Citation: Blood Cancer Journal (2011) 1, e31; doi:10.1038/bcj.2011.27

www.nature.com/bcj

ORIGINAL ARTICLE

Glucocorticoid-induced cell death is mediated through reduced glucose metabolism in lymphoid leukemia cells

E Buentke[1], A Nordström[1], H Lin[1], A-C Björklund[1], E Laane[2], M Harada[1], L Lu[3], T Tegnebratt[4], S Stone-Elander[4], M Heyman[5], S Söderhäll[5], A Porwit[1], C-G Östenson[6], M Shoshan[1], K Pokrovskaja Tamm[1,7] and D Grandér[1,7]

[1]Department of Oncology and Pathology, Cancer Centre Karolinska, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden; [2]Divison of Hematology, Internal Medicine Clinic, North Estonian Regional Hospital, Tallinn, Estonia; [3]Department of Comparative Medicine, KERIC, Karolinska University Hospital, Stockholm, Sweden; [4]PET, Karolinska University Hospital and Department of Clinical Neurosciences, Karolinska Institutet, Stockholm, Sweden; [5]Childhood Cancer Research Unit, Department of Women and Child Health, Astrid Lindgren Children's Hospital, Stockholm, Sweden and [6]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden

Malignant cells are known to have increased glucose uptake and accelerated glucose metabolism. Using liquid chromatography and mass spectrometry, we found that treatment of acute lymphoblastic leukemia (ALL) cells with the glucocorticoid (GC) dexamethasone (Dex) resulted in profound inhibition of glycolysis. We thus demonstrate that Dex reduced glucose consumption, glucose utilization and glucose uptake by leukemic cells. Furthermore, Dex treatment decreased the levels of the plasma membrane-associated glucose transporter GLUT1, thus revealing the mechanism for the inhibition of glucose uptake. Inhibition of glucose uptake correlated with induction of cell death in ALL cell lines and in leukemic blasts from ALL patients cultured *ex vivo*. Addition of di-methyl succinate could partially overcome cell death induced by Dex in RS4;11 cells, thereby further supporting the notion that inhibition of glycolysis contributes to the induction of apoptosis. Finally, Dex killed RS4;11 cells significantly more efficiently when cultured in lower glucose concentrations suggesting that modulation of glucose levels might influence the effectiveness of GC treatment in ALL. In summary, our data show that GC treatment blocks glucose uptake by leukemic cells leading to inhibition of glycolysis and that these effects play an important role in the induction of cell death by these drugs.
Blood Cancer Journal (2011) **1,** e31; doi:10.1038/bcj.2011.27; published online 29 July 2011
Keywords: glucocorticoids; leukemia; glycolysis; cell death

Introduction

Normal and tumor cells differ markedly in their energy metabolism. When glucose, a primary source of energy, is metabolized in normal cells in the presence of oxygen, the process results in the complete oxidation of glucose and involves cytoplasmic glycolysis and the mitochondrial citric acid cycle and oxidative phosphorylation (OxPhos). In contrast, tumor cells rely mostly on glycolysis, that is, conversion of glucose into pyruvate and further into lactate, rather than on mitochondrial OxPhos, even in the presence of adequate oxygen. This phenomenon is referred as to the Warburg effect.[1] This pathway is not as efficient as OxPhos in producing energy. Therefore, to meet their high-energy demands, tumor cells dramatically increase the rate of glycolysis. This is primarily achieved by increased glucose uptake and expression of glucose transporters, GLUTs, at the cell membrane.[2] The increased dependence of cancer cells on glycolysis for energy production is now generally regarded as a novel pathway for targeting in cancer treatment.

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy manifested by the expansion of immature B or T cells. It has been shown that pre-B-ALL cells acquire an altered glucose metabolism, higher expression of the glucose transporter 1, GLUT1, and a particular sensitivity to the glycolysis inhibitor 2-deoxy-D-glucose, 2-DG.[3] Glucocorticoids (GCs) have been used as important therapeutic agents in the treatment of ALL and other lymphoid malignancies for more than 50 years.[4,5] *In vivo* and *ex vivo* GC sensitivity is a major prognostic factor in childhood ALL[6,7] and treatment failure is related to the resistance to GCs. It was demonstrated that GC resistance in precursor-B-ALL is associated with increased expression of genes involved in glucose metabolism[8] and that an increased glycolytic rate in ALL cells is directly related to GC resistance.[9] Importantly, this resistance could be reversed by drugs that inhibit glycolysis, thus suggesting that targeting the glycolytic pathway may be a valuable strategy to modulate GC resistance.[9]

The effect of GCs on lymphoid cells is dramatic and includes G1-phase cell-cycle arrest and apoptosis. Induction of apoptosis has been proposed to be the major effector mechanism for the beneficial effects of GC treatment in lymphoid malignancies.[10] We have also shown that dexamethasone (Dex)-induced activation of the apoptotic response was associated with the sensitivity to the drug in primary ALL samples, and that this *ex vivo* Dex sensitivity correlated with an early response to polychemotherapy.[11]

Endogenous GCs are essential for the maintenance of blood glucose levels. We therefore asked whether a part of the therapeutic effect of GCs in ALL is mediated by affecting glucose metabolism in the leukemic cells. Here, we show for the first time that the GC Dex profoundly affects the leukemic-cell energy metabolism by inhibiting glucose utilization and uptake, and downregulating GLUT1 expression in ALL cell lines. Moreover, Dex-mediated inhibition of (2-18F) 2-fluoro-2-deoxy-D-glucose (FDG) uptake correlated with the *ex vivo* sensitivity to the drug in primary leukemic blasts from pediatric ALL patients, strongly suggesting that Dex-mediated cell killing occurs through inhibition of glucose metabolism.

Correspondence: Dr KP Tamm, Department of Oncology and Pathology, Cancer Centre Karolinska (CCK), R8:03, Karolinska Institutet and Karolinska University Hospital, Stockholm S-171 76, Sweden.
E-mail: katja.pokrovskaja@ki.se
[7]These authors contributed equally to this work.
Received 13 January 2011; revised 13 April 2011; accepted 16 May 2011

Materials and methods

Cell lines, culture conditions and treatment

Two pre-B ALL cell lines, RS4;11 (ATCC, no. CRL-1873, USA), and SupB15 (DSMZ—no. ACC 389, Germany), the T-ALL cell line CCRF-CEM and the ALL cell line Reh-6 (F Albertioni, Karolinska Institutet, Stockholm, Sweden) were used in this study. The cells were cultured in RPMI 1640 medium (Sigma-Aldrich Sweden AB, Stockholm, Sweden) (containing 25 mM Hepes for the RS4 and SupB15 cell lines), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 μg/ml streptomycin and penicillin (Invitrogen AB, Lidingö, Sweden) and maintained in a humidified incubator in 5% CO_2 at 37 °C. Cells were treated with indicated doses of Dex (Oy Organon AB, Helsinki, Finland), for the indicated times. Glucose-free RPMI 1640 medium (Invitrogen) supplemented with 1 g/l and 0.5 g/l of glucose was used in some experiments.

Liquid chromatography–mass spectrometry

Cold methanol (200 μl) was added to frozen RS4;11 cells in Eppendorf tubes together with a few mg of 0.25–0.5-mm glass beads (Retsch GmbH, Haan, Germany) and an internal standard, $^2H_5$-Phenylalanine (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). The tubes were placed in a bead mill (Retsch 301 MM) and shaken at 30 Hz for 30 s. Samples were subsequently spun at 16 000 RPM for 5 min and the supernatant was collected and evaporated to dryness in a Speedvac, and stored at −80 °C until further analysis. For liquid chromatography–mass spectrometry analysis, Agilent binary pump 1200 was coupled to an Agilent 6410 triple quadrupole mass spectrometer, which was operated in electrospray (ESI)-negative ionization mode. For separation, we used hydrophilic interaction chromatography (ZIC-HILIC, 2.1 mm × 100 mm, Merck SeQuant AB, Umeå, Sweden). Mobile phase (A) consisted of H_2O, 25 mM ammonium acetate and (B) of 95:5 acetonitrile: H_2O, 25 mM ammonium acetate. Chromatographic gradient profile was as follows (150 μl/min): 0–1 min 90% (B), 1–30 min, linear gradient 90% (B) to 90% (A), 30–40 min 90% (A), 40–41 min 90% (A) to 90% (B), 41–60 min re-equilibration at 90% (B). Before analysis, the samples were re-suspended in 100 μl 90:10 acetonitrile:H_2O and 10 μl sample was injected on to the column for each analysis. Synthetic standards of all analytes were obtained from Sigma Aldrich (St Louis, MO, USA) and used for identification of optimal multiple-reaction monitoring transition and analyte retention time. The selected multiple-reaction monitoring transitions were (with *m/z* values inside the brackets): glyceraldehyde-3-phosphate (169-79), phosphoenolpyruvate (167-79), lactate (89-43), NAD^+ (662-540), $NADP^+$ (742-620), citrate (191-111) and malate (133-71). The ESI potential was set to −4000 V. The area representing the specific analytes in each multiple-reaction monitoring chromatogram was normalized to that of the internal standard ($^2H_5$-Phenylalanine).

Patients and culture of primary cells

The study included leukemic cells from 19 patients diagnosed with precursor-B ALL according to the World Health Organization classification.[12] Bone marrow (in 17 cases) or peripheral blood (in two cases) were obtained on the day of diagnosis and contained more than 80% leukemic blasts as established during routine diagnostic procedure using the multiparameter fluorescence-activated cell sorting analysis as described before.[13] All patients or their parents were informed of the investigative nature of this study, and informed consent was obtained from each patient/parent in accordance with the ethical committee requirements (Stockholm, Sweden).

Mononuclear cells were isolated from bone marrow or from peripheral blood by centrifugation on a Ficoll/Hypaque (Lymphoprep, Oslo, Norway) gradient and cryo-preserved in liquid nitrogen. Leukemic cells were cultured *ex vivo* at a cell concentration of 1×10^6/ml in RPMI 1640 culture medium as for the cell lines (see above).

Inhibitors and antibodies

The GC-receptor (GR) antagonist RU38486 (RU486) was used at 100 nM. Dimethyl succinate (Sigma-Aldrich) was used at 7.7 mM as an extra energy source. The antibodies against GLUT1 immunostainings were from R&D Systems Europe Ltd (Abingdon, UK), and against GLUT1 and Na^+K^+-ATPase Westerns—from Abcam plc (Cambridge, UK); the allophycocyanin-conjugated (APC) goat anti-mouse Ig was from Pharmingen (Becton Dickinson, Stockholm, Sweden) and fluorescein isothiocyanate-conjugated swine anti-rabbit from DakoCytomation (Glostrup, Denmark). Isotype-matched control antibodies used were from DakoCytomation.

Assessment of apoptosis by immunostainings and flow cytometry

Apoptosis was assessed as redistribution of plasma membrane phosphatidyl serine and changes in mitochondrial membrane potential, $\Delta\Psi$m. Annexin V FLUOS (Roche Diagnostics Gmbh, Mannheim, Germany) and tetramethylrhodamine ethyl ester perchlorate (TMRE, Molecular Probes, Invitrogen AB) or annexin V and propidium iodide (PI) double stainings were performed as previously described.[14,15] The staining for GLUT1 was performed according to the manufacturer's instructions. At least 10 000 events were acquired on a LSR II flow cytometer (Becton Dickinson) equipped with a 355 nm UV laser or a fluorescence-activated cell sorting calibur flow cytometer (Becton Dickinson). Data were analyzed using the FlowJo (Tree Star, Inc., Olten, Switzerland) and Cell Quest software (Becton Dickinson), respectively.

Glucose consumption

Glucose consumption from the cell culture medium was measured using the Amplex Red Glucose kit (Invitrogen, Molecular Probes). Cells were set up at 200 000 or 400 000/ml in triplicates and treated next day for 16 or 24 h with 100 nM (RS4;11, SupB15, Reh cells) or 1 uM (CEM cells) of Dex or 2 mM 2DG. Supernatant samples were frozen from all time points including 0 h (at set up) that served as the base value. The samples were diluted 400 or 1000-fold and the assay was performed according to the manufacturer's instructions. The absorbance was measured at 560 nm using a VERSAmax tunable microplate reader. The values were calculated according to the standard curve and then subtracted from the base line to obtain values of glucose consumption from the growth culture medium by the cells.

Glucose utilization and glucose uptake

Ttritium-labeled glucose and 2-DG were purchased from Sigma-Aldrich Sweden AB. The material was purified from free tritium by addition of water followed by freeze-drying twice. The glucose was then kept frozen at −20 °C until use. Glucose utilization was measured as previously described with minor changes.[16] Briefly, 50 000 of the control or Dex pre-treated cells

were placed in an incubation vial with either 5.5 (low) or 16.7 mM (high) glucose and 1 μCi [5-^{3}H] glucose in 100 μl of Krebs bicarbonate buffer, pH 7.4. Each vial with its content was placed in a scintillation bottle containing 500 μl water, sealed and gassed with O_2/CO_2 (19:1 by volume) for 3 min. After a 90-min incubation at 37 °C, 100 μl 0.1 M HCl was injected into the vial to stop the reaction. The bottle and vial was left at room temperature overnight to let the water in the bottle absorb the ^{3}[H] water produced during glucose metabolism. The next day, the vial with its acidified content was removed, and 10 ml scintillation fluid (Ultima Gold; Packard, Perkin Elmer, Upplands Väsby, Sweden) was added to the bottle. The samples were then assayed for radioactivity in a liquid scintillation spectrophotometer (Tri-Carb 1900 TR liquid scintillation analyzer; Packard). Parallel incubations were performed without cells and with a known concentration of ^{3}[H] water. Glucose uptake was performed as described previously with minor changes.[17] In short, cells were pre-treated with 100 nM Dexa or left untreated for indicated times. A total of 10^6 cells were starved in phosphate-buffered saline at room temperature for 30 min and incubated at 37 °C for 10 min in phosphate-buffered saline containing 5 μM 2-[^{3}H]-2-DG. Scintillation fluid (10 ml) was added and the samples were then assayed for [^{3}H] glucose content in a liquid scintillation spectrophotometer. Average and standard deviation of triplicates were calculated.

^{18}F-2-DG uptake

Aliquots of [^{18}F] FDG produced for daily clinical PET studies at the Karolinska Hospital were used in the experiments. Radiotracer synthesis was performed using a microlab (GE Healthcare, Solna, Sweden) synthesis module, and all tests for radiochemical identity and purity have been approved before release. Cells were grown in a complete medium for 24 h and then treated with Dex for 6–16 h in triplicate. The medium was changed to glucose free and 10 μCi of FDG was added to each sample and incubated for 1 h in 5% CO_2 at 37 °C. Cells were washed three times with ice-cold phosphate-buffered saline and radioactivity was measured on 1480 Automatic gamma counter Wizard3 (Perkin Elmer). Data were normalized to the total protein concentration.

Isolation of membrane-associated proteins and western blotting

Plasma membrane-associated proteins were isolated using a Plasma Membrane Protein extraction kit (BioVision, AH Diagnostics AB, Skärholmen, Sweden). A total of 5×10^8 cells were used for the protein extraction, which was performed as suggested by the manufacturer. For western blot analysis, 15 μg of plasma membrane-associated proteins were separated on 4–12% poly-acrylamid gels (Invitrogen) and western blotting was performed as described.[15] Antibodies against Na^+K^+-ATPase (Abcam) for membrane-associated proteins and against β-actin for the cytosolic fraction were used as loading controls.

Primers and real-time PCR

The following primers for monitoring GLUT1 RNA expression by real-time (RT) PCR were used: GLUT1-R 5′-AAGCGGCC-CAGGATC-3′ and GLUT1-F 5′-CATCAATGCCCCCCA-3′. Primers for human β-actin R 5′-GCGGATGTCCACGTC-3′ and F 5′-GCCCTGAGGCACTCT-3′ were used as control. Briefly, 2×10^6 RS4;11 cells were grown in 5 ml medium in six-well plates for 24 h before treatment with 100 nM Dex for 16 or 24 h. The cells were washed once with phosphate-buffered saline, and then total RNA from each sample was extracted with TRI Reagent from Ambion (Applied Biosystems, Europe BV, Stockholm, Sweden, AM9738). Complementary DNA was synthesized from 1 μg total RNA with SuperScript II Reverse Transcriptase from Invitrogen, SYBR GREEN PCR Master Mix was used for real-time PCR and the 7500 Real-Time PCR system from Applied Biosystems, Europe BV. Standard curves with GLUT1 and β-actin primers were generated using a control sample of complementary DNA from RS4;11 cells.

Statistical analysis

Student's *t*-test (Microsoft Excel), Wilcoxon scores rank-sum (Mann–Whitney) test, Fisher's exact test and Pearson's test were used for statistical analysis. The parameters used for each of these tests are described in the corresponding Figure Legends.

Results

Profound effects of Dex treatment on glucose metabolism

To determine the effects of Dex on glucose metabolism in ALL cells, we screened a Dex-sensitive cell line, RS4;11[(ref. 11)] treated with Dex for 16 and 24 h for a number of metabolic molecules using liquid chromatography and mass spectrometry. A glucose analog, 2-deoxy-D-glucose, 2-DG, was used in parallel to block glucose metabolism.[9] The major initial pathway in glucose metabolism is glycolysis that generates pyruvate. Molecules formed during the conversion of glucose to pyruvate, such as glyceraldehyde 3-phosphate and phosphoenolpyruvate, were significantly decreased after both Dex and 2-DG treatment (Figure 1). In addition, pyruvate is converted to lactate to maintain glycolysis at a high rate via the generation of NAD^+, a reaction essential for cancer cells. Both the amounts of lactate and NAD^+ molecules were significantly decreased after both Dex and 2-DG treatments. In the cell, phosphorylated glucose may also enter the pentose phosphate pathway that is often upregulated in cancer cells. The pentose phosphate pathway generates ribose-5-phosphate and NADPH to support nucleic acid synthesis. There was no increase, but rather a decrease at 24 h of ribose-5 phosphate and a prominent decrease in $NADP^+$ at both 16 and 24 h (data not shown and Figure 1).

Pyruvate is normally converted into acetyl-CoA to serve as a substrate for the citric acid (Krebs) cycle. Citrate is an early, and malate is a late metabolite of the cycle; both were decreased in the Dex or 2-DG-treated RS4;11 cells at 24 h of treatment, indicative of inhibition of the mitochondrial energy metabolism as well (Figure 1).

Most of these metabolites were also reduced upon both Dex and 2-DG treatment of another ALL cell line, CCRF-CEM, that is moderately sensitive to 1 μM of Dex[11] (data not shown). These data show that Dex inhibits glucose metabolism, similar to 2-DG.

Dex inhibits glucose consumption, utilization and uptake

Similar to glucose, 2-DG is taken up by GLUTs, and is phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate, but cannot be metabolized further. Data from the metabolic screen made in the two different cell lines mentioned above showed that Dex affects glucose metabolism in a manner similar

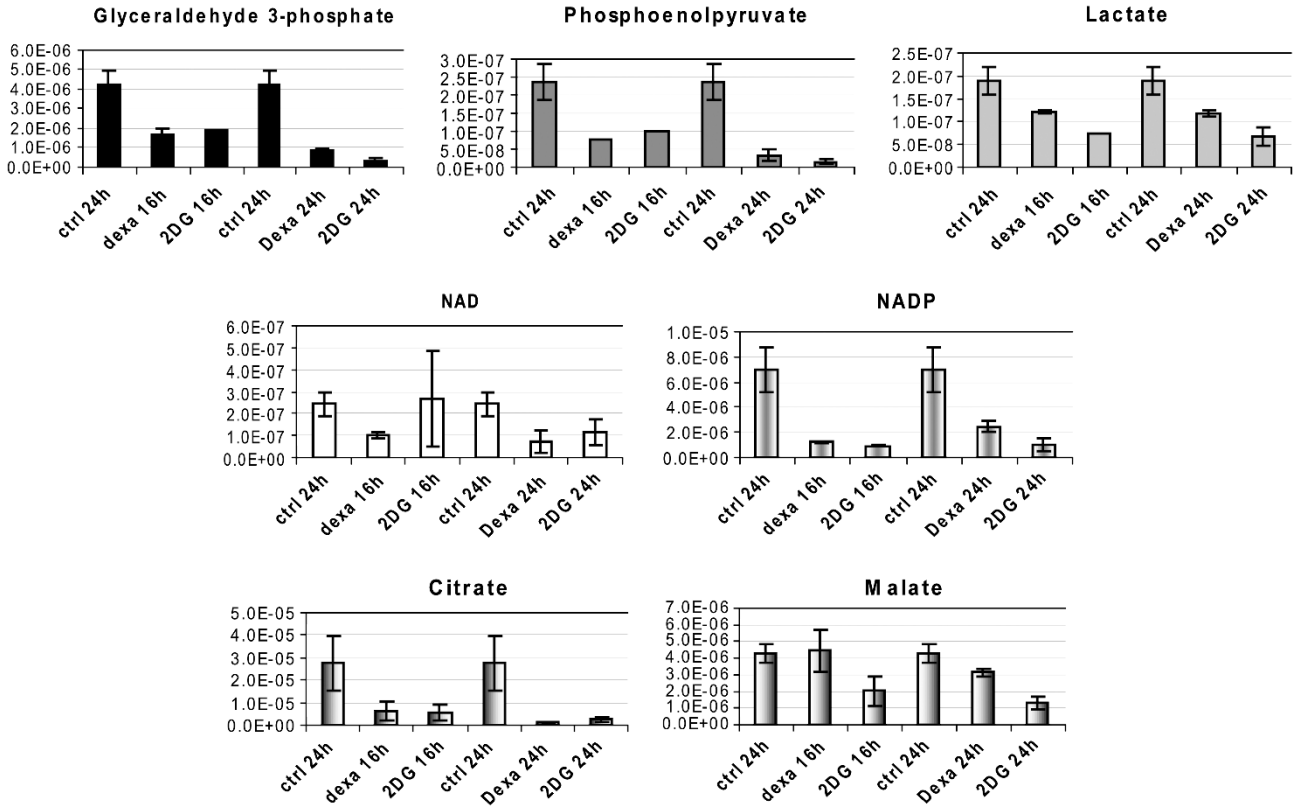


Figure 1 Profound inhibition of glucose metabolism by Dex treatment of RS4;11 cells. RS4;11 cells were left untreated (ctrl) or were treated with 100 nM Dex or 2 mM 2-DG for 16 and 24 h in triplicates; one control was harvested at 24 h. Metabolites were assessed by liquid chromatography and mass spectrometry. The data were normalized to the standard and to the total area of metabolites under the curve and the mean values are presented. Student's *t*-test *P* values for the control vs Dex treatment at 16 or 24 h: glyceraldehyde 3-phosphate, $P<0.01$; phosphoenolpyruvate, $P<0.01$; lactate, $P<0.05$; NAD, $P<0.03$; NADP, $P<0.04$; citrate and malate, $P<0.05$ only at 24 h. All calculations, standard deviations and the Student's *t*-test (two-tailed, two-sample unequal variance) were made in Excel.

to 2-DG. Therefore, we next asked whether Dex affects glucose consumption from the growth medium. ALL cell lines were grown for 24 h in the absence or presence of Dex and the levels of glucose in the growth medium were assessed using the Amplex Red Glucose assay. 2-DG was used to block glucose metabolism in parallel. The basal glucose concentration in the culture medium before the addition of the drugs was measured in each experiment for each cell line. The values following the treatment of each sample were then subtracted from these baseline values to obtain an estimate of glucose uptake from the growth medium. Thus, RS4;11, SupB15, CCRF-CEM and Reh ALL cell lines cultured in the absence of drugs consumed glucose, while glucose consumption in 2-DG-treated cultures was inhibited (Figures 2a–d). Dex inhibited glucose consumption in RS4;11 and SupB15 cell lines but not in Dex-resistant Reh[18] cells (Figures 2a, b and d). Inhibition of glucose consumption was significant in CCRF-CEM when 1 μM and 100 μM concentrations of Dex were used (Figure 2c). To examine whether Dex affects glycolytic flux, glucose utilization in cells cultured in medium containing tritiated glucose was assessed by measuring intracellular amounts of tritiated water.[16] Dex inhibited glucose utilization in RS4;11, and to a lesser extent, in CCRF-CEM cells. Dex did not affect glucose utilization in Reh cells at either 12 or 24 h (Figure 2e). To further understand the mechanism of inhibition of glucose utilization by Dex treatment, we studied glucose uptake using tritium-labeled 2-DG. Similar to glucose utilization, 2-DG uptake was reduced in both RS4;11 and to a lesser extent in CCRF-CEM cells and was unaffected in Reh cells (Figure 2f). Similarly, [2–^{18}F] 2-fluoro-2-deoxy-D-glucose, FDG uptake was inhibited in both RS4;11 and SupB15 already after 6 h of treatment (Figure 2g). FDG uptake was also inhibited in CCRF-CEM treated with 1 and 10 μM of Dex, but was unaffected in Reh cells (data not shown). Thus, the three different methods showed that Dex inhibits glucose consumption, utilization and uptake in RS4;11, SupB15 and CCRF-CEM but not in Reh ALL cell lines. These data also suggested that inhibition of glucose uptake at as early as 6 h after Dex treatment, that is, before any apoptosis or growth arrest would occur,[11] might represent the mechanism of Dex-induced cell death in these cells.

Dex-induced cell death correlates with the inhibition of glucose uptake

The ALL cell lines RS4;11 and SupB15 are sensitive to 100 nM of Dex and undergo extensive cell death after 36 h of Dex treatment while Reh cells are entirely resistant to Dex (Figure 3a and Laane E *et al.*[18] The Dex antagonist RU486 inhibited this response in the sensitive lines, demonstrating the specificity to Dex-induced effects. Thus, inhibition of glucose metabolism and uptake by Dex in RS4;11 and Sup-B15 vs Reh cell lines correlated with the ability of Dex to induce cell death. CCRF-CEM cells start undergoing apoptosis with 10-fold higher concentrations of Dex are used,[11] that is, with 1 and 10 μM (Figure 3b). Thus, even in these less-sensitive cells, inhibition of glucose uptake correlated with cell death induced by higher concentrations of the drug.

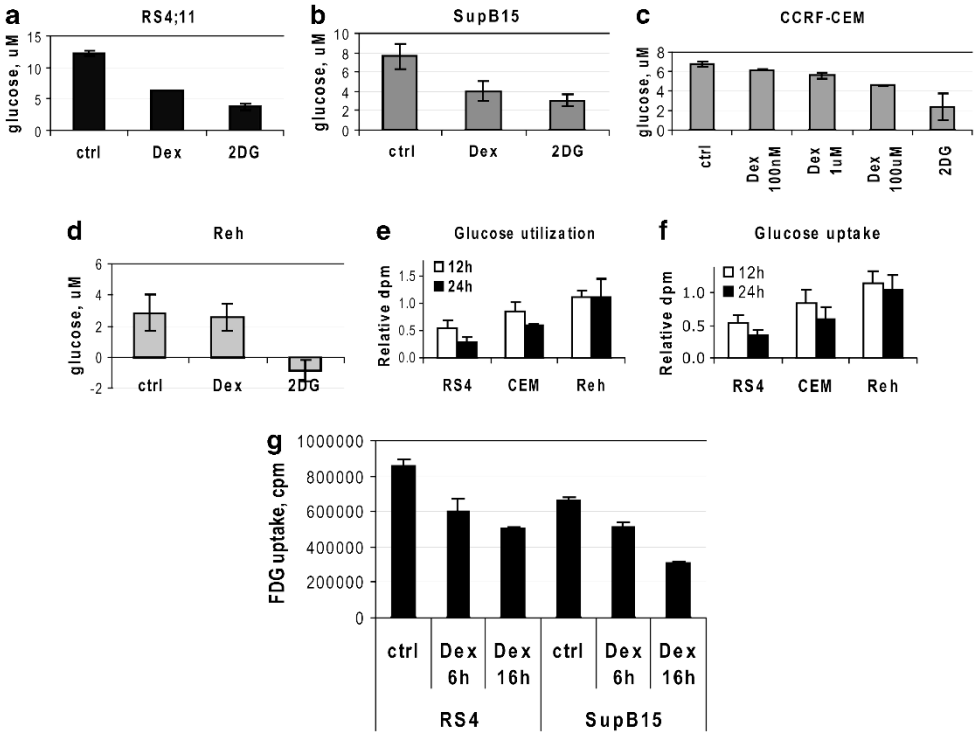


Figure 2 Inhibition of glucose consumption, utilization and uptake by Dex treatment of ALL cell lines. (**a**–**d**) RS4;11, SupB15, CCRF-CEM and Reh ALL cell lines were cultured for 24 h in the absence (ctrl) or presence of 100 nM Dex (or indicated concentrations for CCRF-CEM cells) or 2-DG. Glucose concentration in the medium was measured using the Amplex Red Glucose assay kit. The bars represent glucose consumption calculated by subtracting the values from the baseline glucose concentration in the growth medium measured before the addition of the drugs. Experiments were performed three independent times. Error-bars represent standard deviation. (**e**) RS4;11, CCRF-CEM and Reh cells were cultured in the absence or presence of Dex (100 nM for RS4 and Reh, 100 μM for CEM cells) for 12 or 24 h and incubated for an additional 90 min with [^{3}H]-glucose at 37 °C. Glucose utilization was studied by measuring resulting [^{3}H]-H_2O. The data are a summary of three independent experiments and presented as a ratio to control. (**f**) Cells were treated as in **e**. Glucose uptake was measured after 10 min incubation at 37 °C with [^{3}H]-2-DG. The data represent averages of three independent experiments and are presented as a ratio to control. (**g**) RS4;11 and SupB15 were cultured as in **a** in triplicate and treated with 100 nM Dex for 6 h or 16 h and glucose uptake was measured using FDG.

To further study whether inhibition of glucose uptake is associated with cell death induced by Dex, we analyzed ALL blasts derived from 19 patients with pediatric B-precursor ALL isolated from bone marrow before clinical treatment. FDG uptake and apoptosis were analyzed after 16 and 24 h of Dex treatment, respectively, similarly to the cell lines as described above. The basal level of FDG uptake by the primary ALL cells and the level of inhibition of FDG uptake following Dex treatment varied largely between samples. Notably, the level of cell death induced by Dex treatment *ex vivo* also varied between samples. Figures 3c and d show representative data for six samples: three that are Dex sensitive and three—Dex resistant (Figure 3d). In four of them FDG uptake was inhibited, while in two it was unaffected (Figure 3c). To further analyze the data, we divided the 19 samples into two groups: one, in which cell death was induced 1.2 fold or more as compared to their respective controls (10 samples), and another, in which 24 h of Dex treatment did not induce any cell death (9 samples), and studied the correlation to the inhibition of FDG uptake. FDG uptake was inhibited in 90% of the first group, that is, samples that underwent apoptosis on Dex treatment. In contrast, FDG uptake was inhibited only in 30% of the samples that were insensitive to Dex-induced cell death (Figure 3e). The correlation between cell death and inhibition of FDG uptake was thus significant according to the Fischer's exact test ($P=0.019$). Furthermore, in order to test the association between changes in FDG uptake and cell death in a fully unbiased manner, the level of cell death compared with the level of inhibition of FDG uptake was analyzed by regression analysis and by the Pearson's test. The results demonstrated that inhibition of glucose uptake in primary ALL cells correlated with Dex-induced cell death and might therefore represent a mechanism underlying Dex-induced cell death (Figure 3f, $R=-0.7$).

To study whether inhibition of glycolysis contributed to the Dex-induced cell death, we used dimethylsuccinate that can feed into the Krebs cycle and thus by-pass glycolysis. Dimethylsuccinate could partly, by around 40%, rescue RS4;11 cells from Dex-induced cell death (Figures 3g and h, $P=0.04$). These data provided further evidence that inhibition of glucose metabolism by Dex contributes to the pro-apoptotic effects of this drug in ALL cells.

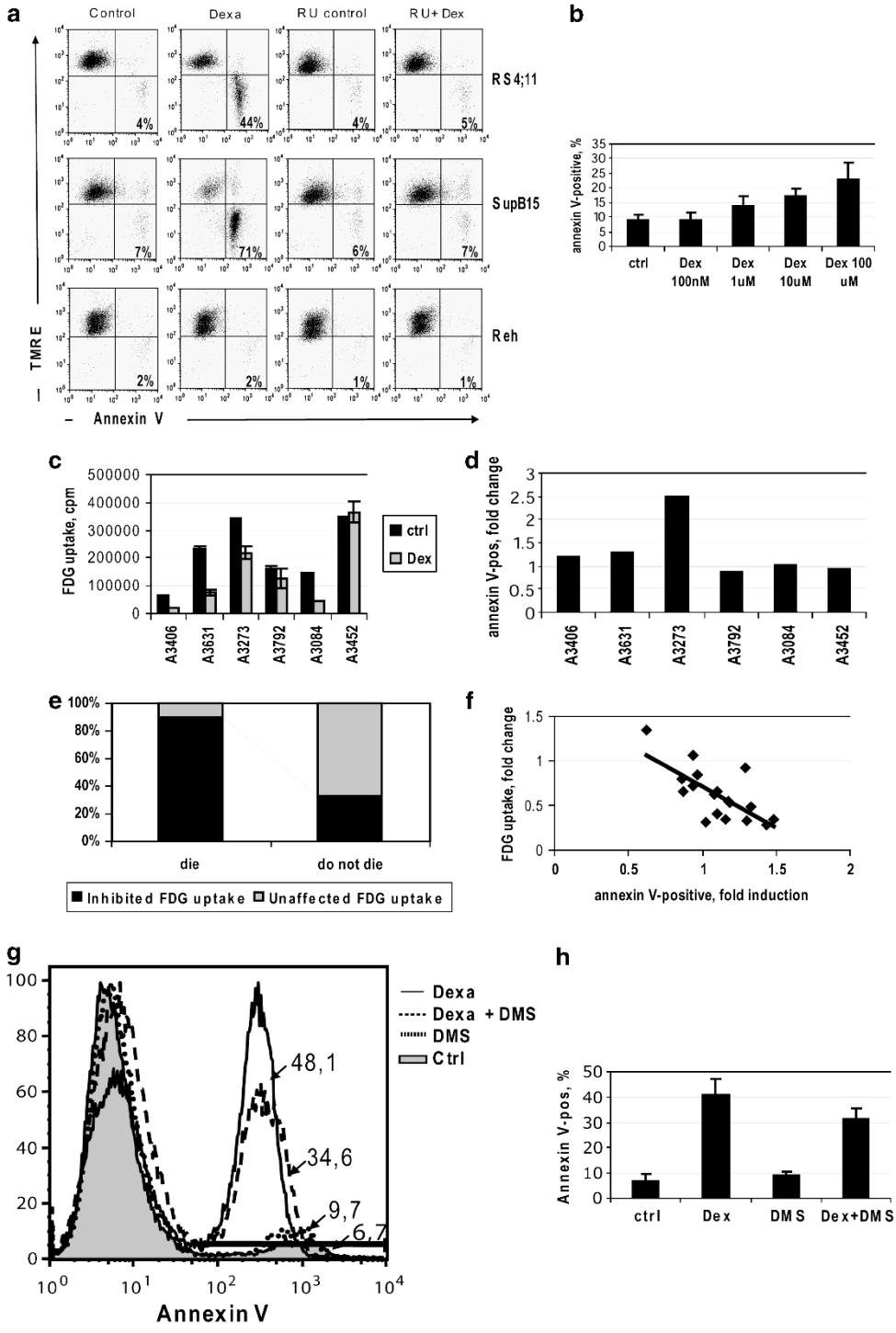
Dex inhibits GLUT1 expression

One of the cellular alterations in transformed cells is an increased rate of glucose uptake that is associated with increased expression of glucose transporters (GLUTs). GLUT1 belongs to the class 1 glucose transporters. It has a high affinity to glucose and has been shown to be expressed at high levels at the membrane of tumor cells, including tumors of lymphoid origin and ALL in particular.[2,3] PI3K signaling and AKT proteins were shown to be essential for the transport of GLUT1 to the cell surface in response to growth factors.[19] We previously found that 16 and 24 h of Dex treatment of RS4;11 cells led to the inhibition of AKT phosphorylation.[18] We therefore tested whether Dex would affect GLUT1 transmembrane expression

in ALL cells. Analysis of cell-surface staining of RS4;11 cells treated with Dex for 24 h and immunoblotting of plasma-membrane-associated proteins after 16 and 24 h of Dex treatment demonstrated a decrease in GLUT1 expression (Figures 4a and b). This was not observed in Reh cells (Figure 4c). Thus, Dex treatment inhibited the levels of membrane-associated GLUT1 in the sensitive but not in the resistant ALL cell line. GLUT1 expression in cancer cells is also regulated at the level of transcription.[2] Indeed, the GLUT1 mRNA levels dropped significantly after 8, 16 and 24 h of Dex treatment, as monitored by real-time PCR (Figure 4d). Thus, Dex inhibits GLUT1 expression and the availability of GLUT1 at the plasma membrane in ALL cells, providing a plausible mechanism for the inhibition of glucose uptake by GCs.

Increased cell death induced by Dex in low glucose concentrations

Our results strongly suggested that inhibition of glucose uptake by Dex leads to the death of ALL cells. It has been shown that 2-DG and prednisolone (or Dex) synergistically inhibit the viability of GC-resistant ALL cell lines, whereas this synergism



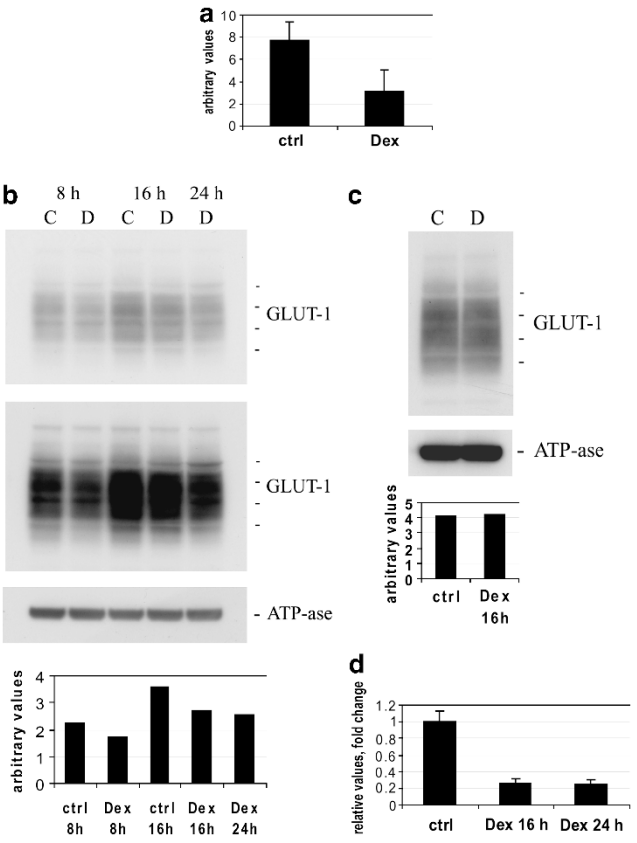


Figure 4 Inhibition of GLUT expression by Dex. (**a**) RS4;11 cells were cultured for 24 h in the absence or presence of Dex. Expression of the glucose transporter GLUT1 was assessed using immunostaining of living cells and analysis by flow cytometry. The bar chart represents the mean fluorescence intensity, MFI, with subtracted background staining. (**b**) RS4;11 cells were either left untreated or were treated with 100 nM of Dex for 8, 16 and 24 h and plasma-membrane-associated proteins were extracted as described in materials and methods section. Protein samples (15 μg/sample) were separated on 4–12% PAG and western blot was performed with anti-GLUT1 antibodies. A shorter (upper panel) and a longer (middle panel) exposure of the same membrane are presented for clarity. Antibodies against Na^+K^+-ATPase (lower panel) were used as control for loading. The chart below western figure represents quantification of the GLUT1 signals as a ratio to ATPase using Aida Image Analyzer, V.4.08. (**c**) Reh cells were cultured, treated for 24 h and processed as in B, except for 1 μM of Dex was used. (**d**) RS4;11 cells were cultured as in **b**; RNA was extracted and mRNA expression of GLUT1 was analyzed by RT-PCR as described in materials and methods section. A representative sample from three independent experiments is shown. The data represent GLUT1 mRNA relative to the β-actin expression levels, in relation to control.

could not be observed in the sensitive cell lines.[9] These data together prompted us to investigate whether the lower glucose levels available to the cells would augment Dex-induced cell death. Dex-sensitive RS4;11 cells were cultured for 1–6 weeks in medium containing twofold or fourfold lower levels of glucose as compared with the normal cell culture medium. Cells grown in 1 g/l or 0.5 g/l of glucose showed no increase in basal apoptosis (Figure 5a). Dex treatment led to a significantly higher cell-death rate in the cultures grown in lower glucose (Figure 5a; $P<0.001$ for either condition). We also asked whether the lower glucose concentration would affect the basal and the Dex-modulated glucose uptake. Culturing cells in the lower glucose concentrations did not significantly change the basal levels of glucose uptake. However, under these conditions, the inhibition of glucose uptake by Dex treatment was more pronounced than under standard cell culture conditions (Figure 5b, $P<0.03$ for either condition). Thus Dex treatment both inhibits glucose uptake and kills ALL cells more efficiently under low glucose conditions.

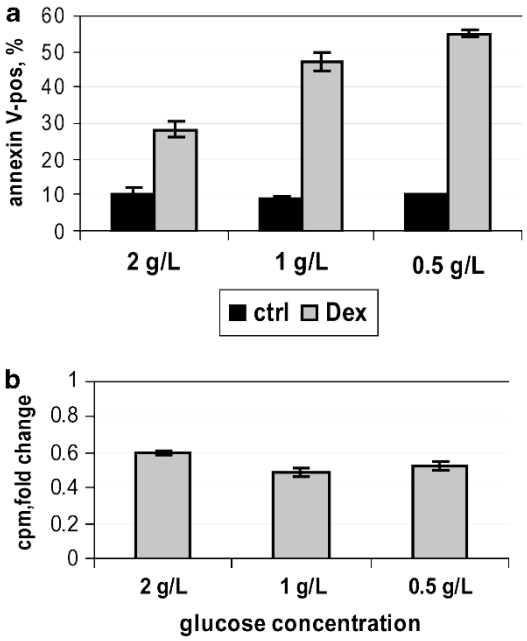


Figure 5 Modulation of glucose uptake and cell death induced by Dex in low glucose. (**a**, **b**) RS4;11 cells were grown in the indicated glucose concentrations over a period of 7–40 days. Cells were either left untreated or were treated with 100 nM of Dex for 36 h. Apoptosis was monitored by AnnexinV/PI stainings (**a**) and glucose uptake was measured using FDG (**b**) as in Figure 2g.

Figure 3 Inhibition of glucose uptake correlates with the ability of Dex to induce cell death in ALL cells. (**a**) RS4;11, SupB15 and Reh cells were cultured in the absence or presence of 100 nM Dex for 36 h and apoptosis was assessed by flow cytometry as accumulation of Annexin V-positive/TMRE-negative cells. To show the specificity of the response, the GC receptor inhibitor RU486 was added to the cells 1 h before the addition of Dex. (**b**) CCRF-CEM cells were cultured in the presence or absence of increasing concentrations of Dex for 48 h and apoptosis was assessed by annexin V staining and flow cytometry. The data represent average of three independent experiments. (**c**) Leukemic cells from ALL patients were cultured *ex vivo* in the presence or absence of 200 nM Dex for 16 h and FDG uptake was measured as in Figure 2g. (**d**) Leukemic cells from ALL patients were cultured as in **c**, but for 24 h and apoptosis was assessed by annexin V staining and flow cytometry. The graph represents a ratio of annexin V-positive cells relative to control. (**e**) Correlation between the inhibition of FDG uptake and cell death *ex vivo* induced by Dex. Primary leukemic cells from 19 ALL samples were cultured, treated and analysed as in **c** and **d**. The samples were grouped according to sensitivity to Dex-induced cell death, as follows: 'die'—samples that show greater than 1.2 fold increase in AnnexinV-positive cells as compared with untreated controls; 'do not die'—samples show no increase in AnnexinV-positive as compared with untreated controls. The $P=0.019$ was calculated using the Fischer exact test and two-sided distribution. (**f**) The data from the experiments described in **c**–**e** were analyzed by regression analysis of the correlation between the level of the inhibition of FDG uptake and the extent of cell death induced by Dex in the primary ALL cells from 18 individual patients *ex vivo*. One of the primary samples was omitted from this analysis due to outlying values. $R=-0.7$; $P=0.02$. (**g**, **h**) DMS overcomes Dex-induced apoptosis. RS4;11 cells were cultured in the absence or presence of 7.7 mM DMS, treated with 100 nM of Dex for 36 h, stained with AnnexinV and analyzed by flow cytometry. The bar chart (**h**) represents average of three independent experiments; $P=0.04$ was calculated using the Wilcoxon rank-sum test for one-sided distribution.

Discussion

In this study, we demonstrated that the GC Dex had profound effects on glucose metabolism in ALL cells. Dex was found to inhibit glucose consumption, utilization and uptake leading to a strong inhibition of glycolysis in ALL cell lines and primary ALL blasts cultured *ex vivo*. GC hormones are normally secreted by the adrenal gland and their effects in multiple tissues have essential physiological roles in glucose, lipid and protein metabolism and contribute to energy homeostasis. Excess plasma levels of GCs may increase endogenous glucose production both directly and indirectly, by antagonizing insulin's metabolic functions. GCs interfere with glucose uptake by skeletal muscle tissue, which is responsible for 80% of glucose uptake from the circulation. This occurs through interference with the insulin-stimulated transport of GLUT4 to the cell surface.[20] Previous studies have not investigated whether GCs affect the glucose uptake/metabolism in lymphoid/leukemic cells. We show in this study that, similarly to the skeletal muscle tissue, Dex reduced the cell surface GLUT1 expression in ALL cells, thus providing a plausible mechanism for the subsequent dramatic effects of Dex on glucose uptake and glycolysis in these cells.

As ALL cells, similar to tumor cells of other origin, have increased glucose uptake and glucose dependence,[3] it was logical to hypothesize that inhibition of glucose uptake would lead to cell death. It was indeed a plausible suggestion as inhibition of FDG uptake was significant already at 6 h after Dex treatment, well before the first signs of apoptosis or of the growth arrest occurred (data not shown). To test this hypothesis, we used both ALL cell lines and primary ALL cells from patients, and measured both the glucose uptake and apoptotic effects induced by the drug. In the cell lines and, most importantly, in the primary ALL cells, inhibition of glucose uptake by Dex correlated with the ability of the drug to kill the cells. Furthermore, Dex-induced cell death was attenuated using dimethylsuccinate that allowed circumvention of glycolysis and directly supported the Krebs cycle, providing more evidence that the inhibition of glycolysis leads to cell death. Interestingly, culturing an ALL cell line in a low glucose medium or with addition of 2-DG slowed down or inhibited growth but did not induce cell death *per se* as compared with Dex treatment. This suggested that inhibition of glucose metabolism is not the sole pro-apoptotic signal induced by Dex. In fact, the combination of low glucose in the culture medium and Dex had an additive effect on cell death in RS4;11 cells. Increased cell death also correlated with a more pronounced inhibition of FDG uptake at half the standard glucose concentration. It was found earlier that 2-DG and prednisolone (or Dex) synergistically inhibit the viability of GC-resistant ALL cell lines.[9] Thus, clearly, inhibition of glucose uptake/metabolism contributes to the GC-induced cell death. On the other hand, our data also suggest that GC-mediated inhibition of glucose uptake is only part of the mechanisms underlying GC-mediated cell death. Our data thus provide evidence that modulating glucose levels in blood might change the effectiveness of GC treatment in ALL.

It was demonstrated that GC resistance in precursor-B ALL is associated with an increased expression of genes involved in glucose metabolism[8] and that an increased glycolytic rate in ALL cells is directly related to GC resistance.[9] In light of our present data, one could speculate that the mechanism(s) of the downregulation of GLUT1 by Dex are impaired in the resistant cells, thus contributing to the resistance to the drug. Importantly, this resistance could be reversed by drugs that inhibit glycolysis, demonstrating that targeting the glycolytic pathway may be a valuable strategy for modulating GC resistance.[9]

Recently, it was demonstrated that an alternative mode of cell death, autophagy, is involved in the response to anti-cancer drugs and that the role of autophagy seems to be cell type and drug specific in that it can either lead to cell survival or to cell death.[21] We have recently shown that Dex induced a strong autophagic response upstream of apoptosis in ALL and CLL cells, and that this was a necessary step for the execution of cell death.[18] We tested whether low glucose or 2-DG exposure would induce autophagy and thus mimic the effect of Dex and potentiate the effects of GCs. However, none of these agents alone induced lipidation and cleavage of LC3 protein, the best marker for autophagy available at present (data not shown). More studies are thus required to analyze the effects of GLUT1 downregulation and glucose starvation on the autophagic response in these cells and to reveal the role of this response in GC-induced cell death of ALL cells.

In conclusion, our study links the inhibitory effects of GCs on glucose uptake to GC-induced cell death, further revealing the mechanisms of the pro-apoptotic action of these drugs in ALL. A detailed understanding of the pro-apoptotic mechanisms will facilitate the design of novel strategies to combat the survival of malignant cells and to overcome resistance to GC treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank the patients and their parents for their acceptance to be included in this study. The study was supported by grants from the Swedish Child Cancer Society, The Cancer Society of Stockholm and the Swedish Cancer Society. AN is supported by The Swedish Research Council (junior research position) and Åke Wiberg Foundation.

References

1 Warburg O. On the origin of cancer cells. *Science* 1956; **123**: 309–314.
2 Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol* 2005; **202**: 654–662.
3 Boag JM, Beesley AH, Firth MJ, Freitas JR, Ford J, Hoffmann K *et al.* Altered glucose metabolism in childhood pre-B acute lymphoblastic leukaemia. *Leukemia* 2006; **20**: 1731–1737.
4 Gaynon PS, Carrel AL. Glucocorticosteroid therapy in childhood acute lymphoblastic leukemia. *Adv Exp Med Biol* 1999; **457**: 593–605.
5 Greenstein S, Ghias K, Krett NL, Rosen ST. Mechanisms of glucocorticoid-mediated apoptosis in hematological malignancies. *Clin Cancer Res* 2002; **8**: 1681–1694.
6 Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood* 1998; **92**: 259–266.
7 Schrappe M, Reiter A, Zimmermann M, Harbott J, Ludwig WD, Henze G *et al.* Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. Berlin-Frankfurt-Munster. *Leukemia* 2000; **14**: 2205–2222.
8 Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM *et al.* Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *N Engl J Med* 2004; **351**: 533–542.

9 Hulleman E, Kazemier KM, Holleman A, VanderWeele DJ, Rudin CM, Broekhuis MJ *et al*. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. *Blood* 2009; **113**: 2014–2021.
10 Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. *Cell Death Differ* 2002; **9**: 6–19.
11 Laane E, Panaretakis T, Pokrovskaja K, Buentke E, Corcoran M, Soderhall S *et al*. Dexamethasone-induced apoptosis in acute lymphoblastic leukemia involves differential regulation of Bcl-2 family members. *Haematologica* 2007; **92**: 1460–1469.
12 Swerdlow SH, Campo E, Lee Harris N, Jaffe ES, Pileri SA, Stein H *et al*. *WHO Classification of Tumours of Haematopoietic and Lymphatic Tissues*. The International Agency for Research on Cancer, IARC: Lyon, 2008.
13 Bjorklund E, Mazur J, Soderhall S, Porwit-MacDonald A. Flow cytometric follow-up of minimal residual disease in bone marrow gives prognostic information in children with acute lymphoblastic leukemia. *Leukemia* 2003; **17**: 138–148.
14 Panaretakis T, Pokrovskaja K, Shoshan MC, Grander D. Activation of Bak, Bax, and BH3-only proteins in the apoptotic response to doxorubicin. *J Biol Chem* 2002; **277**: 44317–44326.
15 Thyrell L, Erickson S, Zhivotovsky B, Pokrovskaja K, Sangfelt O, Castro J *et al*. Mechanisms of interferon-alpha induced apoptosis in malignant cells. *Oncogene* 2002; **21**: 1251–1262.
16 Ling ZC, Hong-Lie C, Ostenson CG, Efendic S, Khan A. Hyperglycemia contributes to impaired insulin response in GK rat islets. *Diabetes* 2001; **50**(Suppl 1): S108–S112.
17 Barata JT, Silva A, Brandao JG, Nadler LM, Cardoso AA, Boussiotis VA. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J Exp Med* 2004; **200**: 659–669.
18 Laane E, Tamm KP, Buentke E, Ito K, Kharaziha P, Oscarsson J *et al*. Cell death induced by dexamethasone in lymphoid leukemia is mediated through initiation of autophagy. *Cell Death Differ* 2009; **16**: 1018–1029.
19 Wieman HL, Wofford JA, Rathmell JC. Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. *Mol Biol Cell* 2007; **18**: 1437–1446.
20 van Raalte DH, Ouwens DM, Diamant M. Novel insights into glucocorticoid-mediated diabetogenic effects: towards expansion of therapeutic options? *Eur J Clin Invest* 2009; **39**: 81–93.
21 Gozuacik D, Kimchi A. Autophagy and cell death. *Curr Top Dev Biol* 2007; **78**: 217–245.