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High concentrations of glucose suppress etoposide-induced cell death of B-cell lymphoma through BCL-6

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ABSTRACT

Glucose is potentially a factor in the resistance to chemotherapy of B-cell lymphomas. In this study we investigated the expression of the glucose induced transcription factor Bcl-6 and the underlying mechanism by which it suppresses B-cell lymphoma cell death. Glucose was found to prevent etoposide-induced tumor cell death. BCL-6 expression was induced by glucose but down-regulated by etoposide. BCL-6 expression was regulated by the interaction of VDUP1 and p53. The molecular mechanism by which glucose prevented etoposide-induced tumor cell death was shown to involve the BCL-6 mediated caspase pathway. Our data suggest that glucose-induced BCL-6 overexpression could abrogate the etoposide chemotherapy effect on tumor cell death.

1. Introduction

Glucose metabolism correlates with cellular proliferation in B-cell lymphoma and influences the response to therapy [1]. Hyperglycemia is known to be an important factor in the resistance to chemotherapy of breast cancer cells [2]. In lymphoma treatment, although etoposide can trigger a process of lymphoma cell death and suppress the energy production of lymphoma cells by impairing mitochondrial function [3], some lymphoma cells can remain resistant to this chemotherapy drug and obtain energy by using glucose. Thus, the possibility of glucose-mediated resistance to etoposide-induced B-cell lymphoma cell death has been proposed.

The Bcl-6 gene encodes a POZ/zinc finger protein that acts as a transcriptional repressor. The gene is involved in a chromosomal translocation in approximately 30% of diffuse large B-cell lymphomas (DLBCL). In gastric lymphomas, high levels of BCL-6 expression predict a favorable prognosis, independent of the Bcl-6 translocation status, translocation partner, or Bcl-6-deregulating mutation [4]. Glucose and serum starvation induce BCL-6 in pancreatic β-cells and decrease cyclin D2 activity and cell proliferation [5]. The level of etoposide controls the fate of the germinal center (GC) B cells by means of Bcl-6, suggesting that Bcl-6 may play a similar role in GC B-cell-like lymphomas. BCL-6 overexpression significantly inhibits the apoptosis caused by etoposide [6] and might link glucose metabolism and cell survival to impact chemotherapy efficacy.

The key tumor-suppressor gene p53 is mutated or lost in approximately 50% of all human cancer cases worldwide. The p53 protein can activate the transcription activity of the Bcl-6 promoter by binding to the Bcl-6 promoter p53RE in vivo [7]. By inducing various cell cycle checkpoints, apoptosis or cellular senescence, p53 can restrict proliferation in response to DNA damage or deregulation of mitogenic oncogenes. Consequently, p53 mutations increase cell proliferation and survival and in some settings promote genomic instability and resistance to certain anti-cancer drugs. Glucose restriction could increase cell death in tumors with p53 impairment. Another tumor-suppressor, vitamin D3 up-regulated protein 1 (VDUP1), can interact with BCL-6 to regulate GC B cells [8]. VDUP1 not only induces oxidative stress [9–12] but also regulates cellular proliferation and the aging process [13]. VDUP1 can be induced by growth arrest stimuli, leading to cell cycle arrest at the G0/G1 phase [14]. In summary, p53 and VDUP1 might be involved in the Bcl-6 mediated cell signal pathway.

In this study, we attempted to determine whether glucose induced Bcl-6 gene expression and suppressed etoposide-induced B-cell lymphoma cell death. The expression of BCL-6 in B lym-

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phoma cells was found to be up-regulated by high levels of glucose, whereas it was down-regulated by etoposide. The underlying mechanism of the glucose-induced BCL-6 role in B-cell lymphoma chemotherapy was explored. The induction of BCL-6 by glucose was synchronized with VDUP1 expression dependent on p53. Glucose prevented etoposide-induced cell death by inducing BCL-6 and suppressing etoposide-induced apoptosis via the caspase pathway.

2. Materials and methods

2.1. Glucose starvation treatment and mRNA stability assays

For glucose starvation experiments, all cells were cultured in complete growth medium. Upon reaching 80% confluence, the cells were washed with pre-warmed PBS and cultured in serum-free medium for different times, depending on the survival of the cell lines. Cells were cultured in glucose-DMEM medium for various times with 20 M etoposide (Sigma–Aldrich) or different concentrations of glucose (5–25 mM). All experiments were repeated three times independently.

2.2. Luciferase reporter assay

The pBcl6-Luc plasmid was transfected into Ramos cells using a Microporator (Invitrogen). The *Renilla* luciferase control vector (Promega) was used to monitor the transfection efficiency. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The firefly luminescence was measured using a luminometer (MicroLumat Plus LB96 V, Berthold Technologies). The *Renilla* luciferase activity was measured as an internal control. The relative luciferase activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

2.3. Chromatin immunoprecipitation (ChIP)

The human p53 deficient cell line HCT116ΔP53 was transfected with a p53 expression plasmid using Lipofectamine 2000 (Invitrogen). Formaldehyde cross-linking and ChIP were performed according to the manufacturer's protocol (Upstate Biotechnology). Immunoprecipitation was performed with protein G-Sepharose and 3 to 5 μg of the following antibodies: anti-p53 antibody (Upstate Biotechnology; BP53–12), anti–RNA polymerase II antibody (Upstate Biotechnology), and normal mouse IgG (Upstate Biotechnology). The latter two antibodies served as positive and negative controls, respectively. PCRs were performed with previously described Bcl-6, CDKN1A (also called p21), and GAPDH primers [7].

2.4. Knockdown of BCL-6 by siRNA

Raji cells were transfected with a control non-targeting siRNA (control siRNA) or with a BCL-6-targeting siRNA (BCL-6 siRNA) using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h of transfection, cells were washed and treated with glucose or etoposide.

3. Results

3.1. Glucose prevents etoposide-induced cell death

To explore whether glucose can prevent apoptosis of B-cell lymphoma cells, we first examined the effect of glucose on the apoptosis caused by etoposide. Ramos cells were cultured in glucose-free medium and treated with or without etoposide and glucose at different times. By observing live cells by microscopy (Fig. 1A), etoposide was found to induce apoptosis. After etoposide treatment (sample name: Eto4 h), the apoptotic cells became smaller and lost their luster. The nuclei of the apoptotic cells were deeply stained and fragmented and showed karyopyknosis. After glucose treatment, the cells seemed viable (sample name: Glu4 h). When the cells were treated with glucose and etoposide at the same time, glucose partially rescued the cytotoxic effect of etoposide (sample name: Eto4 h Glu4 h). Similar results were found by apoptosis assays using flow cytometry (Fig. 1B). In addition, as shown in Fig. 1C, etoposide decreased the number of viable cells in a dose-dependent manner in the presence or absence of glucose. More cells survived with an additional treatment with a higher concentration of glucose, suggesting that glucose might prevent etoposide-induced cell death.

3.2. Bcl-6 expression was induced by high concentrations of glucose

To evaluate genetic alterations associated with high glucose exposure in leukemia or other cancer cells, we ascertained whether Bcl-6 expression was induced in tumor cells by glucose. The following cell lines were exposed to glucose for 3 h: the B-cell lymphoma cell lines (A20, Ramos, Raji, and WIL-2NS), the plasmacytoma cell line J558, the T cell lines (Jurkat and EL-4), the NK cell line NK-92, the non-small cell lung cancer cell line H1299, the HCC cell line HepaG2, the colorectal carcinoma cell lines (HCT and HCTΔP53), and the prostate adenocarcinoma cell line PC-3. The expression of the Bcl-6 gene increased dramatically in the GC-like B lymphoma cell lines Ramos and Raji in the high glucose conditions (25 mM) in a time-dependent manner (Fig. 1D). In addition, the glucose transporter molecule GLUT1 (but not GLUT2) on the membranes was involved in the glucose-induced Bcl-6 signal pathway (Fig. 1E).

3.3. p53 regulated Bcl-6 transcription

To identify the regulatory elements responsible for BCL-6 induction by glucose, we examined the expression of BCL-6 and p53 in Raji cells in the high glucose conditions. Interestingly, we found that p53 expression decreased and BCL-6 expression increased in the high glucose conditions (Fig. 2A). The co-localization of both proteins was shown in the nucleus, predicting the possibility of a physical interaction between Bcl-6 and p53. In addition, after glucose treatment for 4 h, BCL-6 expression increased while p53 expression decreased in Raji cells. After etoposide treatment for 4 h, BCL-6 expression decreased, but p53 expression increased. After treatment with etoposide and glucose together for 4 h, p53 expression was attenuated and the repression of Bcl-6 expression was partly reversed (Supplementary Fig. 1). Our results suggested that a high glucose concentration could prevent etoposide-induced apoptosis via BCL-6 and p53 interaction.

To extend our novel findings, Ramos cells were transfected with serially deleted Bcl-6 promoters and exposed to the high glucose conditions to measure the promoter activity of Bcl-6. A pronounced down-regulation of promoter activity was observed when the P696-1288 construct was used, indicating that regulatory elements located in the 696-1288 region of BCL-6 played a major role in glucose-mediated Bcl-6 induction (Fig. 2B). This result confirmed the presence of a p53 response element (p53RE) in the 696-1288 region of the Bcl-6 promoter [7]. Moreover, the down-regulation of promoter activity was found after co-transfection of Raji cells with the full-length Bcl-6 promoter plasmid and the p53 expression plasmid (Fig. 2C). To measure p53 binding to the Bcl-6 promoter in vivo, primers were designed to amplify a short

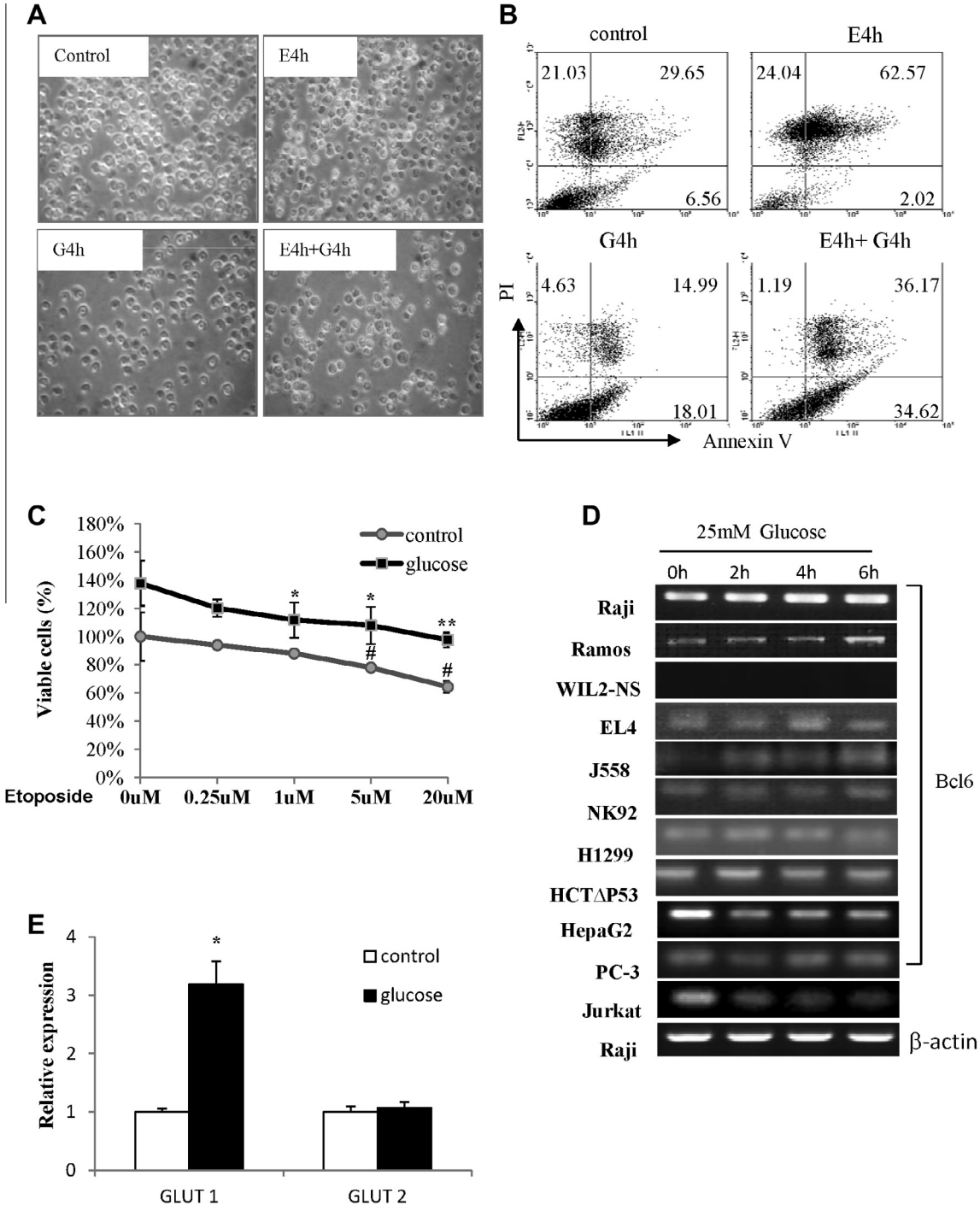

Fig. 1. Glucose prevents etoposide-induced apoptosis via Bcl-6. Raji cells were treated with 20 M etoposide or 25 mM glucose at different times. Control, untreated cells; E4 h, cells were treated with etoposide for 4 h; G4 h, cells were treated with glucose for 4 h; E4 h + G4 h: cells were treated with etoposide and glucose for 4 h. (A) The changes of Raji cell morphology are shown. (B) Scatter plots of the profiles of Annexin V-FITC and PI fluorescence after stimulation as indicated. (C) Cells were treated with 0.25, 1, 5, and 20 M etoposide in the presence or absence of 25 mM glucose. MTT survival assays were performed after treatment for 4 h. Control, viable cells were considered 100%, and results are reported as the percentage of control. Data were expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ Vs normal control. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ Vs glucose control. (D) Several human cell lines were exposed to 25 mM glucose for the indicated times (0 h, 2 h, 4 h, 6 h) or dose-dependent glucose for 3 h, Bcl-6 expressions were examined at the indicated times. Bcl-6 expressions in different cell lines were analyzed by RT-PCR. (E) The expression of the glucose transporter GLUT1, not GLUT2, on the membranes of Raji B cells with or without 25 mM glucose treatment.

segment of the Bcl-6 gene containing the p53RE. This segment was amplified by PCR after immunoprecipitation with an anti-p53 antibody or an anti–RNA polymerase II antibody as a positive control but not when the anti-IgG antibody was used as a negative control. Compared with the results from the p53 deficient cell line HCT116ΔP53 (Fig. 2D, 1st group), an accumulation of the p53RE fragment was detected in the p53 immunoprecipitates from HCT116ΔP53 cells transfected with the p53 expression plasmid (Fig. 2D, 2nd group).

3.4. Bcl-6 expression was synchronized with VDUP1

To investigate genes physiologically related to Bcl6, the expression of VDUP1 was measured after glucose or etoposide treatment.

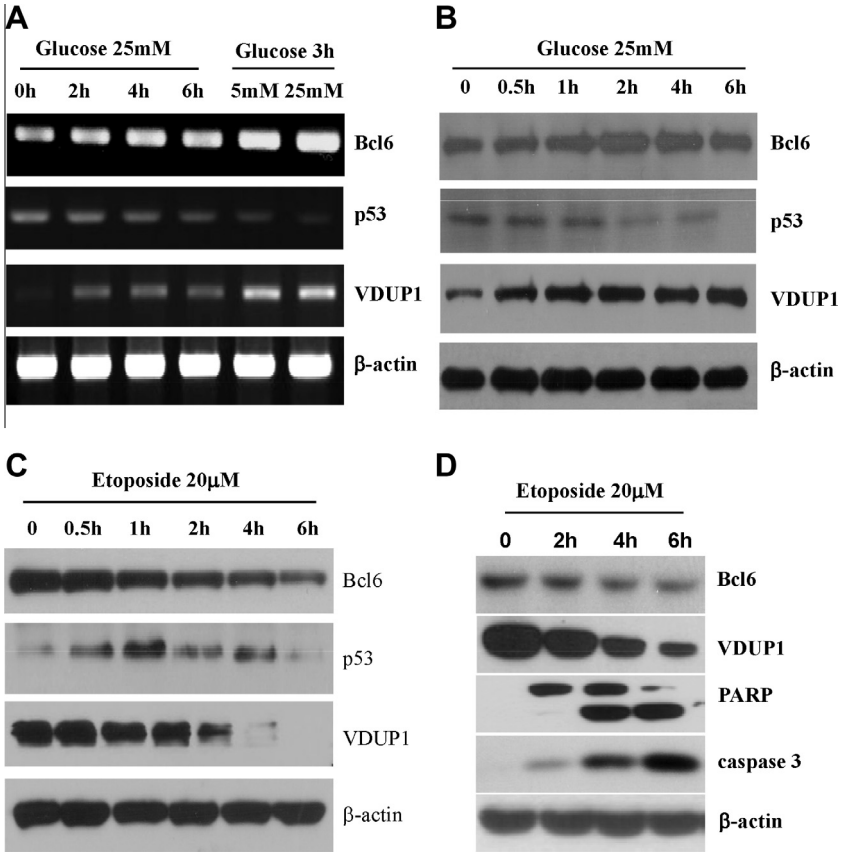

Fig. 2. p53 regulated Bcl-6 transcription. (A) Bcl-6 co-localized with p53 in the nucleus. Raji cells were cultured with or without 25 mM glucose. Cells were stained with anti-BCL-6 and anti-p53 antibodies, followed by secondary Cy3-labeled anti-rabbit (green) and Cy5-labeled anti-mouse IgG (red) antibodies, respectively. Nuclei were stained with DAPI. The BCL-6 expression (green) increased while the p53 expression (red) in the nucleus partially decreased after high glucose treatment. (B) Schematic representation of the Bcl-6 promoter deletion constructs, and the relative promoter activity of the Bcl-6 promoter deletion constructs in Ramos cells in 25 mM glucose at the indicated times. Data were expressed as the mean ± SD of three independent experiments. (C) Bcl-6 promoter activities were measured by luciferase assays in p53 deficient B cells (Ramos cells) co-transfected with the full-length Bcl-6 promoter reporter plasmid and the p53 expression plasmid. (D) ChIP analyses were performed as described in Materials and Methods. HCT116ΔP53 cells were transfected with a p53 expression plasmid (+). Bcl-6 p53RE and CDKN1A (p21) p53 binding sites and the GAPDH exon 4 region were determined by PCR after immunoprecipitation with anti-p53 antibody, anti–RNA polymerase II antibody, or normal mouse IgG. Negative controls were chromatin immunoprecipitated with mouse IgG and samples to which no antibody was added (no Ab). The positive control was chromatin immunoprecipitated with anti–RNA polymerase II antibody. Primers specific for the CDKN1A promoter and GAPDH exon 4 served as positive and negative controls. The PCR results of each ChIP assay were quantitatively compared to the anti–RNA polymerase II control antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Glucose rapidly induced the up-regulation of Bcl-6 and VDUP1 and the down-regulation of p53 at both the transcriptional and translational levels in a time-dependent manner (Fig. 3A and B). Etoposide treatment rapidly caused the down-regulation of BCL-6 and VDUP1 and the up-regulation of p53 in Raji cells, a GC like B cell line (Fig. 3C). Additionally, the decrease of BCL-6 and VDUP1 together with an increase in cleaved caspase 3 and PARP were observed in Raji cells during etoposide treatment, suggesting that etoposide may induce the apoptosis of Raji cells through BCL-6 and VDUP1 (Fig. 3D).

3.5. Glucose induced BCL-6 prevents etoposide-induced apoptosis via the caspase pathway

To assess the possible functional importance of BCL-6 in the glucose mediated suppression of etoposide-induced cell death, we compared the apoptosis of the BCL-6 negative cell line WIL-2NS and the BCL-6 positive cell line Raji after treatment with etoposide or glucose. The expression of BCL-6 was measured after etoposide or glucose treatment in both cell lines (Fig. 4A and B). In the BCL-6 positive Raji cells, BCL-6 expression was induced by glucose (sample name: G4) but suppressed by etoposide (sample name: E4). The repression of BCL-6 expression was reversed by glucose (sample name: E4G8) (Fig. 4A).

Raji cells were transfected with the Bcl-6 siRNA or the control GFP siRNA plasmid in glucose-free medium. The knock-down of BCL-6 expression increased the percentage of apoptotic cells. In the absence of BCL-6 expression, glucose suppressed apoptosis of Raji cells (Fig. 4C). As shown in Table 1, in the glucose-free condition (sample name: control), the BCL-6 siRNA increased the frequency of apoptotic cells (sample name: Bcl-6 siRNA, 15.88 ± 0.91%) compared with the control siRNA (4.84 ± 1.08%, $p = 0.024$), indicating that BCL-6 played an anti-apoptotic function. With high glucose treatment, Bcl-6 siRNA reduced the frequencies of necrotic and apoptotic cells (sample name: Bcl-6 siRNA/Glu8 h, 15.26 ± 0.67%) compared with the untreated Bcl-6 siRNA/control (18.73 ± 1.13%, $p = 0.032$), suggesting that the glucose effect was BCL-6-dependent (Table 1). Treating the BCL-6 silenced cells with glucose and etoposide together caused higher frequencies of necrotic and apoptotic cells than treating with glucose alone (sample name: Bcl-6 siRNA/Glu8hEto4 h, 17.92 ± 1.78% Vs Bcl-6 siRNA/Glu8 h, 15.26 ± 0.67%, $p = 0.043$) (Table 1). Meanwhile, the percentage of viable cells was similar with all treatments.

Caspase 8, the initiator of apoptosis, is synthesized as two isoforms of 55 and 54 kDa, which can be cleaved to intermediate 43 and 41 kDa fragments for activation [15]. Immunoblot assays revealed the cleavage of caspase 8 to the 43 and 41 kDa fragments

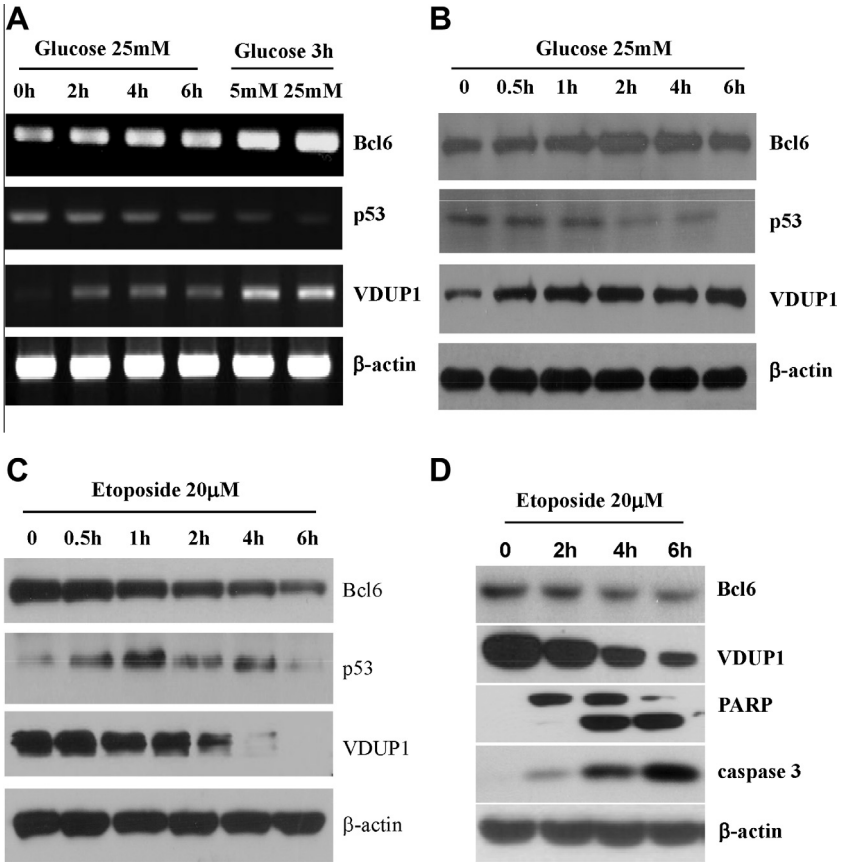

Fig. 3. VDUP1 was synchronized with BCL-6. (A) Raji B cells were exposed to 25 mM glucose for the indicated times (0 h, 2 h, 4 h, and 6 h) or to different doses of glucose for 3 h. The expression of the Bcl-6, p53 and VDUP1 mRNAs was measured by RT-PCR (B) Raji B cells were exposed to 25 mM glucose for 0 h, 0.5 h, 1 h, 2, 4 and 6 h. The expression of the BCL-6, p53 and VDUP1 proteins was measured by immunoblot assay. (C) Raji cells were treated with 20 M etoposide for 0 h, 0.5 h, 1 h, 2, 4 and 6 h. The expression of BCL-6, p53 and VDUP1 proteins was measured. (D) The mouse GC B cells were treated with etoposide for 0, 2, 4 and 6 h. The expression of BCL-6, VDUP1, PARP, and caspase 3 proteins was examined.

after either glucose or etoposide treatment. Quantification of the cleavage of the full-length caspase 8 protein bands is shown (Fig. 4D and E). The activity of caspase 8 did not appear to be influenced by glucose in the absence of Bcl-6 expression.

The effector of apoptosis, caspase 3, is activated after the protein p32 is cleaved into 17 kDa fragments. In the BCL-6 negative cell line WIL-2NS, etoposide stimulated caspase 3 activity, whereas glucose inhibited it. Glucose impaired the stimulation of caspase 3 activity by etoposide (sample name: E4G8 or E8G4). In BCL-6 positive Raji cells, a weaker level of caspase 3 activity was found after etoposide treatment (sample name: E4), while caspase 3 activity was significantly decreased in Raji cells after treatment with etoposide for 4 h and then with added glucose for 4 h (sample name: E8G4) compared with WIL-2NS cells. These data indicate that BCL-6 expression induced by glucose may repress the stimulation of caspase 3 by etoposide (Fig. 4F).

4. Discussion

In the present study, we identified a novel, glucose-initiated signaling pathway that prevented etoposide-induced cell death in B-cell lymphoma. For the first time, we found that glucose rapidly induced up-regulation of Bcl-6 in B-cell lymphoma cells, and we studied the underlying mechanism. In addition, we found that glucose prevented etoposide-induced cell death, and this effect was BCL-6-dependent.

A growing body of evidence indicates that impaired glycolysis may limit the available oxidizable substrate needed to maintain mitochondrial membrane potential and thereby trigger apoptosis [16]. Glucose levels in patients can be a factor in resistance to chemotherapy treatment [17]. Our *in vitro* finding provides a possible explanation for these clinical data. In our study, in high glucose conditions, the decreased necrotic and apoptotic cells in Bcl-6 silenced Raji cells indicated that prevention of cell death by high glucose treatment was BCL-6-dependent. In glucose combined with etoposide, BCL-6 silencing caused more necrotic and apoptotic cells than in glucose alone. Therefore, Glucose may suppress etoposide induced cell death mainly by inducing BCL-6.

Downstream of the glucose-induced BCL-6 signal pathway in B-cell lymphoma cells, we for the first time found that glucose rapidly induced the up-regulation of BCL-6 and VDUP1 and the down-regulation of p53 in Raji cells, indicating that there could be a relationship among Bcl-6, VDUP1 and p53. We found that p53 regulated Bcl-6 transcription directly by binding the 696-1288 region of the Bcl-6 promoter in the high glucose concentration. Moreover, the C-terminal domains of both VDUP1 and p53 were found to participate in the interaction between VDUP1 and p53 (data not shown). Together, in the high glucose condition, increasing levels of VDUP1 might interact with p53 to suppress p53 expression, relieving the Bcl-6 transcription repression by p53. Later, the expression of BCL-6 was up-regulated in the glucose condition.

In agreement with previous studies [18], BCL-6 overexpression inhibited the increase of apoptosis caused by etoposide. Interestingly, with etoposide treatment, the expression of both BCL-6 and VDUP1 was reduced in a time-dependent manner, whereas the levels of cleaved caspase 3 and PARP were increased in Raji

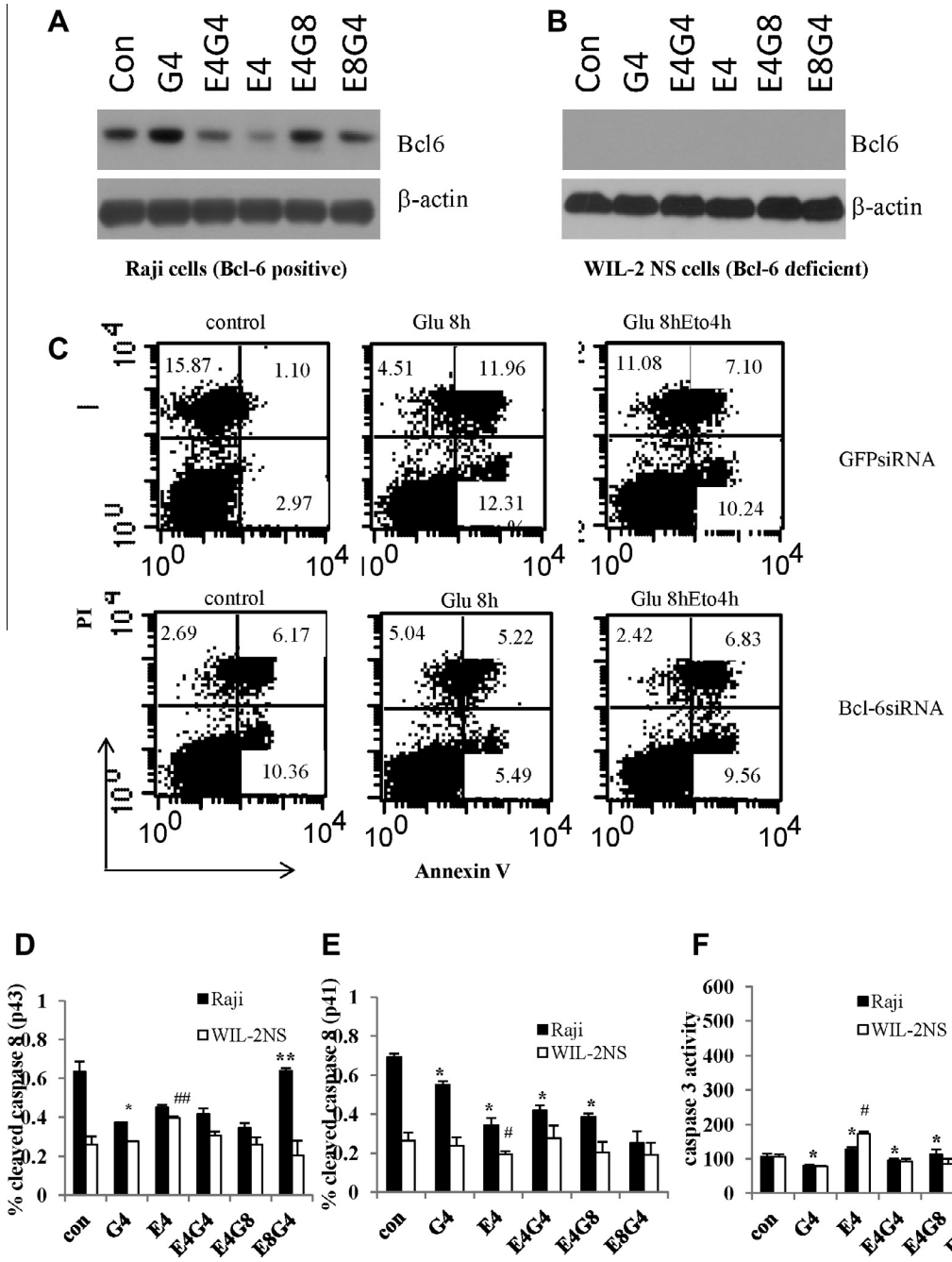

Fig. 4. Glucose induced BCL-6 prevented apoptosis via the caspase pathway, WIL-2NS cells or Raji cells were treated with serum-free media (sample name 1: con), glucose for 4 h (sample name 2: G4 h), etoposide for 4 h (sample name 3: E4), a combination of glucose and etoposide for 4 h (sample name 4: E4G4), glucose for 4 h and subsequently a combination of etoposide and glucose for 4 h (sample name 5: E4G8), and etoposide for 4 h and subsequently a combination of etoposide and glucose for 4 h (sample name 6: E8G4). (A and B) The expression of Bcl-6 in WIL-2NS cells or Raji cells was examined. (C) Raji cells were transfected with GFP siRNA or Bcl-6 siRNA. Then, the transfected cells were treated with glucose for 8 h (Glu8 h) or with glucose for 4 h and subsequently with a combination of glucose and etoposide for 4 h (Glu8hEto4 h). The percentages of apoptotic transfected cells were determined. (D and E) Caspase 8 activation was detected by caspase cleavage (p43 and p41 fragments). WIL-2NS cells or Raji cells were stimulated with the same treatment described above before lysis and immunoblot assay. The percentages of cleaved caspase 8 (p43 and p41 fragments) were calculated by densitometric scanning of the blot. Three independent experiments were carried out. (F) The caspase 3 activity of WIL-2NS cells and Raji cells was measured after the same treatment described above. Data were expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ Vs corresponding control. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ Vs WIL2NS control.

cells. These data indicated that etoposide induced apoptosis of B-cell lymphoma cells by suppressing Bcl-6 expression. Considering that Bcl-6 is down-regulated and p53 is up-regulated in mouse GC B cells, BCL-6 may provide some tolerance to DNA damage by suppressing p53-dependent apoptotic and cell cycle–arrest responses [19].

Apoptosis induced by p53 was identified by caspase activation [20]. Caspase 8 was the principal caspase activated by arsenic trioxide leading to caspase 3 activation and apoptosis in p53 deficient cells [21]. Our results for the first time showed that caspase 3 was activated in the p53 and Bcl-6 null WIL-2NS cells more than in the p53 and Bcl-6 positive Raji cells in etoposide-induced apoptosis. In

Table 1
Glucose effect on etoposide-induced cell death in Bcl-6 siRNA transfected Ramos cells.

	Control		Glucose 8 h		Glucose 8 hEtopside4 h	
	GFP siRNA	Bcl-6 siRNA	GFP siRNA	Bcl-6 siRNA	GFP siRNA	Bcl-6 siRNA
Live cells (%)	80.02 ± 0.06	81.265 ± 0.68	71.99 ± 1.08	84.73 ± 0.67	71.46 ± 0.16	82.07 ± 1.24
Apoptotic cells (%)	4.84 ± 1.08	15.88 ± 0.91	23.42 ± 1.2	10.57 ± 0.19	17.08 ± 0.36	15.47 ± 1.29
Necrosis cells (%)	15.15 ± 1.03	2.85 ± 0.22	3.59 ± 0.11	4.69 ± 0.48	11.45 ± 0.52	2.45 ± 0.49

addition, glucose reduced etoposide-induced cell death by up-regulating Bcl-6 and decreased the activation of caspase 3.

In conclusion, high concentrations of glucose suppress etoposide-induced cell death of B-cell lymphoma by inducing BCL-6 and suppressing etoposide-induced apoptosis via the caspase pathway. Our finding reveals that glucose changes may not only be linked to tumor survival but also to chemotherapy outcome. BCL-6 will contribute to glucose restriction in cellular protection and the improvement of chemotherapy efficacy during cancer treatment.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.096.

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