated for 24h (“C”) or treated with mannose for 24h (“M”).

C. 2x10^3 Rh4 cells were cultured in 96 well-plates for 20h as indicated. ATP levels were measured using ATPlite 1 step kit (Perkin-Elmer) and normalized to cell number in each well to prevent effects of decrease in cell numbers by treatment with 2-DG. Values shown are relative to untreated controls. Graph shows average and SEM of 4 experiments.

D. RH4 cells were treated with 20ng/ml tunicamycin and subjected to western blot and subG1 analysis. Average number of apoptotic cells is shown (n=3).

E, F. Cells were transfected with ATF4 or control siRNA and collected for subG1 (E) or western blot analysis (F). Asterisks denote significant effects. Note that siRNA against ATF4 also reproducibly upregulated basal Mcl-1 levels after transfection.
Fig 3
2-deoxyglucose induces Noxa-dependent apoptosis in alveolar rhabdomyosarcoma

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