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Expression of 11beta-hydroxysteroid dehydrogenase 2 contributes to glucocorticoid resistance in lymphoblastic leukemia cells

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Abstract

Synthetic glucocorticoids (GC) form a crucial first-line treatment for childhood acute lymphoblastic leukemia (ALL). However prolonged GC therapy frequently leads to GC-resistance with an unclear molecular mechanism. 11 β -hydroxysteroid dehydrogenase (11 β -HSD) 2 inactivates GCs within cells. Here, we show the association between GC sensitivity and 11 β -HSD2 expression in human T-cell leukemic cell lines. 11 β -HSD2 mRNA and protein levels were considerably higher in GC-resistant MOLT4F cells than in GC-sensitive CCRF-CEM cells. The 11 β -HSD inhibitor, carbenoxolone pre-treatment resulted in greater cell death with prednisolone assessed by methyl-thiazol-tetrazolium assay and Caspase-3/7 assay, suggesting that 11 β -HSD2 is a cause of GC-resistance in ALL.

Keywords

Glucocorticoid resistance; 11 β -hydroxysteroid dehydrogenase 2; acute lymphoblastic leukemia

Introduction

Glucocorticoids (GCs) have potent pro-apoptotic effects on lymphoid cells at certain stages of differentiation that are widely exploited in the treatment of malignant lymphoproliferative disorders. The synthetic GCs dexamethasone and prednisolone form a crucial first-line treatment for both B- and T-cell subtypes of childhood acute lymphoblastic leukemia [1,2]. However, ALL cells show different degrees of GC sensitivity/resistance at diagnosis and resistance to GCs can arise during therapy [3,4]. In childhood-ALL, *in vitro* GC sensitivity at diagnosis correlated with the *in vivo* response to 7 days prednisolone pretreatment, with a poor response associated with an unfavourable prognosis [5,6,7]. Functional glucocorticoid receptor (GR) levels generally correlate with response to GC treatment. Low GR levels may be a common, though hard to detect, mechanism of GC resistance [4,8,9,10,11]. However, this does not account for all GC resistance in ALL.

11 β -HSD interconverts endogenous active GC (cortisol) and intrinsically inert cortisone (which does not bind to GR) [12]. Two isozymes exist. 11 β -HSD2 (encoded by *HSD11B2*) inactivates GCs, whereas 11 β -HSD1 (encoded by *HSD11B1*) regenerates active GCs from inert 11keto forms [12,13]. Importantly, prednisone (inert) and prednisolone (active) are also interconverted by these enzymes [14]. In contrast, dexamethasone is poorly inactivated [14]. Previous work has shown that 11 β -HSD2 is expressed in some tumours and malignant cells, although it is not expressed in the normal adult tissue from which it is derived [15,16,17]. We have therefore postulated that 11 β -HSD2 is expressed in glucocorticoid-resistant leukemia cells where its activity contributes to glucocorticoid resistance. The aim of this study was to evaluate how 11 β -HSD2 affects GC sensitivity in lymphoblastic leukemia.

Materials and methods

Cell culture

Human T-lymphoblastic leukemia cells, CCRF-CEM (JCRB9023) and MOLT4F (JCRB0021) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin, 100 μ g/mL streptomycin at 37°C, 5% CO₂. Carbenoxolone (CBX, 10 μ M) was added 1h before adding glucocorticoids.

Cytotoxicity assay

Cytotoxicity was assessed using the methyl-thiazol-tetrazolium (MTT) assay and Caspase-3/7 assay. Cells (1 \times 10⁵ cells/well) were cultured in 96-well plates. Glucocorticoid sensitivity/resistance was determined by addition of prednisolone (PRED) or dexamethasone (DEX) (9 nM–280 μ M) for 96 h and cell viability measured by MTT assay, as previously described [18,19]. The 50% lethal dose (LD50) was calculated from the dose–response curve. For caspase assay, cells were treated with prednisolone (1 μ M) or dexamethasone (1 μ M) for 48h. 100 μ l of caspase-Glo 3/7 (Promega, Madison, WI, USA) reagent was then added to each sample, cells were incubated for 1 h and caspase activity measured according to the manufacturer's instructions.

RNA extraction and reverse transcription-PCR analysis

Cells (1 \times 10⁶ cells/well) were cultured for 24 h in the presence or absence of 1 μ M dexamethasone in 12-well plates. RNA was extracted following homogenisation in Trizol (Invitrogen) and resuspended in RNase-free water. Reverse transcription of RNA (1 μ g) used a reverse transcriptase system (Takara) according to the manufacturer's protocol. Specific mRNA levels were quantified by real-time PCR on a LightCycler (Roche, Mannheim, Germany), according to the manufacturer's instructions. Primers used were: 11 β -HSD2, 5'-ATCCGTGCTTGGGGCCTATGGAAC-3', 5'-GACCCACGTTTCTCACTGACTCTGTC-3' and GR α , 5'-ACTTACACCTGGATGACCAAAT-3', 5'-TTCAATACTCATGGTCTTATCC-3'. 18S served as internal control.

Western blotting

Cells were washed twice in cold PBS and lysed with RIPA buffer containing protease inhibitor cocktail. Electrophoresis was carried out on 12% NuPAGE Bis-Tris gels (Invitrogen) with 20 μ g protein loaded. After transfer, blots were probed with antibodies to 11 β -HSD2 (a gift of Dr. Roger W. Brown [20]) or tubulin at 1:10000 dilution overnight at 4°C. After washing, membranes were incubated with goat anti-rabbit IRDye-800CW (for 11 β -HSD2) or goat anti-mouse IRDye-800CW (for tubulin) secondary antibodies (both from

LI-COR) for 1 h at room temperature. Blots were visualized on an Odyssey scanner (LI-COR).

Analysis of 11 β -HSD2 activity

Cells were cultured in 12-well plates in the presence or absence of 10 μ M CBX for 1 h. Cortisol (0.1 μ M) was added and medium was collected after 24 h. Cells were removed from the medium by centrifugation (10,000 \times g, 20 min, 4°C) and cell numbers per well were counted. The collected medium was loaded onto activated Sep-Pak C₁₈ (Waters Corp., MA, USA) cartridges. The column was then washed with 5% methanol and steroids eluted with 100% methanol. The eluate was dried in a vacuum centrifuge and steroids were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described [21]. Enzyme activity is expressed as pmol cortisone produced/ h/ 10⁶ cells.

Statistics

Data were analyzed using ANOVA. Significance was set at $p < 0.05$. Values are mean \pm SEM.

Results

Differential sensitivity to dexamethasone and prednisolone in glucocorticoid-resistant MOLT4F cells

Dexamethasone is poorly metabolised by 11 β -HSD enzymes, whereas prednisone/ prednisolone are readily metabolized [14]. To determine whether glucocorticoid-sensitive CCRF-CEM [22] and glucocorticoid-resistant MOLT4F cells [23] show differential sensitivity to dexamethasone and prednisolone, MTT and caspase 3/7 assays were carried out. Both MOLT4F and CCRF-CEM cells showed measureable sensitivity to dexamethasone, although MOLT4F cells were considerably more resistant than CCRF-CEM cells (Fig. 1A). However, in contrast to CCRF-CEM cells, MOLT4F cells were completely resistant to prednisolone (LD50 >280 μ M). Consistent with these data, prednisolone and dexamethasone (at 1 μ M) were equally effective in the induction of caspase 3/7 activity in CCRF-CEM cells, yet only dexamethasone induced caspase 3/7 activity in MOLT4F cells (Fig. 1B).

High expression of 11 β -HSD2 and low expression of GR alpha in glucocorticoid-resistant MOLT4F cells

The relative resistance to prednisolone suggested the presence of 11 β -HSD2 in MOLT4F cells. Quantitative (q)PCR showed that levels of 11 β -HSD2 mRNA were considerably higher in MOLT4F cells than in CCRF-CEM cells (Fig. 2A). 11 β -HSD1 mRNA was not detectable in MOLT4F cells (data not shown). GR alpha mRNA was also expressed in both CCRF-CEM cells and MOLT4F cells (Fig. 2B). The basal level of GR alpha mRNA was higher in CCRFCEM cells than MOLT4F cells, although this did not achieve significance. In CCRF-CEM cells, GR alpha mRNA was dramatically increased by dexamethasone treatment, whereas no induction of GR alpha occurred in MOLT4F cells (Fig. 2B) to give a 10.5 fold difference in GR alpha mRNA levels between glucocorticoid-sensitive CCRF-CEM and glucocorticoid-resistant MOLT4F cells following dexamethasone treatment (Fig. 2B). Consistent with the levels of 11 β -HSD2 mRNA, western blotting showed higher levels of 11 β -HSD2-immunoreactive protein in MOLT4F cells than in CCRF-CEM cells (Fig. 2C), indicating that 11 β -HSD2 could be inactivating prednisolone to prednisone in MOLT4F cells.

Inhibition of 11 β -HSD2 increases sensitivity to prednisolone in MOLT4F cells

To determine whether 11 β -HSD2 could contribute to prednisolone resistance in MOLT4F cells, cells were pre-treated with the 11 β -HSD inhibitor carbenoxolone (CBX) prior to cytotoxicity assay. CBX pre-treatment significantly increased caspase 3/7 activity following prednisolone treatment in MOLT4F cells (Fig. 3A) and significantly decreased cell survival following prednisolone treatment, assayed by MTT (Fig. 3B), whereas CBX had no effect in CCRF-CEM cells (Fig. 3A and 3B). Cortisol was converted to cortisone by MOLT4F cells, but not by CCRF-CEM cells (Fig. 3C), confirming the presence of functional 11 β -HSD2 in MOLT4F cells with negligible levels in CCRF-CEM cells. Moreover, conversion of cortisol (active natural glucocorticoid) to cortisone (inert natural glucocorticoid) was completely abolished by CBX pre-treatment (Fig. 3C), demonstrating inhibition of 11 β -HSD2 by CBX in MOLT4F cells.

Discussion

Here we have shown that high levels of 11 β -HSD2 are associated with resistance to the synthetic glucocorticoid, prednisolone, in MOLT4F cells, whereas the non-metabolisable glucocorticoid, dexamethasone, could induce measurable apoptosis in these cells. Dexamethasone reportedly has 6 times the glucocorticoid potency of prednisolone [24]. However even a 40-fold higher dose of prednisolone could not induce apoptosis to the levels achieved with dexamethasone, suggesting that the prednisolone resistance in these cells is not simply due to differences in potency of the two synthetic glucocorticoids. The presence of 11 β -HSD2 in MOLT4F cells could plausibly contribute to prednisolone resistance. Indeed, the conversion of active cortisol into inactive cortisone by MOLT4F cells and its inhibition by CBX demonstrates the presence of oxidative 11 β -HSD activity in these cells. This was confirmed by demonstration of 11 β -HSD2 mRNA and protein, but not 11 β -HSD1 mRNA in these cells.

MOLT4F cells showed greater resistance to dexamethasone-induced cell death than did CCRF-CEM cells. This is unlikely to be due to 11 β -HSD2, as dexamethasone is poorly inactivated by this isozyme [14]. However, dexamethasone resistance in MOLT4F cells could, at least in part, be due to the low levels of GR in these cells and failure of GR auto-induction. This is consistent with previous reports of low GR levels and/or a failure of auto-induction as a common though hard to detect cause of GC resistance in lymphoblastic leukemia [4,8,9,10,11,25], exquisitely sensitive to glucocorticoid dose [9]. Our data therefore point to at least two mechanisms of GC resistance in MOLT4F cells: a pre-receptor mechanism due to 11 β -HSD2 expression (dependent on the type of glucocorticoid administered) and a receptor-mediated mechanism; low GR levels and failure of GR auto-induction.

We have previously shown differential GC regulation of 11 β -HSD1 in glucocorticoid-sensitive and glucocorticoid-resistant lymphoblastic leukemia [19]. Levels of 11 β -HSD1, which amplifies glucocorticoid action within cells by converting inert GC to active forms, were increased by GC treatment of glucocorticoid-sensitive acute lymphoblastic leukemia cells, whereas they were decreased or unchanged in resistant cells [19]. This may be yet another mechanism that could contribute to differential glucocorticoid sensitivity of leukemic cells. In contrast to 11 β -HSD1, 11 β -HSD2 is widely expressed during development and is re-expressed in tumours and transformed cell lines [17,26,27]. Indeed, many tissues switch from 11 β -HSD2 to 11 β -HSD1 expression as they differentiate and mature [27,28]. In transfected cells, 11 β -HSD1 expression reduced proliferation, whereas transfection with 11 β -HSD2 increased proliferation rate [26]. Therefore it is possible that the expression of 11 β -HSD1 and 2 produce opposing patterns of cell proliferation.

Alternatively, expression of these enzymes together with GR auto-induction may contribute to the differential GC-sensitivity of early lymphocyte precursors [29,30].

CCRF-CEM and MOLT4F cells may be extreme examples of GC sensitivity/resistance. Nevertheless, they add further support to the hypothesis that threshold levels of GR are required for glucocorticoid sensitivity of lymphoblastic leukemic cells. Moreover, whilst 11 β -HSD2 is unlikely to be a contributory factor in dexamethasone resistance, it may contribute to prednisolone resistance. It is now critical to establish whether 11 β -HSD2 can contribute to, or is a marker of, glucocorticoid resistance in primary cells. If this is true for primary leukaemic cells then dexamethasone would be of greater therapeutic benefit than prednisolone in the initial treatment of lymphoblastic leukemia. Indeed, replacement of prednisolone (or prednisone) by dexamethasone in induction therapy markedly reduced risk of relapse with better clinical outcome [31,32,33]. In addition, further research with primary cells is required to investigate whether levels of GR, 11 β -HSD2 and 11 β -HSD1 could be useful prognostic makers in childhood acute lymphoblastic leukemia.

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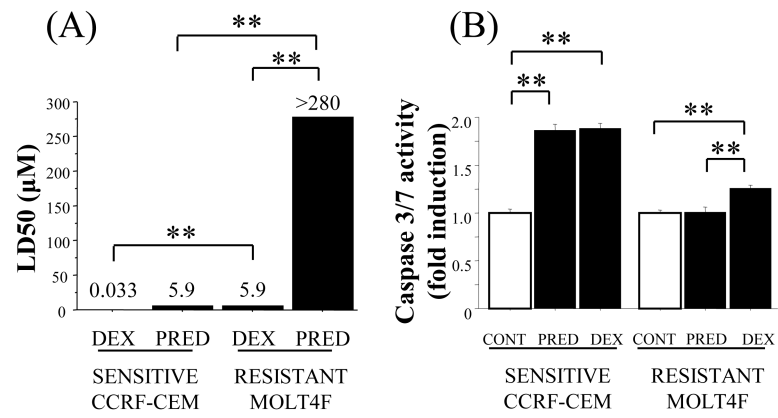


Figure 1.

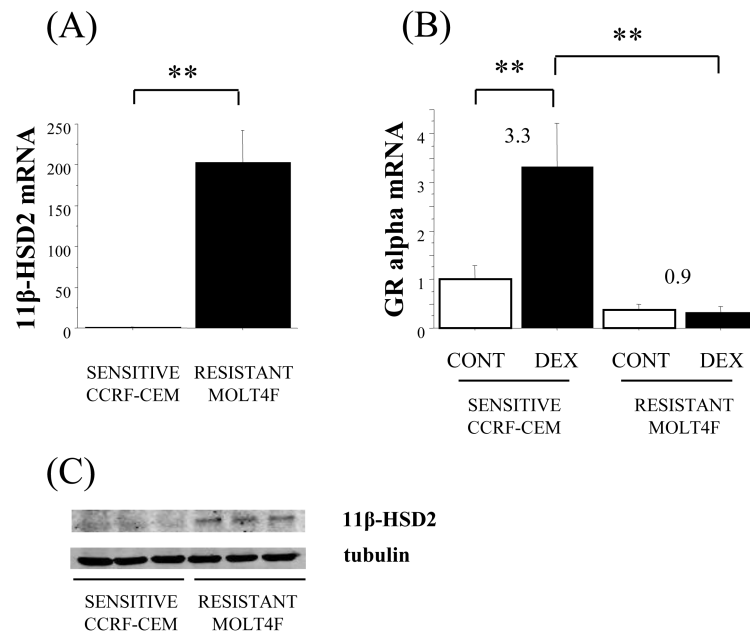


Figure 2.

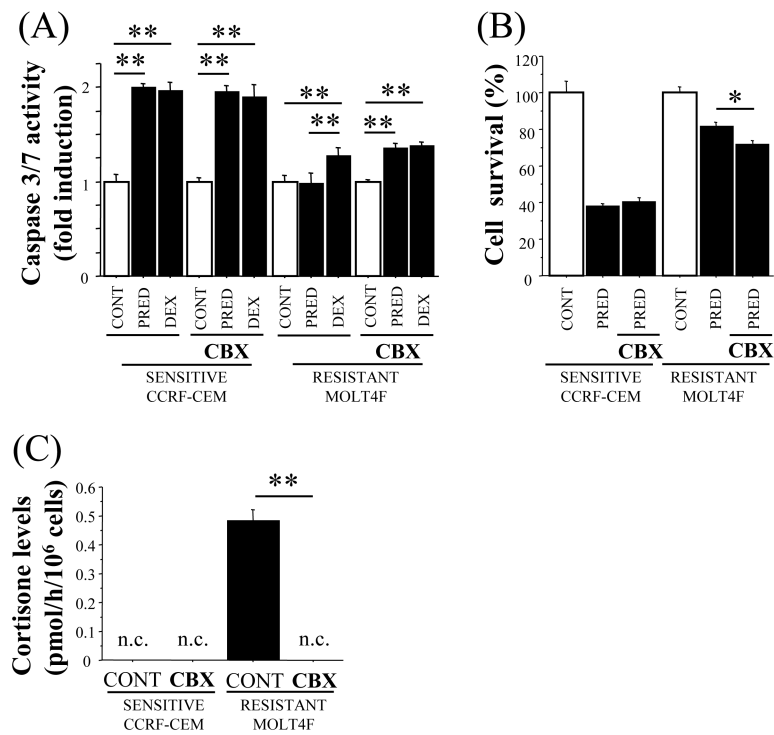


Figure 3.